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Probiotic Supplementation for Neonates with Congenital Gastrointestinal Surgical Conditions

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This thesis is presented for the degree of Doctor of Philosophy of The University of Western
Australia.

School of Medicine

2022

THESIS DECLARATION

I, Clinical Associate Professor Shripada Cuddapah RAO, certify that:

This thesis has been substantially accomplished during enrolment in this degree.

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The research involving human data reported in this thesis was assessed and approved by The University of Western Australia Human Research Ethics Committee. Approval #: [RA/4/1/7743 and RA/4/20/4051). Written patient consent has been received and archived for the research involving patient data reported in this thesis.

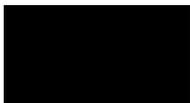
The research undertaken in this thesis did not involve animal experimentation or data.

The following approvals were obtained prior to commencing the relevant work described in this thesis:

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This thesis contains published work and/or work prepared for publication, some of which has been co-authored.

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ABSTRACT

The major congenital gastrointestinal surgical conditions (CGISCs) include oesophageal atresia, gastroschisis, exomphalos, malrotation, duodenal atresia, jejunoileal atresia, Hirschsprung disease, anorectal anomalies, and congenital diaphragmatic hernia. Common issues faced by neonates with CGISCs are feed intolerance, delayed exposure to breast milk, lack of adequate skin to skin contact with their mothers, infections, the use of parenteral nutrition, gastric acid suppressants and multiple courses of antibiotics. These factors could adversely affect their gut microbiota (dysbiosis) and short-chain fatty acids, physical growth, and neurodevelopment.

Our retrospective study (n=413) found that infections, suboptimal neurodevelopment, and postnatal growth restriction are significant issues in neonates with (CGISCs). Our prospective study (n=73) demonstrated that neonates with CGISCs had less bifidobacteria and short-chain fatty acids (SCFAs) in their stools by two weeks of age compared to healthy infants.

We then hypothesised that probiotic supplementation might improve their clinical outcomes by increasing the abundance of bifidobacteria and SCFA levels and decreasing the abundance of pathogenic bacterial families. However, our systematic review found limited evidence on the role of probiotics in neonates with CGISCs. Hence, we conducted a pilot randomised trial (RCT) (n=61), which found that probiotic supplementation with a three-strain bifidobacteria decreased the relative abundance of potentially pathogenic families of *Clostridiaceae*, *Enterobacteriaceae*, *Enterococcaceae*, *Pseudomonaceae*, *Staphylococcaeae*, *Streptococcaceae*, and *Yersiniaceae* and increased the relative abundance of the *Bifidobacteriaceae* family. The pilot RCT also found that the probiotic supplemented group had higher faecal SCFA levels and better head growth. Based on the experience gained from our studies, we provided guidelines for conducting definitive trials in this field. The suggestions included sample size estimation, methods to minimise the influence of potential confounders,

outcomes of interest, probiotic strain selection, storage, dose, duration and microbial quality assurance, stool sample collection, storage, and analysis, and reporting of results. We believe that following these guidelines will increase the internal validity of future RCTs in this field and hence confidence in their results. If such multicentre RCTs confirm that probiotic supplementation improves the *clinical outcomes* of neonates with CGISCs, it will be incorporated into their routine clinical management protocols. Overall, the results of this PhD project have the potential to change clinical practice and improve the outcomes of neonates with CGISCs.

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AUTHORSHIP DECLARATION: CO-AUTHORED PUBLICATIONS

This thesis contains work that has been published and/or prepared for publication.

Details of the work:

Common Congenital Gastrointestinal Surgical Conditions in Neonates

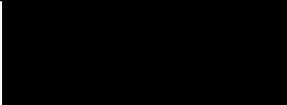
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Chapter 1

Student contribution to work:

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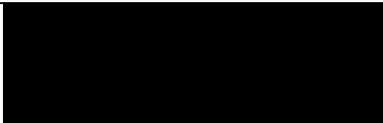
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Physical Growth of Neonates with Congenital Gastrointestinal Surgical Conditions

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 Dr Shripada Rao (student) conceptualised the retrospective study design, created the data collection sheet, verified the data entered by the co-author, conducted the statistical analysis with help from Prof Bulsara (statistician), wrote the first draft of the manuscript, and updated it after receiving feedback from co-authors and submitted it to a peer-reviewed journal.

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Early neurodevelopmental outcomes of congenital gastrointestinal surgical conditions: a single-centre retrospective study

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Chapter 3

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Dr Shripada Rao conceptualised and designed the study, coordinated, and supervised data collection done by the first author, verified the data for accuracy, interpreted the results of the statistical analyses, drafted the initial manuscript, revised it after receiving feedback from co-authors, submitted it to a peer-reviewed journal, updated the manuscript after receiving feedback from reviewers and made the final submission to the journal.

Batta V, **Rao S**, Wagh D, Tan JKG, Gollow I, Simmer K, Bulsara MK, Patole S. Early neurodevelopmental outcomes of congenital gastrointestinal surgical conditions: a single-centre retrospective study. *BMJ Paediatr Open*. 2020 Aug 11;4(1):e000736. doi: 10.1136/bmjpo-2020-000736. PMID: 32821861; PMCID: PMC7422631.
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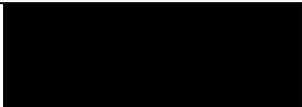
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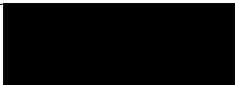
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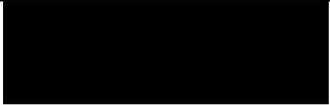
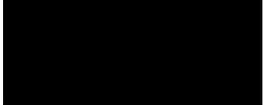
Chapter 6

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Dr Shripada Rao (student) conceptualised and designed this prospective study, applied for, and obtained ethics approval, recruited participants into the study, acquired data, analysed, and interpreted clinical data; liaised with scientists for stool microbial analysis and short-chain fatty acid assays, drafted the article and revised it after receiving feedback from co-authors, submitted to the journal, and addressed reviewers' comments before final submission to the journal for publication.

Rao SC, Esvaran M, Patole SK, Simmer KN, Gollow I, Keil A, Wemheuer B, Chen L, Conway PL. Gut microbiota in neonates with congenital gastrointestinal surgical conditions: a prospective study. *Pediatr Res*. 2020 Dec;88(6):878-886. doi: 10.1038/s41390-020-0824-7. Epub 2020 Mar 16. PMID: 32179871; PMCID: PMC7223116.

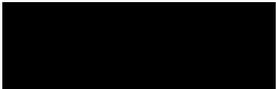
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Rao S, Simmer K, Patole S. Probiotic supplementation in neonates with major gastrointestinal surgical conditions: a systematic review. *J Matern Fetal Neonatal Med.* 2018 Jun;31(11):1517-1523. doi: 10.1080/14767058.2017.1317738. Epub 2017 Apr 25. PMID: 28391755.

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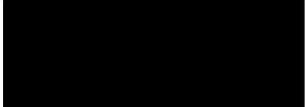
Chapter 9

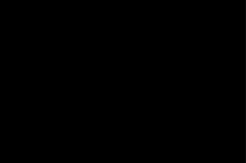
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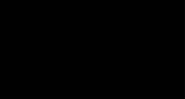
Dr Shripada Rao (student) conceptualised and designed this RCT, applied for and obtained ethics approval, recruited participants into the study, liaised with scientists in Sydney and Singapore to facilitate lab analysis of stool microbiota and short-chain fatty acids, collected clinical data and conducted its statistical analysis, drafted the article, revised it after receiving feedback from co-authors, submitted to the journal, updated it after addressing comments from peer reviewers before final submission to the journal.

Rao S, Esvaran M, Chen L, Keil AD, Gollow I, Simmer K, Wemheuer B, Conway P, Patole S. Probiotic supplementation in neonates with congenital gastrointestinal surgical conditions: a pilot randomised controlled trial. *Pediatr Res.* 2022 Jan 3:1–10. doi: 10.1038/s41390-021-01884-x. Epub ahead of print. PMID: 34980887; PMCID: PMC8722408.

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Probiotic supplementation in neonates with major gastrointestinal surgical conditions: guidelines for future research

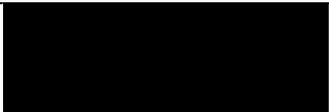
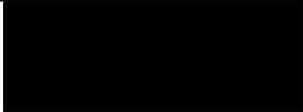
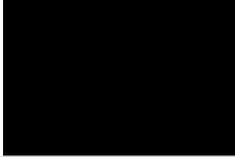
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Preface to Chapter 1

Common Congenital Gastrointestinal Surgical Conditions in Neonates

This chapter describes the anatomical and clinical features, surgical options, medical management, short-term complications, and long-term outcomes of neonates with various congenital gastrointestinal surgical conditions (CGISCs). The important conditions that are covered include oesophageal atresia, gastroschisis, exomphalos, malrotation, duodenal atresia, jejunoileal atresia, Hirschsprung disease, anorectal anomalies, and congenital diaphragmatic hernia. The common issues faced by neonates with CGISCs are feed intolerance, delayed exposure to breast milk, lack of adequate skin to skin contact with their mothers, surgeries and invasive procedures, infections, the use of parenteral nutrition, gastric acid suppressants and multiple courses of antibiotics. All these factors could adversely affect their gut microbiota and short-chain fatty acids, physical growth, and neurodevelopment.

Eleven articles co-authored by the PhD candidate have been cited in this chapter.

CHAPTER 1

Common Congenital Gastrointestinal Surgical Conditions in Neonates

ABSTRACT

The major congenital gastrointestinal surgical conditions (CGISCs) include oesophageal atresia, gastroschisis, exomphalos, intestinal malrotation/volvulus, congenital duodenal obstruction, jejunoileal atresia, Hirschsprung disease (HD), anorectal malformations and congenital diaphragmatic hernia. This chapter describes their anatomical and clinical features, surgical and medical management, short-term complications, and long-term outcomes.

The common issues faced by neonates with CGISCs are feeding intolerance, delayed exposure to breast milk, lack of adequate skin to skin contact with their mothers, surgeries and invasive procedures, infections, the use of parenteral nutrition, gastric acid suppressants and multiple courses of antibiotics, postnatal growth restriction and adverse neurodevelopmental outcomes.

Introduction: The major congenital gastrointestinal surgical conditions (CGISCs) include oesophageal atresia, gastroschisis, exomphalos, intestinal malrotation/volvulus, duodenal atresia, jejunoileal atresia, Hirschsprung disease (HD), anorectal malformations and congenital diaphragmatic hernia. This chapter describes their anatomical and clinical features, surgical options, medical management, short-term complications, and long-term outcomes.

Oesophageal Atresia

In Oesophageal atresia (OA), there is an interruption to the continuity of the oesophagus. OA is usually associated with a fistulous connection to the trachea¹. Common types of OA² are Type A: Isolated OA without tracheo-oesophageal fistula (TOF) (7%); Type B: OA with proximal TOF (3%); Type C: OA with distal TOF (85%); Type D: OA with both proximal and distal TOF (double fistula, 1%); and Type E: H-type TOF without OA (4%).

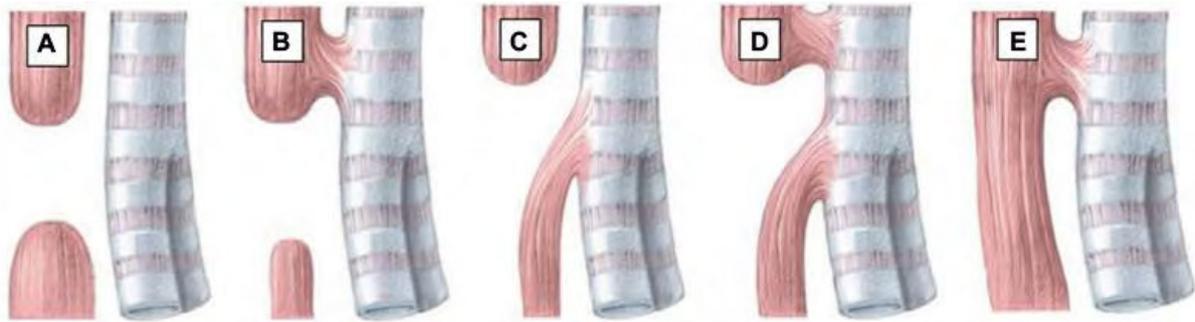


Figure 1: Anatomical classification of oesophageal atresia according to Gross. (Reproduced from Parolini F et al. ². *Pediatric Health Med Ther.* 2017 Jan 18; 8:1-7) with permission from Dove Press Ltd.

The diagnosis of OA is suspected on antenatal ultrasound if polyhydramnios is present, and the stomach bubble is small or absent. Newborn infants with OA have excessive mucousy oral secretions because they cannot swallow saliva. Hence, they require frequent oropharyngeal suctioning to clear the airway. When the diagnosis is suspected, an attempt should be made to insert a nasogastric tube. If the infant has OA, resistance will be felt at about 9-10 cm from the lips, and the tube will not advance further in the oesophagus.

A plain X-ray of the chest and abdomen will show the tip of the tube stuck at thoracic vertebral levels T2–T4. If there is distal TOF (the most typical OA), gas will be present in the stomach and intestine (figure 2A). If a stomach bubble is absent, it suggests the presence of isolated OA without TOF (figure 2B).

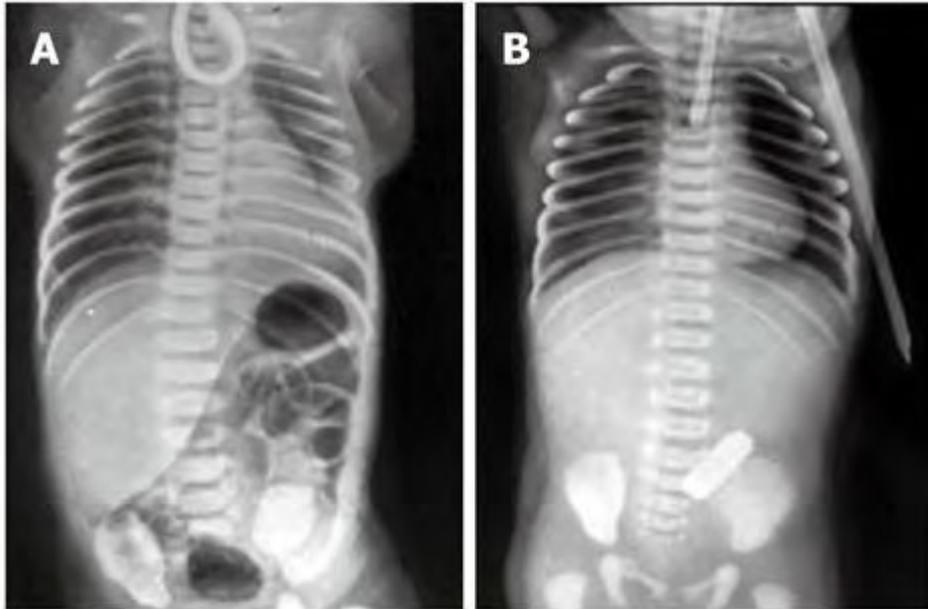


Figure 2: Plain X-rays of the chest and abdomen of two neonates with OA. A: The non-progression of an orogastric tube in the blind oesophageal pouch and the presence of air in the stomach is suggestive of OA with distal TOF; B: The radiopaque tube in the blind oesophageal pouch and the absence of air in the stomach is suggestive of OA without TOF (Reprinted with the Creative Commons Attribution Non-Commercial (CC BY-NC 4.0) license. Pinheiro et al. *World J Gastroenterol.* 2012 Jul 28;18(28):3662-72³

Associated anomalies: The VACTERL association includes vertebral, anorectal, cardiac, tracheo-oesophageal, and renal or radial abnormalities and limb defects.

Management of Oesophageal Atresia: A double lumen Replogle tube (size 10) should be inserted into the upper oesophageal pouch once the diagnosis is confirmed. The first lumen is to aspirate saliva continuously under low-pressure suction to minimise the risk of aspiration pneumonia. Normal saline is instilled through the other lumen of the Replogle tube into the upper oesophageal pouch to keep it moist. Preoperatively, the infant is managed with intravenous fluids and broad-spectrum antibiotics. It is mandatory to do a preoperative echocardiogram to identify the side of the aortic arch because the surgical approach (right or left thoracotomy) depends on the side of the aortic arch. An echocardiogram will also help in diagnosing any associated cardiac anomalies.

Before surgery, in the operation theatre, it is vital to do laryngo-tracheo-bronchoscopy to evaluate the upper airways and detect the rare upper pouch TOF, which could otherwise go unnoticed². Surgery involves closure of the tracheal end of the TOF with sutures, mobilisation of proximal and distal oesophageal pouches, and performing end-to-end anastomosis (Figure 3). A trans-anastomotic tube is placed at the time of surgery to act as a splint for the anastomotic site and facilitate the early commencement of enteral feeds in the postoperative period.

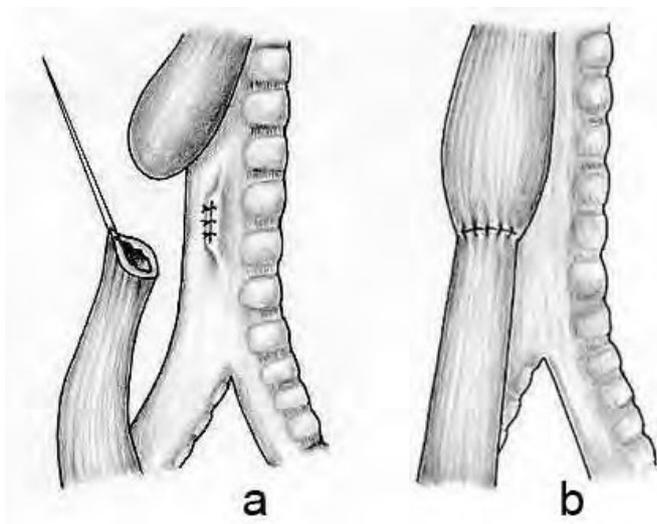


Figure 3: Surgical repair of OA with distal TOF (Reproduced from Spitz L¹. *Oesophageal atresia. Orphanet J Rare Dis.* 2007 May 11; 2:24. Permission to reproduce the figure was not required as it was published under the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>).

The infant is ventilated for 48-72 hours and then extubated. While there is controversy as to whether CPAP could be used to prevent post-extubation atelectasis in these infants, our retrospective study found CPAP to be safe and beneficial⁴.

Post-Operative complications of Oesophageal Atresia: Important post-operative morbidities are gastro-oesophageal reflux, anastomotic leak, strictures, and tracheomalacia⁵. These morbidities could prolong hospital stay, and may necessitate recurrent oesophageal

surgeries, and tracheostomy. Prophylactic long-term anti-reflux medications are widely used even though there is no substantial evidence that they reduce the risk of anastomotic strictures⁶⁻⁸.

Long-term outcomes of Oesophageal Atresia: Mawlana et al.⁹ evaluated the long-term outcomes of 253 infants with OA-TOF at 12-36 months of age. They reported an overall mortality of 8.3%, mainly due to associated severe cardiac anomalies. Neurodevelopmental evaluations (n=182) found 76% were within the normal range, whereas some delay was seen in 24%, especially in expressive and receptive language domains. Hearing or visual impairment was noticed in nine and five infants, respectively. Several specialist interventions were required, primarily related to speech and language. Gastrostomy tubes were necessary for 47 patients, and 15% continued to have postnatal growth restriction⁹.

Leibovitch et al.¹⁰ followed 65 infants with OA-TOF into the adolescent period and reported significant morbidities such as gastro-oesophageal reflux disease (GORD), recurrent episodes of pneumonia, hyperactive airway disease, and reduced quality of life¹⁰

Acher et al.¹¹ surveyed 445 patients or parents of children with OA/TOF. The mean age of patients was 8.7 years (0-61 years). Out of 409, 221 (54%) reported dysphagia, of whom only 77 (34.8%) reported resolution by five years of age. Out of 381 patients, 290 (76%) reported symptoms of GORD. Anti-reflux surgery was required in 22% of patients with GORD (15% of all patients). They concluded that TOF/OA patients have long-term dysphagia and GORD, adversely affecting their quality of life¹¹.

Newton et al.¹² assessed neurodevelopmental outcomes at two years of age of 38 infants with OA. There was a trend towards a higher referral rate for children with TOF than controls (52.6% vs 34.2%; p=0.071). They concluded that more than half of TOF patients experience neurodevelopmental delays requiring referral for early intervention¹².

In summary, infants with OA/TOF suffer from significant morbidities that persist into later childhood and adulthood¹³.

Gastroschisis

It is a full-thickness defect of the anterior abdominal wall, which results in extrusion of abdominal viscera into the amniotic cavity.¹⁴ The defect is commonly to the umbilicus's right (Figure 4) and is usually detected antenatally.



Figure 4: Newborn infant with gastroschisis (Reproduced from Bhat V et al.¹⁵ *Children (Basel)*. 2020 Dec 17;7(12):302, MDPI publishers; permission to reproduce was not required as per open access Creative Common CC BY license).

Management of gastroschisis: Immediately after birth, the lower half of the body is placed in a large, sterile plastic bag with a drawstring to protect the bowel and minimise fluid losses. The infant is nursed in a lateral position with the intestines supported to prevent twisting of the mesentery or compression of the bowel against the margins of the fascial defect¹⁴. A nasogastric tube is inserted and put under free drainage to decompress the stomach and intestines. Maintenance IV fluids and antibiotics are commenced. Normal saline boluses are given judiciously to treat hypovolemia and metabolic acidosis.

The two major approaches to the repair of gastroschisis are a). immediate closure, and b). silo placement (figure 5). The advantages of immediate closure are that feeds could be started within 24-48 hours after birth and graded up quickly as tolerated. However, primary closure carries a risk of abdominal compartment syndrome, iatrogenic gut necrosis, and unplanned return to theatre¹⁶. On the contrary, with silo reduction, the bowel contents are gradually reduced over 3-4 days, and hence the risk of abdominal compartment syndrome is minimised. However, it could delay the commencement of enteral feeds.



Figure 5: Silo reduction of gastroschisis (Reproduced from Bhat V et al.¹⁵ *Children (Basel)*. 2020 Dec 17;7(12):302, MDPI publishers; permission to reproduce was not required as per open access Creative Common CC BY license).

Postoperative care of gastroschisis: Once the final closure of the gastroschisis defect is done, the infant is ventilated for 24-48 hours and given analgesia with morphine or fentanyl infusion. Enteral feeds are commenced as soon as possible in the postoperative period. Our group showed that early commencement of enteral feeds is associated with early attainment of full feeds in a systematic review. Forty-two observational studies on gastroschisis (4,835 infants) were included in that review. Meta-regression results indicated that each day delay in commencing enteral feeds was associated with an additional 1.4 days delay (95% CI: 0.95, 1.85) in achieving full enteral feeds, 2.05 days (95% CI: 1.50, 2.59) delay to the duration of PN, and 1.91 days (95% CI: 1.37, 2.45) to the duration of hospital stay¹⁷.

Simple versus Complex gastroschisis: It is essential to categorise gastroschisis as simple or complex immediately after birth because prognosis depends on the classification.^{18,19} Complex gastroschisis (cGS) is associated with at least one of the following pathologies at birth: intestinal atresia, perforation, necrotic segments, or volvulus. Simple gastroschisis (sGS) is not associated with these additional pathologies²⁰. The systematic review by Bergholz et al¹⁸ reported that mortality of infants with cGS (16.6%) was significantly higher than sGS (2.1%); RR: 5.39 [2.42, 12.01], $p < 0.0001$). It also found that infants with cGS take longer to achieve full enteral feeds and have a more extended duration hospital stay. The risk of sepsis, short bowel syndrome, and necrotising enterocolitis was higher in cGS¹⁸.

Raymond et al²¹ assessed the outcomes of 566 neonates with gastroschisis. Overall survival was 95%. The median duration of hospital stay was 37 days. Sepsis occurred in 107 infants. The median duration of parenteral nutrition was 27 days. Complex gastroschisis, prematurity, and VLBW (<1500 g) were associated with worse outcomes such as infections, short gut syndrome, prolonged duration of PN, and hospital stay²¹.

The incidence of catheter-associated bloodstream infections (CLABSI) is relatively high in infants with gastroschisis. A recent study found the incidence of CLABSI to be 81 out of

2032 central line insertions (3.9%) in patients with gastroschisis compared to 4449 out of 298,862 [1.48%] in children with various other conditions (OR 2.83, 95% CI 2.26-3.54, $p < 0.001$). Average costs were more significant in gastroschisis patients with CLABSI, increasing from \$281,779 to \$421,970 ($p = 0.008$). The average length of stay increased from 31 days to 38 days with CLABSI ($p < 0.001$)²². In another study, the incidence of sepsis was 107/566 (19%)²¹.

Long term neurodevelopmental outcomes of gastroschisis:

Our group²³ retrospectively reviewed all cases of gastroschisis in Western Australia born between 1997 and 2010. Out of 128 pregnancies with fetal gastroschisis, 117 infants were live-born, of whom 112 (95.7%) survived to one year. 19% had z scores of < -1.28 for weight at birth (< 10 th centiles) compared with 30% at one year. The mean GQ was 99 (SD 9.8) at one year of age. Suboptimal neurodevelopmental outcomes were noted in eight. Complex gastroschisis and acquired complications were associated with adverse long-term consequences. We concluded that a large proportion of infants with gastroschisis have suboptimal weight gain during the first year. The incidence of adverse developmental outcomes appears to be low²³. Similar encouraging short-term neurodevelopmental results have been reported by South et al²⁴ and Gorra et al.²⁵

On the contrary, Lap et al.²⁶ found that median verbal intelligence quotient and global executive functioning scores of children with gastroschisis ($n=16$) were lower than controls ($n=32$). Children with gastroschisis were more often classified as borderline or abnormal than controls regarding response inhibition, selective visual attention, sustained auditory attention, and fine motor skills (50% vs 0%).

Giudici et al.²⁷ enrolled 62 infants with gastroschisis, of which 52 were assessed at one year, 34 at three years, and 17 at six years of age. Normal outcomes at one, three and six years

were growth (80%, 85% and 80%), neurology-psychomotor development index (64%, 50% and 82%), audiology (100%, 76% and 76%), vision (98%, 94% and 89%) and language (55%, 62% and 65%). The rehospitalisation rates were 30%, 0.3% and 0, and the surgical re-intervention rates were 9%, 0.3% and 12%.

In summary, infants with gastroschisis are at higher risk of adverse long-term outcomes, with complex gastroschisis more susceptible. Hence multidisciplinary follow-up of infants with gastroschisis is essential²⁸.

Exomphalos (Omphalocele)

Exomphalos is an abdominal wall defect involving the umbilical ring through which small and large intestine, liver, spleen, and occasionally gonads herniate. The fused membrane covering the herniated viscera consists of three layers: the outer amnion, the middle Wharton's jelly, and the inner peritoneal layer²⁹.

Exomphalos is associated with older maternal age and trisomy 13, 18, 21, and Beckwith Wiedemann syndrome³⁰. Anomalies of cardiac, gastrointestinal, genitourinary, and musculoskeletal, and central nervous systems are common. Diagnosis can be made on antenatal ultrasound in the first trimester of pregnancy.

The defect is <5 cm in small exomphalos, and the sac usually contains a few intestinal loops.

In giant exomphalos, the defect is >5 cm, and the sac contains more than 75% of the liver.

Management of exomphalos: Multidisciplinary discussion during the antenatal period is essential to ensure optimal outcomes. During such meetings, information about the defect's size, herniated liver presence, associated anomalies and syndromes and pulmonary hypoplasia, and parents' preference are considered. The main focus would be on the continuation or termination of pregnancy and the type of delivery (cesarean vs vaginal). Spontaneous vaginal delivery is feasible with minor defects. In contrast, a cesarean section is

essential for giant exomphalos to prevent dystocia and damage to the fetal liver or rupture of the sac.

Immediate postnatal care includes delivery room resuscitation and placing the baby's lower half in a sterile bowel bag or covering the lesion with saline gauze. Other management components include decompression of the stomach using a nasogastric tube, IV fluids, and antibiotics. An urgent echocardiogram is essential to rule out cardiac anomalies and to assess pulmonary hypertension. An ultrasound of the abdomen is performed to rule out renal anomalies. A blood sample should be collected for karyotype or microarray analysis.

The goal of surgery is to achieve fascial and skin coverage while avoiding abdominal compartment syndrome. The standard approaches are (1) Immediate (primary) repair; (2) Staged repair with delayed primary closure, and (3) Delayed repair (paint and wait)³¹

Small exomphalos is treated with a primary repair under general anaesthesia. The sac is opened carefully, and the viscera is completely reduced. Subsequently, the umbilical vessels are ligated and divided, followed by the closure of the fascia and the creation of neo-umbilicus (Figure 6).



Figure 6: Small omphalocele and the results of primary closure of the defect. Reproduced with from Skarsgard ED. *Semin Pediatr Surg.* 2019 Apr;28(2):89-94 ³¹ with permission from Elsevier Publishers.

For moderate and large exomphalos still amenable to primary repair, the usual practice is a primary midline fascial closure with umbilical cord preservation (Figure 7).



Figure 7: A–C - Following midline fascial closure, dermal advancement sutures are used to approximate the skin edges to the cord, leading to a normal-appearing neo-umbilicus. Reprinted from Skarsgard ED. *Semin Pediatr Surg.* 2019 Apr;28(2):89-94³¹ with permission from Elsevier Publishers.

Extensive defects are managed with staged closure with a silo (Figure 8) or the paint and wait approach (Figure 9).

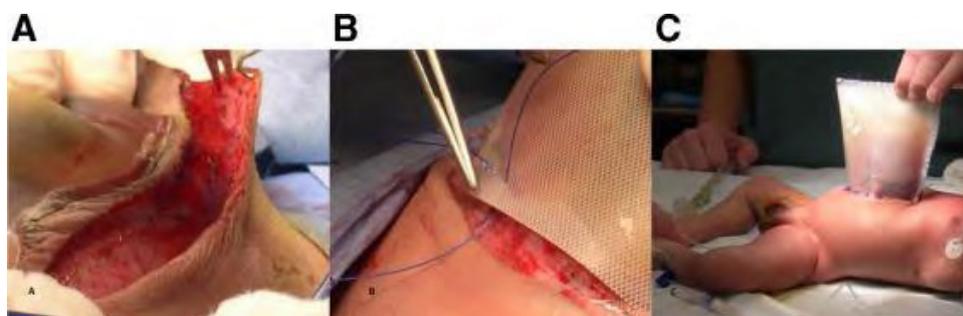


Figure 8: (A) Dissection of fascia in the subcutaneous plane. (B) Suturing silastic mesh to the fascial edge (C) Final construction of a “custom” silo to facilitate gradual visceral reduction with amnion preservation. Reprinted from Skarsgard ED. *Semin Pediatr Surg.* 2019 Apr;28(2):89-94³¹ with permission from Elsevier Publishers.

The ‘Paint and wait approach’ is used in very large defects to avoid abdominal compartment syndrome and respiratory distress (Figure 9). The escharotic agent (silver sulfadiazine cream

or povidone-iodine solution) is applied daily to the whole exposed sac surface with a supportive gauze wrap until cicatrization and epithelialisation are complete. Delayed closure of the abdominal wall is undertaken when the infant is 9-18 months of age (Figure 10)).

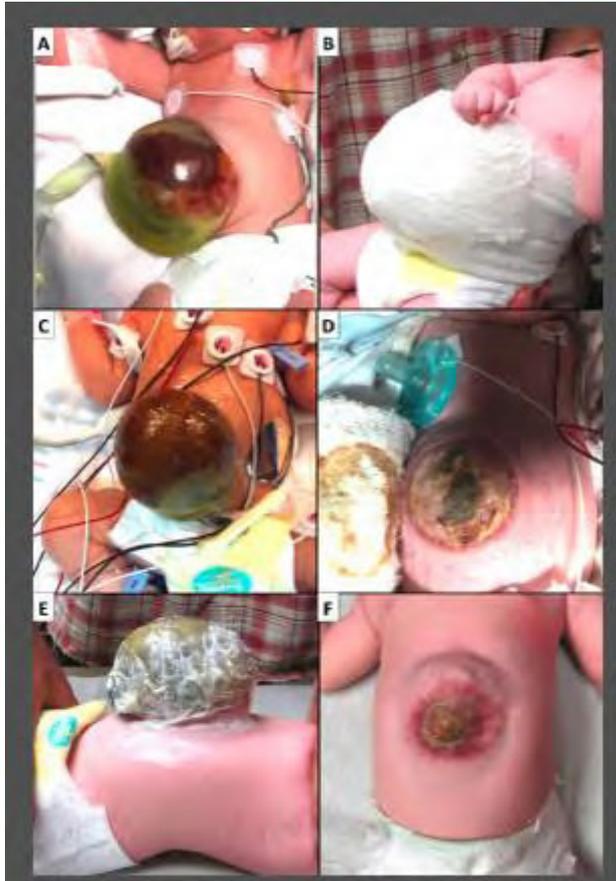


Figure 9: *“Paint and wait” approaches. A) Giant omphalocele containing liver and bowel. B) Supportive gauze dressing after application of the escharotic agent. C) Povidone-iodine treatment, one day after initiation. D) Granulated sac after daily povidone-iodine dressings. E) Silver sulfadiazine treatment, one day after initiation. F) Escharotic sac after daily silver sulfadiazine dressings. Reprinted from Wagner JP et al.³². *Semin Pediatr Surg.* 2019 Apr;28(2):95-100, with permission from Elsevier Publishers.*

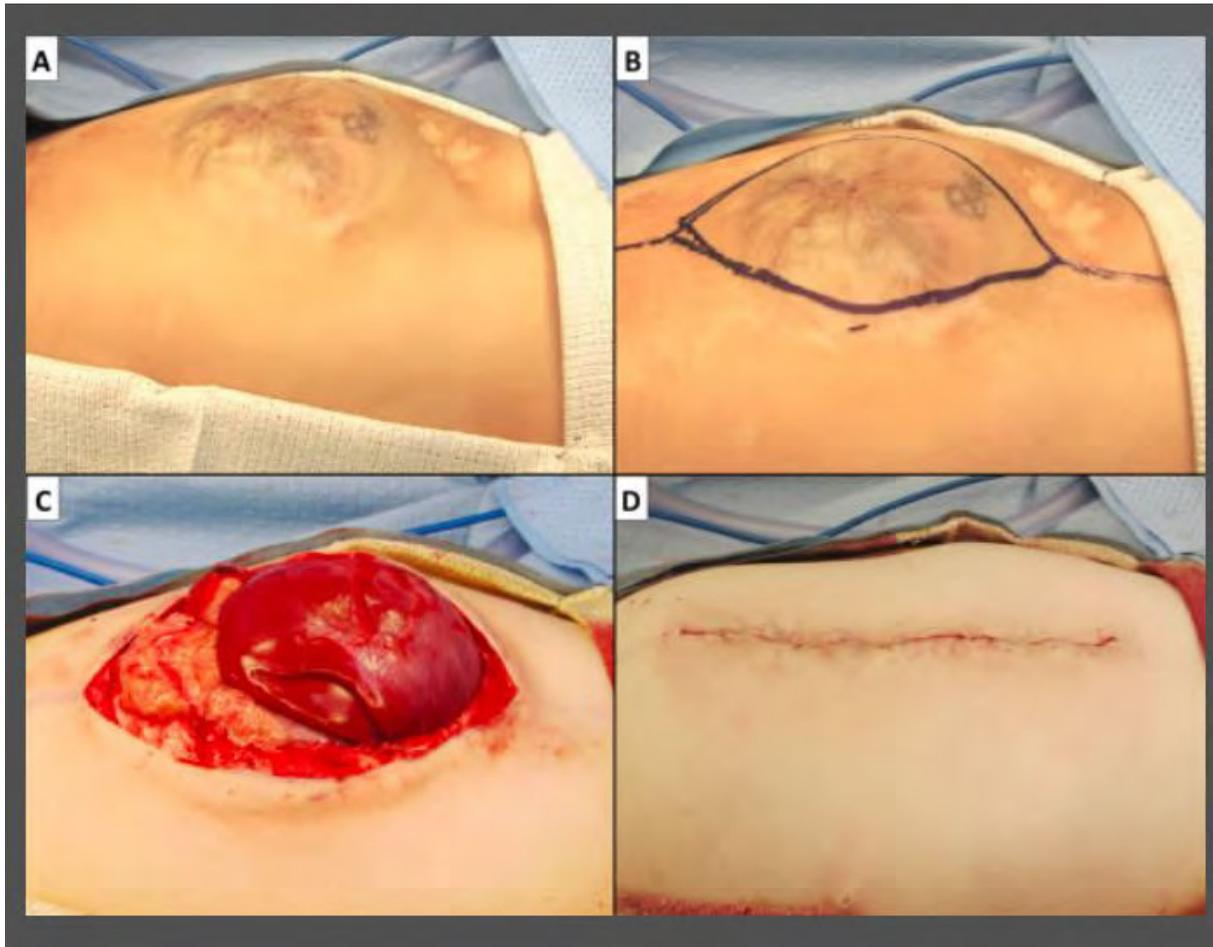


Figure 10: Delayed operative repair after “paint and wait” approach. A) Fully epithelialised sac with normal abdominovisceral proportion. B) Intended incision site marked. C) Viscera liberated; redundant sac resected. D) Completed abdominal closure. Reproduced from Wagner JP et al.³². *Semin Pediatr Surg.* 2019 Apr;28(2):95-100 with permission from Elsevier Publishers.

Long-term outcomes of Exomphalos: The survival rates depend on the size of the lesion and the presence of associated anomalies. Infants with isolated exomphalos have survival rates of nearly 90%. Mortality rates could be as high as 40% in infants with chromosomal abnormalities. Giant exomphalos is associated with even higher mortality rates, prolonged feeding intolerance, prolonged hospitalisation, and adverse neurodevelopmental outcomes³³.

Malrotation of Intestines and Volvulus

Normal rotation of the intestines results from steps between 4 and 10 weeks of gestation. They transform the straight primitive gut into the following configuration: (1) duodenojejunal junction (DJJ) fixed in position by the ligament of Trietz (LOT) to the left of the spine and at the level of the pylorus; (2) caecum fixed to the right lower quadrant, and (3) small intestine supported by the superior mesenteric artery (SMA) and superior mesenteric vein (SMV) contained within a broad-based mesentery³⁴.

An error during this complex process can lead to intestinal malrotation, and the subsequent abnormal fixation results in congenital adhesions known as Ladd's bands. These adhesion bands originate on the cecum and can obstruct the duodenum. The base of the mesentery in malrotation is very narrow. It hence can easily rotate on its axis, causing the loop of the intestine to twist around itself, leading to intestinal obstruction (volvulus). Midgut volvulus is accompanied by acute ischemia of the intestines due to obstruction to blood flow through the superior mesenteric artery. If not treated urgently, it can lead to severe infarction of the intestines, shock, and death. Survivors develop short bowel syndrome due to extensive resection of the intestines. Therefore, a high index of suspicion, timely diagnosis, and prompt treatment are critical.

Clinical Presentation of malrotation: A cardinal sign of malrotation and midgut volvulus is bilious vomiting³⁵. Late signs are abdominal distension, bloody stools, abdominal wall erythema, peritonitis, and shock due to bowel ischemia. The majority of cases present within the first month of life.

Confirmation of diagnosis of malrotation: Upper gastrointestinal (UGI) contrast study is the gold standard investigation to diagnose malrotation³⁶. A water-soluble contrast is administered orally or via a nasogastric tube. The aim is to demonstrate a normal position of the duodenum and DJ flexure because this marks the position of the LOT. If this is normally

located, it rules out malrotation. The normal duodenum can be C-shaped or distorted C-shape or U-shape (see figure 11). The first part of the duodenum is intraperitoneal and lies to the right of the spine at the same level as the pylorus. The second part descends to the right of the spine, the third part crosses the midline to the left of the spine, and the fourth part ascends to the DJ flexure, which is sited to the left of the spine at the same level as or higher than the pylorus. In lateral projection, the second to fourth parts of the duodenum are posterior to the stomach since they are retroperitoneal (figure 12). The findings in malrotation are: (1) Location of DJJ to the right of the vertebral column or below the level of the pylorus; (2) duodenal redundancy; and (3) corkscrew appearance of the DJJ (Figure 13, 14)

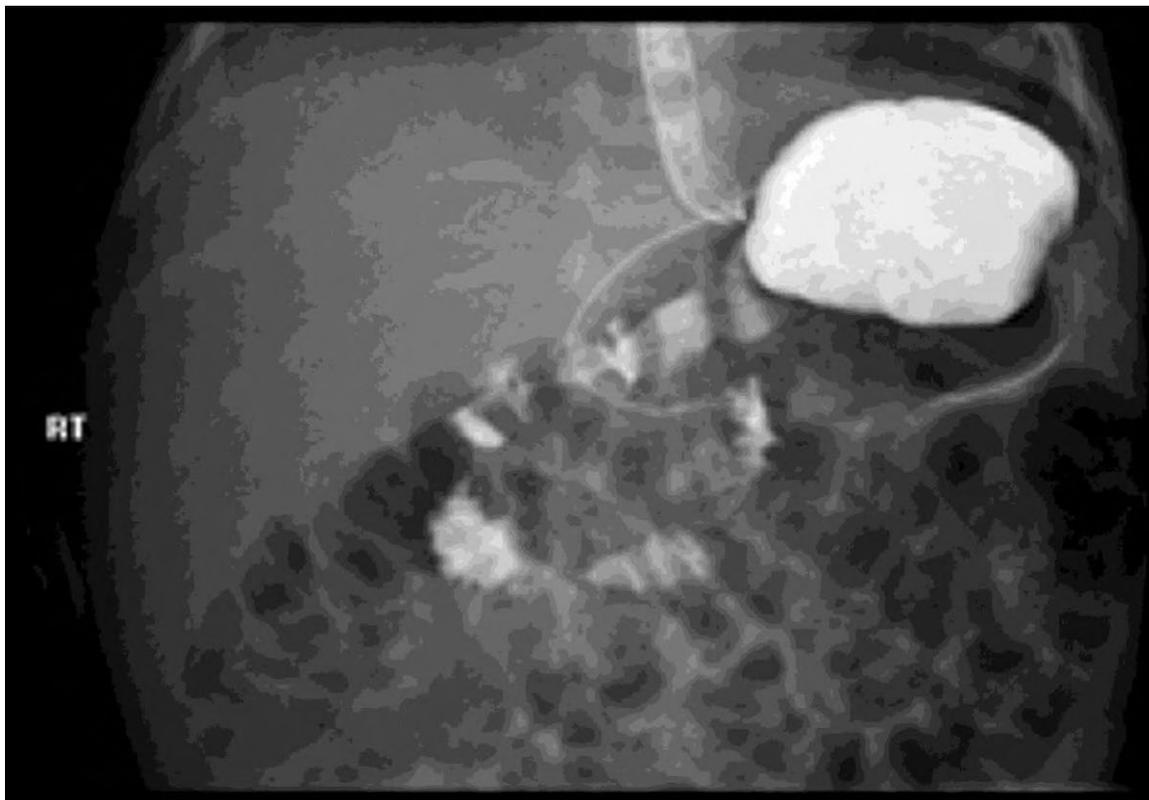


Figure 11: Upper gastrointestinal contrast study frontal view: Normal position of the duodenojejunal junction to left of the spinal pedicle and at the level of the pylorus. Reproduced with permission from Birajdar et al. *J Paediatr Child Health*. 2017 Jul;53(7):644-649. Publisher: John Wiley and Sons³⁶.

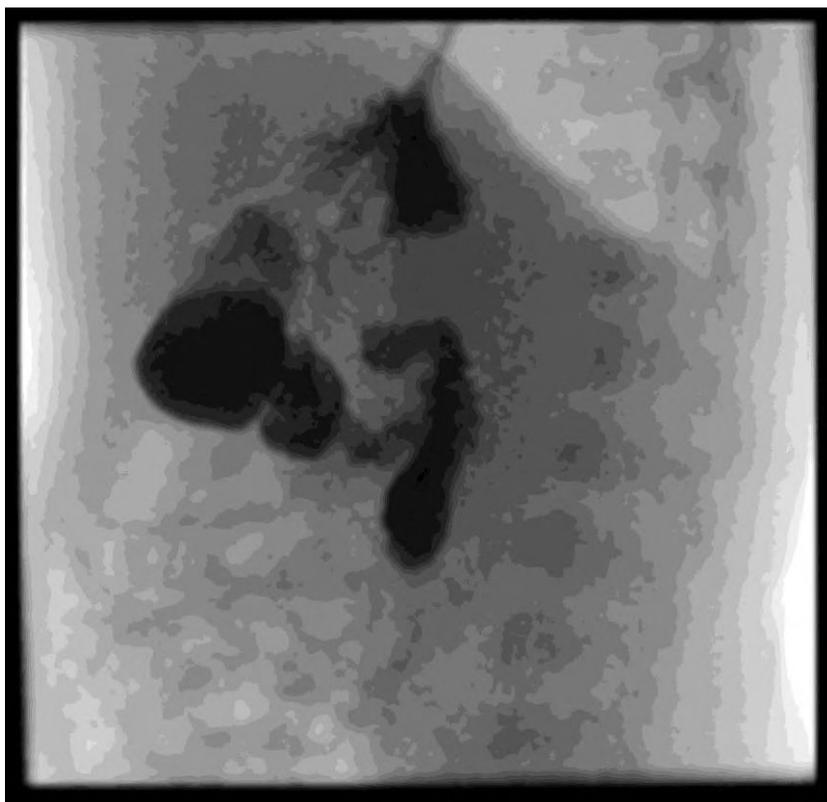


Figure 12: Normal upper gastrointestinal contrast study oblique view: Posteriorly located duodenojejunal junction. Reproduced with permission from Birajdar et al. *J Paediatr Child Health*. 2017 Jul;53(7):644-649. Publisher: John Wiley and Sons³⁶.



Figure 13: Malrotation. On frontal film, the duodenojejunal junction is located to the right of the spinal pedicle and below the pylorus. Reproduced with permission from Birajdar et al. *J Paediatr Child Health*. 2017 Jul;53(7):644-649. Publisher: John Wiley and Sons³⁶.



Figure 14: Malrotation and volvulus. On the lateral view, the duodenojejunal junction is located anteriorly with the 'corkscrew' downward path of the distal duodenum. Reproduced with permission from Birajdar et al. *J Paediatr Child Health*. 2017 Jul;53(7):644-649. Publisher: John Wiley and Sons³⁶.

Abdominal ultrasound is a valuable investigation, but the gold standard investigation is the upper GI contrast study. On ultrasound, the SMA is typically to the left of the SMV, and reversal of this position is suggestive of malrotation. But ultrasound has a false-positive rate of up to 21%, leading to unnecessary surgery. Ultrasound also has high false-negative rates³⁷, resulting in false reassurance with dire consequences. On the contrary, the "whirlpool" appearance of the mesentery on ultrasound is highly suggestive of volvulus³⁸. A meta-analysis that included 16 studies (1640 participants) found a pooled sensitivity of 87.42 (95%

CI: 81.0–92.2) and specificity of 98.6% (95% CI: 97.8–99.2) of whirlpool sign to diagnose volvulus.³⁹

Surgical Treatment of intestinal malrotation: Once diagnosed, urgent surgical correction is essential to prevent catastrophic outcomes due to intestinal volvulus, ischemia, and necrosis. The standard surgery is Ladd's procedure in which the bowel is de-torsed in a counter-clockwise direction, and Ladd's bands causing obstruction are resected. The base of the mesentery is broadened by placing the cecum in the left upper quadrant with the colon in the left abdomen and the small bowel in the right quadrant of the abdomen. For patients with midgut volvulus and necrotic intestines, a nonviable gut is resected after reducing volvulus.

Neurodevelopmental outcomes of infants with intestinal malrotation: Very few studies have reported on the long-term neurodevelopmental outcomes of infants with malrotation. In our retrospective study⁴⁰, 33 infants were assessed using the Griffiths Mental Development Scales (GMDS) at one year. The mean general quotient (GQ) of the study population was 98 (SD 7.33), which was similar to the population norms (100.2, SD 12.8). None of the infants developed cerebral palsy, tone abnormality, sensorineural deafness, or blindness. The physical growth of these infants was satisfactory. In summary, the one-year developmental outcomes and physical development of neonates with intestinal malrotation were satisfactory⁴⁰.

Congenital Duodenal Obstruction

Congenital duodenal obstruction (CDO) represents about 60% of intestinal atresias. The estimated incidence is around 1.22 cases per 10 000 live births^{41,42}. CDO could be intrinsic or extrinsic. Intrinsic obstruction is due to atresia or stenosis or web, whereas extrinsic obstruction is due to the annular pancreas, Ladd's bands in malrotation, and occasionally duplication cysts⁴³. Of these, the commonest is duodenal atresia. The majority of duodenal obstructions are distal to the ampulla of Vater in the second portion of the duodenum.

Patients with atresia present in the immediate newborn period, whereas duodenal membranes and partial obstruction can present later in infancy (Figure 15).



Type I - Membrane only



Type II - Fibrous Cord



Type III - Complete Interruption

Figure 15: Types of duodenal atresia. Reproduced from Bethell et al. ⁴¹. *Arch Dis Child Fetal Neonatal Ed* 2020; 105:178-183 (Creative Commons CC BY 4.0 license permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. <https://creativecommons.org/licenses/by/4.0/>).

Clinical presentation of duodenal atresia: Most infants with duodenal atresia present with bilious vomiting within the first 48 hours of life⁴³. In about 20%, the vomit is not bile-stained as the obstruction is proximal to the ampulla of Vater. Approximately 30% of infants with DA will have trisomy 21, and up to 25% with DA have structural cardiac anomalies^{44,45}.

Antenatal diagnosis: Duodenal atresia is suspected if there is polyhydramnios, and the suspicion is strengthened if there is a double bubble appearance on fetal ultrasound.

Imaging studies: The classical picture of duodenal atresia is a double bubble appearance on the X-ray of the abdomen⁴⁴ (Figure 16). If the classic double bubble appearance is not seen or the baby appears unwell, it is essential to do an upper GI contrast to exclude malrotation and confirm the duodenal atresia⁴².



Figure 16: Abdominal radiography shows the markedly distended stomach and proximal duodenum ('double bubble') with a lack of gas throughout the rest of the gastrointestinal tract. Reproduced with permission from Choudhry MS et al.⁴⁴ *Pediatr Surg Int.* 2009 Aug;25(8):727-30. Publisher: Springer Nature

Management of duodenal atresia: Initial management includes nasogastric drainage, nil by mouth, and IV fluids. Surgical repair is with duodenoduodenostomy, where the proximal and

distal duodenal pouches are opened and joined, thereby bypassing the atretic segment⁴⁶. A duodenal membrane can be dealt with using a vertical duodenotomy, web resection, and transverse closure.

A trans-anastomotic tube can be inserted at the surgery to facilitate enteral feeds⁴⁷.

In a recent retrospective study of 59 infants with duodenal atresia, TAT tube was associated with a significant reduction in the duration of parenteral nutrition [6 (0-11) vs 12 (8-19) days, $p=0.006$], and a median cost saving of £622.26 per patient⁴⁷. Our recent meta-analysis concluded that evidence is limited regarding the efficacy and safety of intraoperative TAT placement in neonates with CDO and recommended the need for well-designed RCTs⁴⁸.

The annular pancreas occurs due to incomplete rotation of the ventral pancreatic bud. It results in a persistent partial or complete ring encircles the 2nd portion of the duodenum. This encircling pancreatic ring prevents subsequent growth of the underlying duodenum; thus, there is a fixed focal underlying duodenal stenosis or atresia that would not be relieved by simply removing the pancreatic ring^{43,49}. The clinical symptoms and treatment of the annular pancreas are similar to duodenal atresia, with duodeno-deodenostomy being the procedure of choice⁵⁰.

Short-term outcomes of duodenal atresia: With contemporary management, mortality rates are very low⁴¹. Death is usually due to the associated lethal anomalies such as cardiac defects. The complications are rare and include wound infection, dehiscence, anastomotic leaks, and strictures. Central line-related complications occur in nearly 20% of cases. Feeding intolerance, prolonged dependence on parenteral nutrition, and hospital stay for more than four weeks is common⁴¹.

Long term outcomes of duodenal atresia:

Vinycomb et al.⁵¹ assessed the quality of life of 38 children who had undergone neonatal surgery for congenital duodenal atresia. The median age of participants was 6.7y (range 2.7-

17.3y). Seven participants had trisomy 21. They found that children with duodenal atresia in the neonatal period have a QoL comparable to a healthy population. Children with trisomy 21 were more likely to have reduced QoL⁵¹.

Jejuno-Ileal atresia

Jejuno-ileal atresia (JIA) is the most common type of intestinal atresia and with an incidence of 1 in 5000 to 1 in 14,000 live births⁴⁵. Different types of JIA are described in the table 1 below and figure 17.

Table 1: Types of intestinal atresia: Louw's classification with Grosfeld's modification⁴⁵

Type of Atresia	Description
I	Internal membrane, serosa in continuity, no mesenteric defect
II	Serosal discontinuity, the cord between proximal and distal ends
IIIa	Serosal discontinuity with mesenteric defect
IIIb	Serosal discontinuity with mesenteric defect and Apple peel deformity
IV	Multiple atresias

(Reprinted from Adams et al⁴⁵., Early Hum Dev. 2014 Dec;90(12):921-5, with permission from Elsevier Publishers).

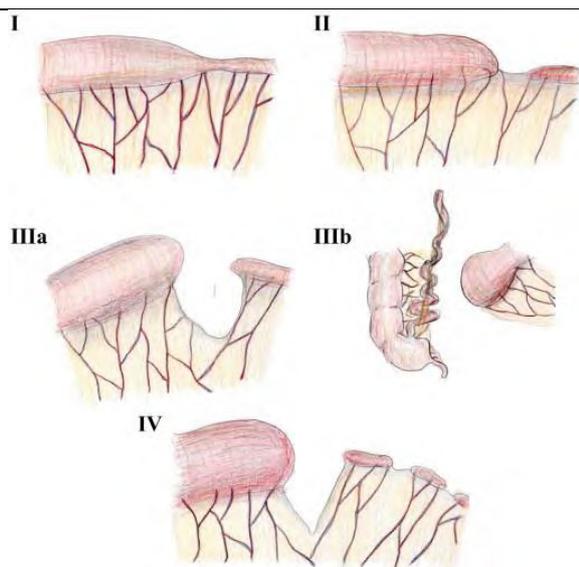


Figure 17: Classification of intestinal atresias (reprinted from Adams et al. ⁴⁵, Early Hum Dev. 2014 Dec;90(12):921-5, with permission from Elsevier Publishers).

Type IIIb and IV atresias are usually associated with a significantly short length of intestines. JIA can be associated with cystic fibrosis, colonic atresia, duodenal atresia, malrotation, and volvulus.

Antenatal diagnosis of JIA: The finding of dilated loops of bowel and polyhydramnios on antenatal ultrasound should raise the suspicion of JIA, but the predictive ability is low with detection rates of 0.51 (0.38, 0.63)⁵².

JIA's clinical features: Neonates with JIA present with bilious vomiting, abdominal distension, and delayed passage of meconium. Pale plugs of meconium are passed after rectal washouts with normal saline.

Investigations for JIA: Plain x-ray of the abdomen will reveal dilated intestinal loops with air-fluid levels and absence of gas in the distal bowel (Figure 18). Calcified meconium might be visible on x rays if antenatal bowel perforation. An upper GI contrast may be necessary to rule out malrotation. Contrast enema may be required to differentiate distal atresia from meconium ileus, Hirschsprung disease, or hypoplastic left colon. Upon contrast enema, atresia can be excluded if the contrast refluxes into dilated gas-filled bowel loops.



Figure 18: Plain X-ray of the abdomen demonstrating multiple dilated bowel loops in ileal atresia (reprinted from Adams et al.⁴⁵, *Early Hum Dev.* 2014 Dec;90(12):921-5, with permission from Elsevier Publishers).

Treatment of JIA: Management involves decompression of the stomach using the nasogastric tube, IV fluids, and surgical repair. The standard surgical procedure involves resection and end-to-end anastomosis. Some cases require the creation of jejunostomy or ileostomy, especially if there are multiple atresias. Parenteral nutrition is administered postoperatively, and enteral feeds are commenced in small volumes and increased as tolerated. While mortality rates are very low, prolonged feed intolerance, dependency on PN, and sepsis are common morbidities.^{53,54}

Apple-peel atresia (APA), or Type-IIIb intestinal atresia (Figure 17), is characterised by a massively dilated jejunum or ileum, absence of the distal superior mesenteric artery and the dorsal mesentery, and a small calibre distal small intestine coiled around its only blood supply⁵⁵. APA is associated with an increased risk of the short-gut syndrome, prolonged feed intolerance, and hospital stay⁵⁵.

The prognosis of JIA depends on the total length of the remaining intestine and the presence or absence of an intact ileocaecal valve. Infants with APA have worse outcomes compared to those without APA.^{56,57} If the length of the remaining intestines is less than 25-30 cm, the short gut syndrome will ensue, with attendant complications. However, recent studies have shown that survivors of APA have good long-term neurodevelopmental outcomes⁵⁸

Hirschsprung Disease

Hirschsprung disease is characterised by the absence of ganglion cells in the myenteric and submucosal plexuses of the distal intestine, which results in functional obstruction due to failure of peristalsis⁵⁹. Failure of the distal intestines to relax to allow the passage of stool leads to functional obstruction and secondary dilatation of the bowel proximal to the aganglionic segment⁶⁰. In the majority of the cases (80%), the aganglionosis is limited to the rectum and sigmoid colon (short segment), but it can extend to the proximal colon (long segment) or involve the entire large intestine (total colonic aganglionosis; TCA)) or even the

small intestine (total intestinal aganglionosis; TIA). Hirschsprung disease has an incidence of approximately 1 in 5000 live births.

Clinical Presentation: Symptoms begin in the neonatal period in more than 90% of cases, and in the majority, the diagnosis is made during the first three months of life, whereas <1% are diagnosed during adult life⁶⁰. Clinical features are delayed passage of meconium, abdominal distension, bilious vomiting, and feed intolerance⁵⁹. Delayed passage of meconium beyond the first 24 hours is a characteristic feature of HD and is present in about 90% of cases. In some patients, HD may present with caecal or appendiceal perforation⁶¹.

Investigations: Abdominal X-rays reveal dilated loops of bowel throughout the abdomen. A water-soluble contrast enema shows a transition zone between the dilated normal and collapsed aganglionic bowel (Figure 19).

Rectal biopsy is the gold standard to diagnose Hirschsprung disease.⁶² Pathognomonic findings in HD are the absence of ganglion cells and the presence of hypertrophic AChE-positive fibres within the submucosal and mucosal layers of rectal biopsies. The tissue for histopathological examination is obtained by rectal suction biopsy. The tissue must contain sufficient submucosa to enable the pathologist to confirm the diagnosis with confidence⁶⁰

The goal of surgery is to remove the aganglionic bowel and reconstruct the intestinal tract by connecting the normally innervated bowel just above the anus so that normal sphincter function is preserved⁵⁹. Serial intraoperative seromuscular frozen section biopsies are obtained and immediately examined to facilitate decisions regarding the level of the proximal resection. The resection is usually performed 2 to 3 cm proximal to the first biopsy showing normal ganglion density. At the distal-most normal point biopsy, a 'doughnut' of the bowel is carefully scrutinised before normal circumferential innervation (NI) is pronounced.

The commonly performed procedures are Duhamel or Transanal endorectal pull-through (TERPT; Suave or Swenson) (Figure 19). All three techniques can utilise laparoscopy to take

biopsies to identify the transition zone and mobilise the colon. In the Duhamel technique, a section of the aganglionic rectum is left connected to a segment of the ganglionic colon (side-to-side) as a pouch reservoir. In contrast, in the TERPT technique, a very low direct anastomosis is made just above the dentate line⁶³.

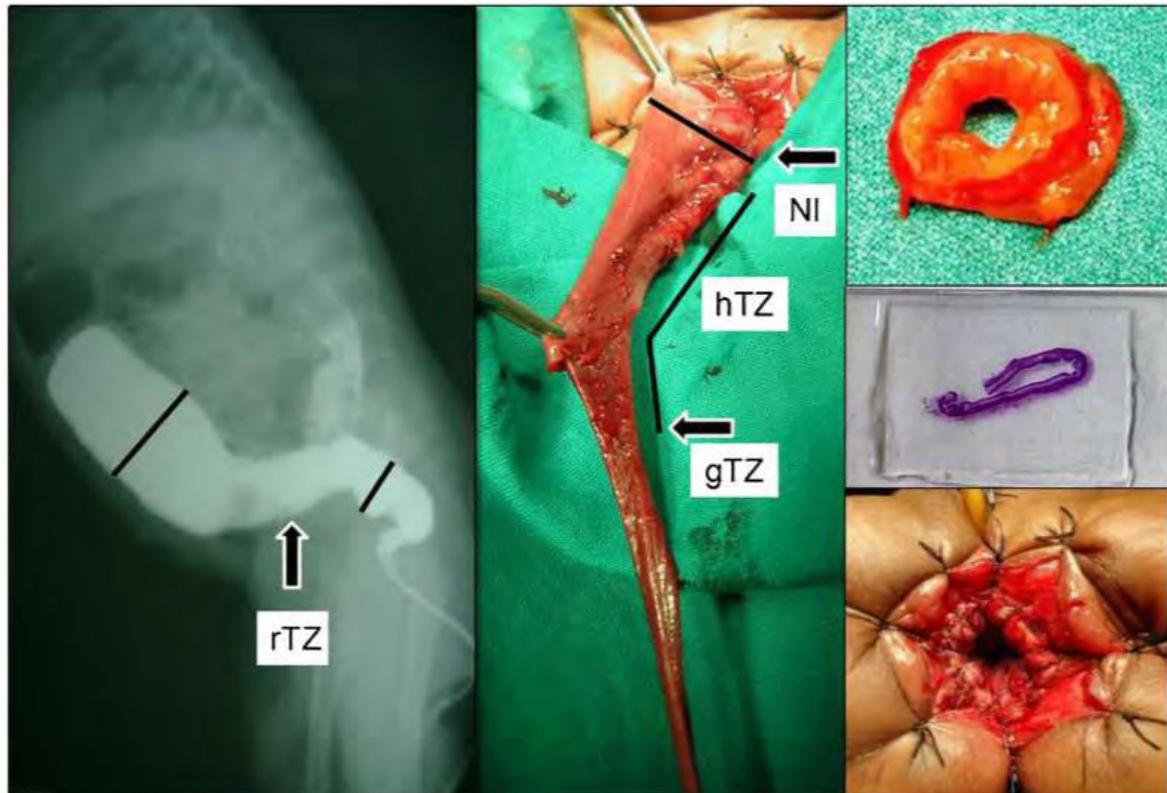


Figure 19: Single-stage transanal endorectal pull-through for rectosigmoid HD. The contrast enema (left panel) shows a characteristic radiological transition zone (rTZ) of rectosigmoid Hirschsprung disease confirmed at rectal biopsy. At transanal endorectal mobilisation (middle panel), note the corroborating gross transition zone (gTZ). The histological transition zone (hTZ) is mapped by serial seromuscular biopsies, and normal innervation (NI) of the entire circumference of the proximal 'doughnut' (right panel, top, and middle) is ensured. The aganglionic and transition zone bowel is excised before the final anastomosis of the ganglionated colon just above the dentate line (right panel, bottom) (Reproduced with permission from Das K et al. *Indian J Pediatr.* 2017 Aug;84(8):618-623⁶⁴. Publisher: Springer Nature).

Postoperative recovery is usually uneventful. The infant can generally be discharged home within a few days. Skin breakdown due to loose stools is a common problem in the

immediate postoperative period. It can be minimised by applying barrier cream to the perianal area. Subsequently, regular anal dilatations are done by parents or the surgeon using calibrated dilators to prevent the development of anal stenosis.

Hirschsprung-associated enterocolitis is the most important cause of morbidity and mortality in patients with HD⁶⁵. It can occur in the pre-operative or post-operative period or any time during a patient's life with HD.⁶⁶ The clinical features could be mild or severe and include abdominal distension and tenderness, fever, tachycardia, diarrhoea, or constipation. In extreme cases, the patient will have features of septic shock such as hypotension, obtundation, peritonitis, and perforation⁶⁶. Children with mild symptoms can be managed on an outpatient basis with rectal washes with saline and oral metronidazole. Severe cases should be admitted and managed with IV fluids, IV antibiotics, and rectal irrigations. Regular rectal washouts while waiting for surgery are thought to help prevent HAEC⁶⁵. Long term use of oral metronidazole has also been tried to prevent HAEC.⁶⁶

Many studies have shown that children with HD have altered gut microbiota, which may lead to HAEC^{67,68}. Hence probiotics have been tried to prevent the occurrence of HAEC. A recent systematic review that included 198 patients with HD from 5 studies found that HAEC incidence with/without probiotics was 22.6 and 30.5%, respectively, but the reduction was not statistically significant (OR 0.72; 95% CI 0.37-1.39; P = 0.33)⁶⁹.

Long-term outcomes of infants with Hirschsprung disease: Multiple studies have been published regarding the bowel outcomes and quality of life in children and adults treated for HD in the neonatal period.

In a prospective study of 15 children with HD at a mean age of 10.5 years, Mille et al. reported subtle decreased performances in some areas of intelligence⁷⁰. In another study of 53 children with HD, Hedbys et al. reported a 20% incidence of cognitive deficits.⁷¹ Sood et al. measured Quality of Life (QoL) outcomes in 58 adolescents with HD⁷². The overall QoL in

the study population was similar to healthy controls. However, faecal incontinence, constipation, and dysfunctional elimination were all negatively correlated with QoL scores. They concluded that in children with HD, ongoing bowel dysfunction negatively impacts their QoL⁷².

Onishi et al.⁷³ reported that the QoL was satisfactory overall, but incontinence and soiling occurred in 18.7%. Neuvonen et al. followed 146 children with HD into adulthood and reported that 42% had occasional soiling and 12% had frequent soiling. Constipation occurred in 9%, and recurrent postoperative enterocolitis occurred in 44% of patients⁷⁴.

The same group⁷⁵ evaluated the bowel function and QoL of 79 infants with HD into adulthood using a survey questionnaire and compared them to healthy controls. 75% of patients were socially continent (vs 98% of controls; $P < 0.001$). With age, soiling, faecal accidents, rectal sensation, and ability to withhold defecation improved to levels comparable to controls by adulthood. PedsQL domains in childhood were equal to controls. Adults exhibited lower emotional scores, and limitation of personal and sexual relationships⁷⁵.

Our group conducted a retrospective study of neonates with Hirschsprung disease in Western Australia⁷⁶. Fifty-four infants were identified (40 with short and 11 long segment and three total colonic aganglionosis); A primary pull-through procedure was performed in 97% and 21% of neonates with short- and non-short-segment Hirschsprung disease, respectively; HAEC occurred in 14 (26%) infants. Griffiths Mental Development Scale scores (1 year) were available in 31 of 45 non-syndromic survivors: mean general quotient (94.2, SD 8.89) was significantly lower than the population mean ($P = .007$). Physical growth appeared adequate in non-syndromic infants⁷⁶.

Taken together, the overall evidence suggests that Hirschsprung disease is associated with gut-related morbidities, suboptimal neurodevelopment, and decreased quality of life in a significant number of patients.

Congenital Diaphragmatic Hernia

The diaphragm development begins at four weeks gestation and is completed by 12 weeks. In congenital diaphragmatic hernia (CDH), one of the components of the diaphragm does not develop properly, leading to a defect through which abdominal viscera herniate into the thoracic cavity. It impedes the normal development of lungs, alveoli, terminal bronchioles, and pulmonary vasculature.⁷⁷ The CDH is classified based on the position of the defect as posterolateral (Bochdalek hernia), anterior (Morgagni hernia), and central defect, the commonest being posterolateral (75%). The CDH occurs on the left side in the majority (85%), less commonly on the right side (13%), and rarely bilaterally (2%)⁷⁷. CDH could be isolated or occur in association with various syndromes.

The diagnosis of CDH can be made antenatally if abdominal contents are noted in the thorax on fetal ultrasound. Prenatal diagnosis enables adequate counselling and planning. However, in nearly 40%, CDH is diagnosed only in the postnatal period. Clinical features of CDH are respiratory distress, scaphoid abdomen, absence of breath sounds on the ipsilateral side, and mediastinal shift to the opposite side. Postnatally, diagnosis can be confirmed with chest and abdominal x-ray, which will show the presence of intestines in the hemithorax (Figure 20).



Figure 20: Left side congenital diaphragmatic hernia.

Postnatal management involves endotracheal intubation immediately after birth and mechanical ventilation, a nasogastric tube to decompress the stomach, IV fluids, gentle ventilation, monitoring for pulmonary hypertension, inhaled nitric oxide if necessary, and repair of the defect once the infant is stable. In infants with severe PPHN, Extracorporeal Membrane Oxygenation (ECMO) could result in survival and improved outcomes.

Surgery can be performed either thoracoscopically (Figures 21-23) or open surgery. A minor diaphragmatic defect can be closed primarily, whereas a larger defect requires a prosthetic patch⁷⁸. Large defects are preferably repaired openly, using a Gore-Tex patch sewn directly to the diaphragm in an interrupted, horizontal mattress fashion.

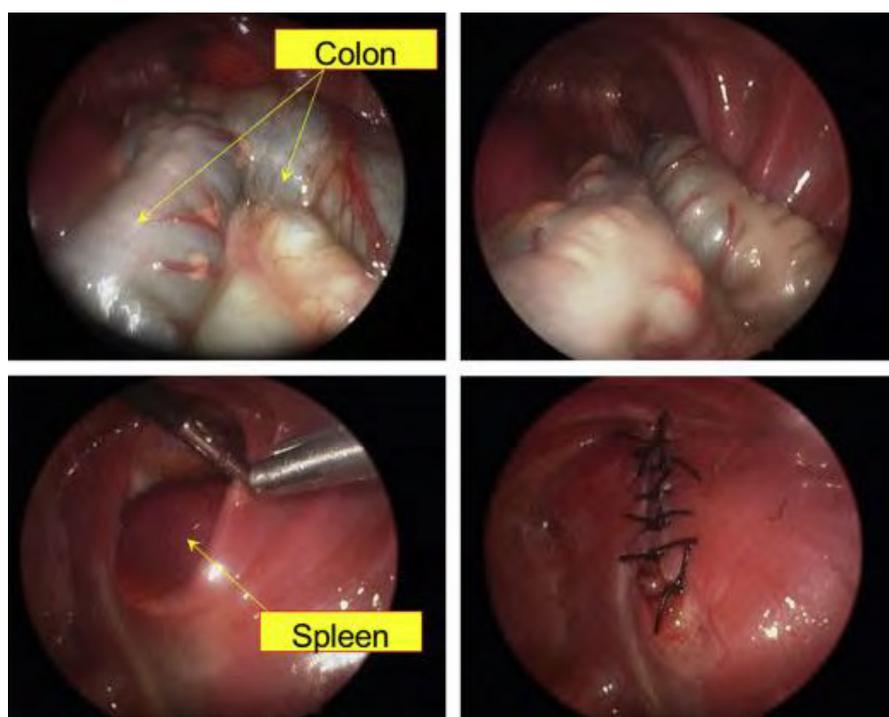


Figure 21: Reduction of viscera and primary closure of diaphragm. Reprinted with permission from Clifton MS et al.⁷⁸ *Clin Perinatol.* 2017 Dec;44(4):773-779. Publisher: Elsevier⁷⁸.

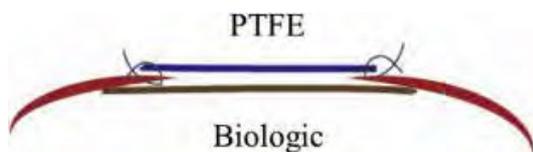


Figure 22: Polytetrafluoroethylene (PTFE) is used to bridge the gap with a larger biologic underlay. Reprinted with permission from Clifton MS et al. *Clin Perinatol.* 2017 Dec;44(4):773-779. Publisher: Elsevier⁷⁸.



Figure 23: Completed composite mesh repair of diaphragmatic hernia. Reprinted with permission from Clifton MS et al. *Clin Perinatol.* 2017 Dec;44(4):773-779. Publisher: Elsevier⁷⁸

Survival and long-term outcomes of congenital diaphragmatic hernia:

While survival rates for CDH have increased in recent decades, mortality rates are still very high. A recent observational study of 975 CDH infants (2004 to 2013) from four centres in the UK and Europe found mortality rates of 28.1%⁷⁹. In a cohort from California, among 577 infants with CDH, 31% died during infancy⁸⁰. In another study from England (2003-2016), of the 2336 live-born infants with CDH, 26% died without undergoing surgery. Of 1491 that underwent surgery, mortality was 8.4%. In total, the mortality rate among live-born babies with CDH was 31.2%⁸¹. Lee et al. from Western Australia have reported a mortality rate of 30% among liveborn infants with isolated CDH without other anomalies and 56% in those with associated anomalies. They also noted that in the recent epoch (2011-2016), mortality rates were as low as 19%.⁸²

The common long-term problems are gastroesophageal reflux, hernia recurrence, chest infections, and mild restrictions to exercise tolerance. Some infants develop chronic pulmonary hypertension with poor outcomes. Survivors of CDH may have significant long-term medical and psychosocial issues⁸³. Patients with CDH are also known to have a lower health-related quality of life in adolescence and adulthood⁸⁴.

The reported incidence of adverse neurodevelopmental outcomes is variable. Church et al. reported that on Peabody Developmental Motor Scales, the mean [95% CI] gross motor quotient was 87 [84-91], fine motor quotient 92 [88-96], and total motor quotient 88 [84-93], representing below average, average, and below-average functioning, respectively⁸⁵.

In another study, Antiel et al. reported that 51% of infants scored 1 SD below the population mean in at least one domain, and 13% scored 2 SD below the population mean on Bayley Scales of Infant Development (BSID-II) scales⁸⁶. Our study found that 14.7% of infants with CDH had suboptimal neurodevelopmental outcomes at one year of age⁸⁷.

Some studies have shown that right side CDH may carry higher morbidity and mortality than left-side CDH^{88,89}. In a retrospective study, our group compared the outcomes of right-sided versus left-sided CDH. Forty-nine cases of CDH were operated during the ten years (2002-2012). Of these, ten cases were R-CDH and 39 L-CDH. R-CDH required patch repair more commonly than L-CDH because of larger defect size. Postoperative mortality was similar between the two groups (1/10 right versus 5/39 left CDH). Hernia recurrence was higher in R-CDH (5/10 vs 6/39; $p=0.03$). At one year, the median GQ on Griffiths assessment was slightly higher at 98 for L-CDH (IQR 86 to 104.25) and 91 for R-CDH (IQR 76.5 to 93)⁹⁰.

Anorectal Malformations

The incidence of anorectal malformations (ARMs) is approximately 1 in 2,000 to 1 in 5000 live births⁹¹. Various classifications have been used, the most common being the Pena system, which is described below:

Males	Females
Recto-Perineal Fistula	Recto-Perineal Fistula
Recto-Bulbar Urethral Fistula	Recto-Vestibular Fistula
Recto-Prostatic Urethral Fistula	Persistent Cloaca with a short common channel (<3 cm)
Recto-Vesical Fistula	Persistent Cloaca with a long common channel (>3 cm)
Imperforate Anus without Fistula	Imperforate Anus without Fistula
<i>Complex and unusual defects:</i>	<i>Complex and unusual defects</i>
Rectal atresia	Rectal atresia
Imperforate anus with presacral mass	Posterior cloaca; Imperforate anus with pre-sacral mass

A careful inspection of the perineal area will provide clues to the type of malformation (Figures 24-26). In the recto-perineal fistula, meconium will be seen exiting the perineal skin.

The presence of the perineal fistula usually suggests a low-level imperforate anus.

If there is meconium in the urine, it is due to the recto-urethral fistula. In female infants with recto-vestibular fistula or cloaca (Figure 24), meconium will be seen exiting the vaginal vault.



Figure 24: Imperforate anus with persistent cloaca in a female infant. Reproduced from Levitt et al⁹². *Orphanet J Rare Dis.* 2007 license Jul; 26; 2:33 (Permission was not required since it was an open-access article distributed under the terms of the Creative Commons CC BY license).



Figure 25: Recto-bladder neck fistula in a male infant. Reproduced from Levitt et al⁹², *Orphanet J Rare Dis.* 2007 Jul; 26;2:33 (Permission was not required since it was an open-access article distributed under the terms of the Creative Commons CC BY license).

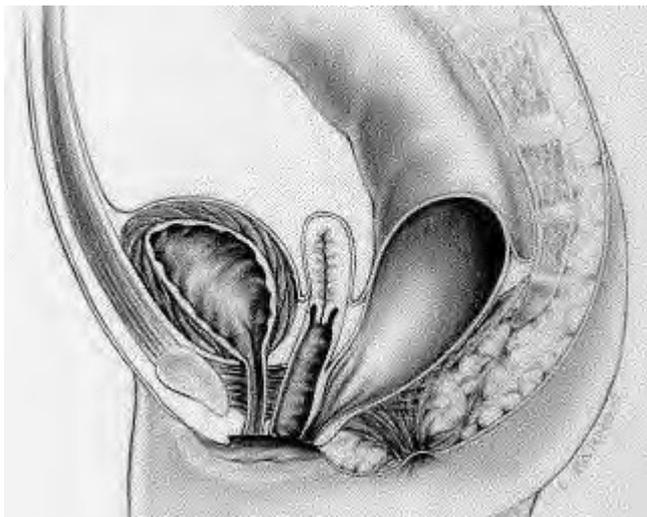


Figure 26: Rectovestibular fistula in a female infant. Reproduced with permission from Levitt et al.⁹². *Orphanet J Rare Dis.* 2007, 2: 33-10.1186/1750-1172-2-33 (Permission was not required since it was an open-access article distributed under the terms of the Creative Commons CC BY license).

Investigations for anorectal malformations: In infants with anorectal malformations, a cross-table lateral radiograph with the patient in the prone position is taken when the infant is more than 24 hours of life. It will demonstrate gas in the rectum and its position in relation to the coccyx. If the rectal gas extends distal to the coccyx, it can be considered as a low-level

imperforate anus. On the other hand, if it stops at a higher level, it is regarded as a high-level anomaly.

Other investigations in infants with anorectal anomalies: Nearly 45% of infants with ARM have associated anomalies of one or more of the following systems: Vertebral, Cardiac, Tracheoesophageal, Renal and genitourinary and Limb (VACTERL), CNS or chromosomes⁹³ Hence infants with ARMs should undergo x-ray, ultrasound, and MRI of the spine, echocardiogram, renal ultrasound, x rays of the limbs and x-ray of the abdomen with a nasogastric tube in situ.

Management of anorectal anomalies: If the cross-table lateral film shows air in the rectum to be located below the coccyx, a primary posterior sagittal ano-rectoplasty (PSARP) can be undertaken in the neonatal period. If the gas in the rectum does not extend beyond the coccyx, a sigmoid colostomy is performed⁹¹, and 2-3 months later, a PSARP is performed. The ligation of fistulae is undertaken during definitive repair with PSARP. Cloacal malformations need advanced surgical techniques to fashion the vagina, urethra, and rectal openings as separate orifices.

Anal dilatations are commenced a few days after the definitive repair to avoid strictures. Dilations are performed twice daily by the parents at home, and the size of the dilator is increased weekly until the rectum reaches the desired size. Once the rectum reaches the desired size, the colostomy is closed.

Long-term outcomes of anorectal anomalies: Satisfactory continence outcomes are usually achieved in mild ARMs.⁹⁴ Even for ARMs with moderate to high complexity, PSARP results in better outcomes than older techniques. The advantage of PSARP is the repair of the malformation under direct vision and precise repositioning of the bowel within the external sphincter funnel that was not possible with older approaches⁹⁴. Other factors that affect the achievement of adequate bowel control are the presence of spinal abnormalities and sacral

development. High-level ARMs can be associated with spinal anomalies such as tethered cord in 25% of cases⁹⁵. This could affect the control of sphincter function and continence. Hence follow-up with urologists, spinal rehabilitation, and neurosurgeons are crucial in such cases.

Cholestasis in neonates with congenital gastrointestinal surgical conditions: Given that neonates with CGISC have varied periods of feed intolerance, parenteral nutrition (PN) is routinely administered to them. Whilst it improves physical growth, PN-associated cholestasis (PNAC) is a severe complication of prolonged administration. The incidence is very high among infants with short gut syndrome and intestinal failure⁹⁶. The risk factors for parenteral nutrition-associated cholestasis (PNAC) are more prolonged duration of PN, prematurity, sepsis, lack of trophic enteral nutrition, and bacterial overgrowth⁹⁶⁻¹⁰¹. Some of the preventative strategies are early commencement and advancement of enteral feeds, and use of fish oil-based lipid emulsions.^{102,103}

Neurodevelopmental outcomes of neonates with congenital gastrointestinal surgical conditions: A recent systematic review¹⁰⁴ that included 895 children from 23 studies with conditions such as diaphragmatic hernia, gastroschisis, exomphalos, and oesophageal atresia found delayed cognitive development in 23% (3%-56%) and delayed motor development in 25% (0%-77%). It also found those children had a cognitive score 0.5 SD below the population means (Mental Development Index 92 ± 13 , $P < 0.001$) and motor score 0.6 SD below average (Psychomotor Development Index 91 ± 14 ; $P < .001$).

Overall, neonates with CGISC suffer from significant morbidities such as prolonged feed intolerance, healthcare-associated infections, gastroesophageal reflux, strictures and stenoses, PNAC, slow weight gain, and prolonged hospital stay. A significant proportion of them is at risk of long-term adverse outcomes such as cerebral palsy, learning disabilities, behavioural problems, and decreased quality of life. While some morbidities are directly related to the

underlying surgical condition and hence not preventable, every effort should be made to minimise the potentially preventable morbidities such as infection, suboptimal nutrition, and PNAC. Established strategies for minimising such morbidities include early commencement of enteral feeds¹⁷, standardisation of feeding advancement¹⁰⁵, strict hand hygiene, and aseptic precautions¹⁰⁶⁻¹⁰⁸. Unfortunately, despite such best practices, morbidities continue to impose a significant health burden on this cohort. Hence, additional strategies are required to improve their outcomes.

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Preface to Chapter 2

Physical Growth of Neonates with Congenital Gastrointestinal Surgical Conditions

The previous chapter emphasized that neonates with congenital gastrointestinal surgical conditions (CGISCs) suffer from feed intolerance, sepsis, and other morbidities. Such morbidities can put them at risk of suboptimal growth in the immediate postnatal period. Hence, we conducted a retrospective study to evaluate the physical growth of neonates with CGSIC during their stay in our neonatal intensive care unit (NICU). This chapter presents the results of that retrospective study.

Four hundred and four neonates with CGSICs were included, of which 13 died. Study infants' median gestation and birth weight were 38 weeks and 3015 grams, respectively. At discharge, the weight-for-age, and head circumference-for-age z scores were significantly lower than at birth. Overall, 222/ 386 (57.5%) had postnatal growth restriction (PNGR) at discharge.

On multivariable logistic regression analyses, higher gestational age, small for gestational age at birth, female gender and longer durations of parenteral nutrition were associated with lower odds of PNGR, whereas the prolonged duration of hospital stay was associated with higher odds.

We concluded that neonates with CGISCs develop PNGR during their stay in the NICU. The importance of inventing strategies to improve these infants' nutrition and physical growth is highlighted.

The results of this study have been submitted to a peer-reviewed journal.

CHAPTER 2

Physical Growth of Neonates with Congenital Gastrointestinal Surgical Conditions during hospitalization: A Retrospective Study.

Objectives: Neonates with congenital gastrointestinal surgical conditions (CGISCs) suffer from various morbidities that can put them at risk of postnatal growth restriction (PNGR). We evaluated the physical growth of neonates with CGISCs during their stay in the neonatal intensive care unit of our hospital. **Study design:** Retrospective study of neonates with CGISCs born at $>34^{0/7}$ weeks between January 2005 and December 2014. The primary outcome was *PNGR for weight or head circumference (HC)* at discharge, defined as a z score decrease >0.8 from birth. **Results:** A total of 404 infants with CGISCs were included, of which 13 died.

The median gestation was 38.0 weeks (IQR: 36.0 to 39.0) and the median birth weight was 3015 grams (IQR: 2595, 3407). Compared to values at birth, there were statistically significant decrements in mean weight-for-age z scores [(-0.19 (SD 1.02) vs -1.03 (SD 1.09); $p=0.000$] and HC-for-age z scores [0.08 (SD 1.04) vs -0.31 (SD 1.10); $p=0.000$] at discharge. Overall, 222/386 (57.5%) had *PNGR for weight or HC* at discharge. On multivariable logistic regression analysis, higher gestational age (OR: 0.78; 95% CI: 0.68 to 0.91), small for gestational age at birth (OR: 0.45; 95% CI: 0.21 to 0.99), and longer durations of parenteral nutrition (OR 0.90; 95% CI: 0.85, 0.96) were associated with lower odds of PNGR, whereas prolonged duration of hospital stay (OR 1.12; 95% CI: 1.07 to 1.19) was associated with higher odds.

Conclusions: Neonates with CGISCs develop significant PNGR during hospitalization. Strategies to optimize their nutrition are needed.

Introduction: Neonates with congenital gastrointestinal surgical conditions (CGISCs) often have gastrointestinal dysmotility, recurrent surgeries, postoperative ileus, infections, and other morbidities. The risk of suboptimal growth in the immediate postnatal period is thus high in this population of infants. A recent study reported that infants with gastroschisis suffer from postnatal growth restriction (PNGR), with nearly 30% experiencing a >1.0 decline in weight for age z scores between birth and discharge¹. Another study reported that 55% of infants with gastroschisis developed weight or length growth failure (decrease in z scores >0.8 from birth) at discharge.² One could hypothesise that similar PNGR could also affect infants with other CGISCs such as oesophageal atresia, malrotation, duodenal atresia, small and large intestinal atresia, and Hirschsprung disease. Considering the emerging data in the field²⁻⁵, we studied the physical growth of 404 neonates with major CGISCs in our neonatal intensive care unit (NICU). We also aimed to identify risk factors for PNGR in these infants.

Study design: It was a retrospective cohort study of all infants born at $>34^{0/7}$ weeks' gestation between 1 January 2005 and 31 December 2014 with CGISCs who underwent neonatal surgery at Perth Children's Hospital (PCH), the only regional tertiary paediatric hospital in Western Australia. All neonates in the state who require surgery are admitted to the neonatal intensive care unit (NICU) of PCH. Our previous paper reported on the one-year neurodevelopmental outcomes of neonates with CGISCs from the same cohort of patients⁶. The objective of the current paper was to report on their physical growth during their stay in the NICU. We hypothesised that these infants develop PNGR during their NICU stay.

Methods: It was a retrospective study based on a chart review of neonates with CGISCs admitted to the NICU of PCH.

The study infants were identified by interrogating the neonatal database of the department. Infants with known chromosomal anomalies and syndromes were excluded. Clinical characteristics of the study infants were abstracted from their medical records by one author and verified for accuracy by the second author. Baseline characters, including weight and head circumference (HC) at birth and discharge, were recorded and their corresponding z scores calculated. All eligible infants meeting the pre-specified criteria were included to minimise bias in selection.

The primary outcome of interest for this study was the prevalence of *PNGR for weight or HC*, defined as a z score decrease of >0.8 from birth⁷. The z scores for weight and HC at birth were calculated using the Fenton growth charts⁸ and at discharge using the WHO charts⁹ through the publicly accessible PediTools website of clinical calculators¹⁰.

Other outcomes were a. Prevalence of different grades of PNGR, which were defined as follows: No PNGR: (z score decrease of <0.8); Any PNGR: z score decrease of ≥ 0.8); Mild PNGR: z score decrease of 0.8 to 1.19); Moderate PNGR: z score decrease of 1.2 to 1.99); Severe PNGR: discharge z score decrease of ≥ 2 .

b) comparison of z scores for weight at discharge vs birth c) comparison of z scores for HC at discharge vs birth. d. Prevalence of PNGR for weight, e. Prevalence of PNGR for HC and f. Prevalence of PNGR for weight and HC. We were also interested in identifying any risk factors for PNGR in this cohort of infants.

Nutrition practice: The standard practice of our unit for these infants is to commence intravenous 10% glucose upon admission and adjust the infusion rate based on blood glucose and other parameters to achieve an adequate fluid and electrolyte balance and glucose homeostasis. Parenteral nutrition with amino acids, intravenous lipids, and micronutrients is usually started after 24-48 hours of admission. Enteral nutrition is commenced as soon as possible in the post-operative period and gradually increased as tolerated by the infant.

Freshly expressed breastmilk is preferred for feeding. When the quantity of mother's milk is inadequate, standard formula milk is used. Use of hydrolysed or semi hydrolysed formula milk is restricted to cases with significant feeding intolerance or excessive fluid losses through the enterostomy. In conditions such as Hirschsprung disease, feeds are given even in the preoperative period if the infant is stable and can tolerate feeds.

Statistical analysis: Statistical analysis was done using the STATA 16.0 software (Stata Corp. 2019 Stata Statistical Software: Release 16. College Station, TX; Stata Corp. LP). The summary statistics for normally distributed continuous variables were expressed as mean and standard deviations; those with skewed distribution were expressed as the median and interquartile range (IQR). Categorical variables were expressed as frequency and percentage. Paired sample t-tests were conducted to compare the z scores at birth vs z scores at the time of discharge for weight and HC. To identify risk factors for PNGR, univariable and multivariable logistic regression analyses were carried out to derive unadjusted and adjusted odds ratios and 95% CIs. A two-tailed p-value of <0.05 was considered statistically significant for all results.

Reporting: The results of this study were reported using the STROBE guidelines (Strengthening the Reporting of Observational Studies in Epidemiology guidelines)¹¹.

The institutional ethics committee approved this retrospective study as a quality activity (10446).

Results: 404 infants with various CGISCs were included in the study, of which 13 died. The predominant conditions were gastroschisis, oesophageal atresia, Hirschsprung disease, congenital diaphragmatic hernia, malrotation, and anorectal anomalies (Table 1).

Table 1: Major congenital gastrointestinal surgical conditions in neonates

Surgical conditions	N (%)
Gastroschisis	93 (23%)
Malrotation	48 (11.9%)
Oesophageal atresia with or without tracheoesophageal fistula	44 (10.9%)
Hirschsprung disease	44 (10.9%)
Congenital diaphragmatic hernia	42 (10.4%)
Anorectal anomalies	39 (9.7%)
Congenital duodenal obstruction	19 (4.7%)
Intrauterine gut perforations	9 (2.2%)
Jejunioileal atresia	16 (4%)
Exomphalos	14 (3.5%)
Meconium ileus	12 (3%)
Multiple gut anomalies	10 (2.5%)
Large bowel atresia	5 (1.2%)
Short bowel syndrome due to congenital gut anomalies	5 (1.2%)
Benign cysts and tumors	3 (0.7%)
Biliary atresia	1 (0.3%)
Total	404 (100%)

The median gestation was 38.0 weeks (IQR: 36.0 to 39.0) and the median birth weight was 3015 grams (IQR: 2595 to 3407). The clinical characteristics of study infants are presented in table 2.

Table 2: Characteristics of study infants

Variable	Median or N (%)	IQR	Range	N
Gestational Age (weeks)	38	36, 39	34, 42	404
Birth Weight (grams)	3015	2595, 3407	1664, 5060	404

Gestational diabetes	34 (8.4%)			
APH	14 (3.5%)			
PIH	19 (4.7%)			
Caesarean	159 (39.4%)			
Cord PH	7.31	7.25, 7.37	6.99, 7.54	137
SGA	50 (12.4%)	-	-	404
Male: Female	58%: 42%	-	-	404
Birth length (cm)	49	47, 51	40, 58	394
Birth HC (cm)	34	33, 35	29, 47	400
Apgar at 5 minutes	9	9,9	4, 10	400
Surgery under GA	1	1,2	1,5	404
Age at first surgery (days)	2	1, 5	0, 25	402
Day after admission when surgery done	1	0,3	0, 16	402
day after admission when TPN commenced	1	1,2	0, 16	333
Duration of TPN	10	6, 17	1, 128	330
Length of stay (days)	17	11, 25	1, 153	404
PCA at discharge (weeks)	40.7	39.4, 42.3	34.6, 59.9	404
HABSI	27 (6.7%)	-	-	404
HAI	52 (12.9%)	-	-	403
Cumulative days of antibiotics	5	4, 8	1, 56	397
Death before discharge	13 (3.2%)	-	-	404

Abbreviations: SGA: Small for gestational age (i.e., birth weight less than 10 centiles); HC: Head circumference; HAI: Healthcare-Associated Infections HABS: Healthcare-Associated Bloodstream Infections; IQR: Inter-quartile range; GA: General Anaesthesia; PCA: Post-conception age.

For the entire cohort, compared to values at birth, there was a statistically significant decrement in the mean weight-for-age z scores at discharge [(-0.19 (SD 1.02) vs -1.03 (SD 1.09); p=0.000]. There was also a significant decrement in the mean HC-for-age z scores at discharge [0.08 (SD 1.04) at birth vs -0.31 (SD 1.10) at discharge; p=0.000]. This significant drop in z scores was observed among almost all the subgroups of CGISCs. Table 3 compares z scores for birth weight versus discharge weight z scores and z scores for birth HC versus discharge HC.

Table 3: Comparison of z scores at birth vs at discharge

Surgical conditions	Number of infants	Birth weight z scores Mean (SD)	Discharge weight z score Mean (SD)	P-value	Birth HC z scores Mean (SD)	Discharge HC z score Means (SD)	Number of infants	P-value
All infants	390	-0.19 (1.02)	-1.03 (1.09)	0.000	0.08 (1.04)	-0.31 (1.10)	381	0.000
Gastroschisis	89	-0.46 (1.01)	-1.33 (1.08)	0.000	-0.35 (1.01)	-0.52 (1.24)	85	0.255
Malrotation	46	0.02 (1.04)	-0.69 (1.13)	0.000	0.26 (1.02)	-0.07(1.12)	43	0.027
Oesophageal atresia	41	-0.84 (0.83)	-1.41 (0.90)	0.000	-0.07 (0.98)	-0.50 (0.81)	41	0.002
Hirschsprung disease	43	0.13 (0.66)	-0.74 (0.72)	0.000	0.17 (0.90)	-0.16 (1.11)	44	0.013
CDH	41	-0.15 (1.04)	-1.12 (1.20)	0.000	0.36 (1.03)	-0.31 (1.12)	39	0.0001
Anorectal anomalies	39	-0.33 (1.14)	-0.97 (1.16)	0.000	-0.10 (1.00)	-0.34 (1.05)	39	0.043
CDO	19	0.26 (0.73)	-0.43 (0.80)	0.000	0.32 (1.03)	-0.05 (1.14)	19	0.047
Intrauterine gut perforations	9	0.74 (1.39)	-0.50 (1.58)	0.009	0.63 (1.16)	-0.09 (0.77)	9	0.005
JIA	16	0.04 (0.91)	-0.73 (0.94)	0.005	0.59 (0.97)	-0.19 (1.18)	15	0.076
Exomphalos	14	-0.43 (1.04)	-0.99 (1.04)	0.001	0.08 (0.87)	-0.17(1.00)	14	0.155

Meconium ileus	12	0.61 (0.59)	-0.65 (0.65)	0.000	0.55 (1.08)	0.14 (1.00)	12	0.245
Multiple gut anomalies	9	-0.16 (0.85)	-1.71 (1.43)	0.000	0.16 (0.72)	-0.90 (0.76)	8	0.009
Large bowel atresia	5	-0.61 (0.57)	-1.48 (0.79)	0.008	-0.48 (1.69)	-0.48 (1.09)	5	0.992
Short bowel Syndrome	3	0.12 (0.12)	-2.21 (1.96)	0.184	0.86 (1.21)	-1.20 (0.71)	4	0.013
Abdominal cysts	3	1.47 (1.10)	-0.32 (0.53)	0.144	0.87 (1.25)	0.34 (0.88)	3	0.274
Biliary atresia	1	0.31	-0.77	NA	0.51	0.77	1	NA

Abbreviations: CDH: Congenital Diaphragmatic Hernia; CDO: Congenital duodenal obstruction; JIA: Jejunioileal atresia; SD: Standard deviation; P values are based on the paired sample t-test

Primary outcome: Overall, 222/386 (57.5%) had the primary outcome of interest (PNGR for weight or HC), 185/390 (47.4%) had PNGR for wight, 106/381 (27.8%) had PNGR for HC, and 69/377 (18.3%) had PNGR for weight and HC. Table 4 provides data on the prevalence of various grades of PNGR in study infants.

Table 4: Grades of PNGR in study infants

Growth parameter	No PNGR (Birth z score minus discharge z score<0.8)	Any PNGR (Birth z score minus discharge z score≥0.8)	Mild PNGR (Birth z score minus discharge z score=0.8 to 1.19)	Moderate PNGR (Birth z minus score-discharge z score=1.2 to 1.99)	Severe PNGR (Birth z score minus discharge z score>2)
Weight or HC	164 (42.5%)	222 (57.5%) Primary outcome	101(26.1%)	91 (23.6%)	30(7.8%)
Weight	205 (52.6%)	185 (47.4%)	98 (25.1%)	67 (17.2%)	20 (5.1%)

HC	275 (72.1%)	106 (27.8%)	47 (12.3%)	42(11.0%)	17 (4.5%)
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The prevalence of primary outcome (PNGR for weight or HC) in individual surgical conditions ranged from 37 to 100% (Table 5).

Table 5: Prevalence of PNGR for weight or HC in individual CGISCs

Surgical conditions	N	%
All infants	222/386	57.5
Gastroschisis	53/88	60.2
Malrotation	21/44	47.7
Oesophageal atresia	17/41	41.5
Hirschsprung disease	27/43	62.8
CDH	25/40	62.5
Anorectal anomalies	16/39	41
CDO	11/19	57.9
Intrauterine gut perforations	8/9	88.9
JIA	10/15	66.7
Exomphalos	6/14	42.9
Meconium ileus	9/12	75
Multiple gut anomalies	8/9	88.9
Large bowel atresia	4/5	80
Short bowel Syndrome	4/4	100
Abdominal cysts	2/3	66.7
Biliary atresia	1/1	100

Abbreviations: CDH: Congenital diaphragmatic hernia; JIA: Jejunoileal atresia; CDO:

Congenital duodenal obstruction; PNGR: Postnatal growth restriction

Association between neonatal risk factors and *PNGR for weight or HC*

On univariable logistic regression analysis, higher gestational age, SGA were associated with lower odds of PNGR, whereas delay in commencing PN, longer duration of PN and prolonged hospital stay were associated with higher odds.

On multivariable logistic regression analysis, higher gestational age, SGA, and longer duration of PN were associated with lower odds of PNGR. In contrast, prolonged duration of hospital stay was associated with higher odds of PNGR. Sepsis was not associated with PNGR (Table 6).

Table 6: Factors associated with *PNGR for weight or HC*:

Variable	Unadjusted OR (95% CI)	P-value	Adjusted OR (95% CI)	p-value
Gestational age	0.78 (0.69, 0.87)	0.000	0.78 (0.68 to 0.91)	0.002
Female gender	0.94 (0.63, 1.42)	0.783	0.61 (0.36 to 1.01)	0.054
SGA at birth	0.52 (0.28, 0.98)	0.043	0.45 (0.21 to 0.99)	0.047
Time to commence PN after admission	1.16 (1.02, 1.33)	0.025	1.09 (0.94, 1.27)	0.238
Duration of PN	1.02 (1.01, 1.05)	0.011	0.90 (0.85, 0.96)	0.001
HAI	1.82 (0.89 to 3.69)	0.099	1.12 (0.48, 2.61)	0.789
LOS	1.05 (1.03, 1.07)	0.000	1.12 (1.06 to 1.19)	0.000

Abbreviations: SGA: Small for gestational age; PN: Parenteral nutrition HAI: Healthcare-associated infections; LOS: Length of hospital stay.

Sensitivity analysis:

There is a suggestion that the definition of PNGR cannot be applied for infants in the first two weeks of life, given the physiological loss of weight during this period⁷. Hence, we conducted a sensitivity analysis by excluding 129 infants discharged ≤ 14 days of life. The results showed an even higher prevalence of *PNGR for weight or HC* [175/259 (67.6%)].

Regression analyses results were similar to the primary analysis in that higher gestational age, female gender, SGA and longer duration of PN were associated with lower odds of PNGR and prolonged duration of hospital stay was associated with higher odds of PNGR.

(Table 7). **Table 7: Factors associated with *PNGR for weight or HC* after excluding infants who were discharged within the first two weeks of life**

Variable	Unadjusted OR (95% CI)	p-value	Adjusted OR (95% CI)	p-value
Gestational age	0.86 (0.74, 0.99)	0.043	0.81 (0.68, 0.98)	0.028
Female gender	0.73 (0.43, 1.23)	0.237	0.54 (0.30, 0.98)	0.044
SGA at birth	0.43 (0.20, 0.90)	0.026	0.28 (0.11, 0.70)	0.006
Time to commence PN after admission	1.15 (0.99, 1.34)	0.058	1.09 (0.93, 1.28)	0.272
Duration of PN	1.01 (0.99, 1.03)	0.243	0.91 (0.86, 0.96)	0.002
HAI	1.58 (0.71, 3.54)	0.258	1.61 (0.63, 4.16)	0.320

LOS	1.03 (1.01, 1.05)	0.004	1.10 (1.04, 1.17)	0.000
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Abbreviations: SGA: Small for gestational age; HAI: Healthcare-associated infections; LOS: Length of hospital stay.

Discussion: This retrospective study of 404 neonates with CGISCs found that nearly 58% had PNGR for weight or HC at discharge. The findings were consistent across almost all major surgical groups. Our results are similar to recent studies that reported that neonates with CGISCs suffer from PNGR during their initial hospital stay.^{1,3,12-15}

Similar to reports by Strobel et al² and Hong et al,¹ we found that higher gestational age was associated with lower odds of PNGR. Contrary to our expectation, but similar to Strobel et al³, infants who were SGA at birth had lower odds of developing PNGR. Future studies should explore this area further.

Hong et al¹ reported that infections were associated with PNGR in infants with gastroschisis, whereas our study did not, even when we restricted the analysis to gastroschisis (data not shown). It could be because the blood culture positive sepsis rates were lower in our cohort (6.6% vs 23%). There is some evidence that inflammation can result in slow growth velocities^{16,17}, and hence every effort should be made to reduce infection and inflammation in these infants.

Parenteral nutrition is essential to achieve adequate growth but can be associated with increased risk of sepsis and cholestasis. Hence, in many situations, clinicians tend to cease PN early to minimise those risks accept relatively slower weight gain. It probably explains the finding that longer duration of PN was associated with lower odds of PNGR in our study.

Observational studies in preterm infants without surgical conditions have shown that PNGR is associated with poor neurodevelopmental outcomes¹⁸⁻²⁰. Hence aggressive parenteral and enteral nutrition has been encouraged in them²¹⁻²⁴. In a recent cohort study in preterm infants

(<30 weeks gestation), Roze et al. performed a propensity score-matched analysis comparing 396 infants who had high amino acid intake (3.51-4.50 g/kg/d) at seven days after birth with 379 infants who did not. They found that high amino acid intake at seven days after birth was associated with an increased likelihood of having higher cognitive scores at five years.

In our study, delayed commencement of parenteral nutrition was associated with increased odds of PNGR on univariable analysis but not on multivariable.

However, some recent RCTs have reported that preterm infants who received early-high doses of parenteral amino acids had smaller HC and higher blood urea levels²⁵⁻²⁷. A Cochrane review that compared higher versus lower amino acid intake in parenteral nutrition for preterm infants found that higher intake reduces the incidence of PNGR, but the evidence was insufficient to show an effect on neurodevelopment²⁸. Furthermore, higher amino acid intake was associated with potentially adverse biochemical effects resulting from an excess amino acid load, including azotaemia²⁸. The results of the recently published RCT in 1440 critically ill children (0-17 years) show that early initiation of parenteral nutrition was associated with increased risk of hospital-acquired infections and delay in discharge from the intensive care unit (ICU)²⁹. Subgroup analysis of the 209 neonates showed that when compared early commencement of parenteral nutrition (i.e., within 24 hours of admission to the ICU), late commencement (i.e., after seven days of admission) was associated with earlier discharge from the ICU but increased the risk of hypoglycaemia³⁰. Another potential side effect of aggressive parenteral nutrition is the risk of refeeding syndrome and the associated morbidities³¹⁻³⁴.

An underlying assumption in our study and similar studies is that infants should regain their birth percentile by discharge even after the postnatal weight loss and surgical stress. This assumption needs to be tested in well-designed clinical trials and large prospective observational studies. In addition, one should not forget that underlying genetic potential could also play an essential role in the postnatal growth of such infants.

Overall, an essential question that the clinicians and researchers should answer is what nutrition support is needed by neonates undergoing major surgeries. One of the multimodal components of the ERAS guidelines (Enhanced Recovery After Surgery)³⁵ in adults is the intake of protein and energy-rich supplements in the postoperative period. Many observational studies have reported improved outcomes in adults undergoing surgery after implementing the ERAS guidelines^{36,37}. On the other hand, large RCTs comparing early versus late PN in critically ill adults and children with medical or surgical conditions have reported worse outcomes with early PN.^{29,38} Given these uncertainties related to the timing, dose, and duration of PN, well designed RCTs are urgently needed in neonates with CGISCs.

Similar to many other studies, we found that lower gestational age was associated with increased odds of PNGR. Hence, wherever feasible, every effort should be done to deliver them as close to term as possible.

While reviewing relevant literature, we realised that various definitions of PNGR have been used: a difference of 1SD between birth and discharge¹, a difference of 0.8 SD^{2,3}, less than -1.28 (10th percentile)¹³, whereas some have simply used statistically significant ($p < 0.05$) difference between birth and discharge z scores. Such heterogeneous definitions will make it difficult to compare the results. Hence consensus definitions are urgently warranted.

The strengths of our study are the large sample size of 404 infants, the inclusion of many types of surgical conditions, and subgroup and sensitivity analyses. The major limitation is the retrospective design. Since it was conducted in a high-income country, the results may not be generalisable globally³⁹.

In summary, PNGR is common in late preterm and term neonates with CGISCs. Strategies to optimise their nutrition while avoiding the potential adverse effects of aggressive nutrition are urgently needed.

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Preface to Chapter 3

Early Neurodevelopmental Outcomes of Congenital Gastrointestinal Surgical Conditions: A Single Centre Retrospective Study

The previous chapter reported that neonates with congenital gastrointestinal surgical conditions (CGISCs) have postnatal growth restrictions of weight and head circumference. This chapter has reported the one-year neurodevelopmental outcomes of 262 infants from the same cohort. A total of 43/262 (16.4%) infants had suboptimal neurodevelopmental outcomes. Taken together, it confirms that strategies are needed to improve the physical growth and neurodevelopmental outcomes of these high-risk infants.

The results of this study were published in BMJ Open Paediatrics August 2020; PMID: 32821861.

Early neurodevelopmental outcomes of congenital gastrointestinal surgical conditions: a single-centre retrospective study

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ABSTRACT

Background Evidence is emerging that surgery in the neonatal period is associated with increased risk of suboptimal neurodevelopmental outcomes (SNDO). The aim of this study was to describe neurodevelopmental outcomes (at 1 year) of neonatal surgery for congenital gastrointestinal surgical conditions (CGSC) and to explore risk factors.

Methods Retrospective study (2005–2014) of infants born ≥ 34 weeks gestation with CGSC and admitted to the surgical neonatal intensive care unit of Perth Children's Hospital, Western Australia. Clinical details and 1-year developmental outcomes based on Griffiths Mental Developmental Assessment Scales were collated from the database and by reviewing the medical records of study infants. SNDO was defined as one or more of the following: a general quotient less than 88 (ie, >1 SD below mean), cerebral palsy, blindness or sensorineural deafness. Univariable and multivariable logistic regression analyses were carried out to explore risk factors for SNDO. A total of 413 infants were included, of which 13 died. Median gestation was 37.6 weeks (IQR: 36.4–39.1). Information on developmental outcomes was available from 262 out of 400 survivors. A total of 43/262 (16.4%) had SNDO. On univariable analysis, lower z scores for birth weight, prolonged duration of antibiotics, increased episodes of general anaesthesia and prolonged duration of hospital stay were associated with SNDO. On multivariable analysis, lower z scores for birth weight and prolonged hospital stay were associated with increased risk of SNDO.

Conclusions Late preterm and term infants undergoing neonatal surgery for CGSC may be at risk for SNDO. Studies with longer duration of follow-up are needed to further evaluate the role of potentially modifiable risk factors on their neurodevelopmental outcomes.

INTRODUCTION

Survival following neonatal surgery has improved in the recent years, but short-term and long-term complications continue to have significant effects on these infants and their families.¹ A recent population-based study that compared developmental outcomes of 124 neonates undergoing non-cardiac surgery versus 92 who underwent

What is known about the subject?

- ▶ Surgery in the neonatal period may have an adverse effect on neurodevelopment outcomes.
- ▶ Infection and excessive inflammation are harmful to the developing brain.

What this study adds?

- ▶ Nearly 16% of late preterm and term infants who underwent neonatal surgery for congenital gastrointestinal conditions had suboptimal neurodevelopment at one year of age.
- ▶ Lower z scores for birth weight and prolonged hospital stay were associated with increased risk of suboptimal neurodevelopmental outcomes.
- ▶ C reactive protein levels and infections were not associated with suboptimal neurodevelopmental outcomes at 1 year of age.

cardiac surgery and 162 healthy infants found that cardiac surgery carried the highest risk of developmental delay, but infants undergoing non-cardiac surgeries also had 7%–14% incidence of developmental delay.²

Factors associated with poor developmental outcomes in neonates undergoing surgery include low birth weight,³ chromosomal anomalies, growth restriction,⁴ prolonged hospital stay,⁵ need for Extracorporeal Membrane Oxygenation,^{6 7} chronic lung disease,⁸ increasing number of surgeries⁵ and low socioeconomic status.⁹ One factor that has not been adequately explored in neonates undergoing surgery is the influence of infection and inflammation. Exploring this area is important because infection and excessive inflammation are potentially harmful to the developing brain.^{10–14}

We conducted this retrospective study to evaluate 1-year developmental outcomes of late preterm and term infants who underwent

surgery for congenital gastrointestinal surgical conditions (CGSC) in our unit and to explore the potential risk factors. Another aim of the study was to analyse the impact of inflammation on neurodevelopmental outcomes of those infants.

METHODS

This was a retrospective cohort study of all late preterm and term infants born at $\geq 34^{0/7}$ weeks gestation between January 2005 and December 2014 with CGSC who underwent surgery in the neonatal period at the tertiary neonatal intensive care unit (NICU) of Perth Children's Hospital, Western Australia.

The following conditions were included in the study—gastroschisis, exomphalos, duodenal atresia, malrotation, jejunoileal atresia, large bowel atresia, meconium ileus, Hirschsprung disease, multiple gut anomalies, gut perforations/stenoses, short bowel syndrome, biliary atresia, anorectal anomalies and benign abdominal cysts. We included oesophageal atresia and congenital diaphragmatic hernia because they also involve the gastrointestinal tract and have long-term gastrointestinal complications.

Infants were identified by interrogating the departmental database. Infants with chromosomal anomalies and syndromes known to adversely affect developmental outcomes were excluded. Infants born at < 34 weeks gestation were excluded because they carry a higher risk of adverse developmental outcomes due to prematurity compared with late preterm and term infants.

Clinical characteristics of study infants were extracted from their medical records by one author (VB) and verified for accuracy by a second author (SR). Two neonatologists with expertise in developmental follow-up (DW and JKG) collated the results of 1-year outcomes based on Griffiths Mental Development Scales (GMDS-II) from the departmental database. The GMDS-II assesses development in five areas: locomotor, personal and social, hearing and speech, eye and hand coordination, and performance. The five subscales are assessed and scored separately and then combined to provide an overall general quotient (GQ) reflecting the child's developmental performance level relative to the general population. On these scales, a combined GQ of 100.2 (SD 12) is considered normal.¹⁵ The GMDS-II is a well-recognised tool for identifying neurosensory disability and is used widely.^{16 17}

Outcome of interest for this study was suboptimal neurodevelopmental outcomes (SNDO) at 1 year of age. SNDO was defined as one or more of the following: (1) a GQ of < 88 (ie, > 1 SD below mean) on GMDS-II,¹⁵ (2) cerebral palsy (based on assessment by neurologist or developmental paediatrician) (3) blindness (visual acuity of $< 6/60$ in the better eye) and (4) sensorineural deafness (based on audiometry assessment) requiring hearing aids.

Healthcare-associated infection (HAI)-included urinary tract infection (UTI) or healthcare-associated

blood stream infection (HABSI), meningitis or surgical site infection or any type of viral infection. HABSI was defined as positive blood culture on a sample taken 48 hours after admission to the NICU. UTI was defined as positive culture based on a sample collected from suprapubic sample or in-and-out catheter. Meningitis was diagnosed based on positive culture on cerebrospinal fluid (CSF) samples collected with aseptic precautions. The diagnosis of wound infection was based on the presence of erythema/oedema/induration at the surgical site and positive culture on the wound swab. Respiratory viral infection was diagnosed based on PCR on postnasal aspirate samples taken in infants who presented clinical symptoms of respiratory illness.

C reactive protein (CRP) was used as the marker of inflammation. We stratified the CRP levels based on the timing in relation to the surgical procedure. Empirically, a CRP done in the preoperative period was considered to be a surrogate marker of early onset sepsis, whereas CRP performed within 72 hours of surgery was considered to be related to the degree of surgical injury and CRP performed after 72 hours of surgery to indicate hospital acquired infection.

Statistical analysis was done using the STATA V.16 software (StataCorp). The summary statistics for normally distributed continuous variables were expressed as mean and SD; those with skewed distribution were expressed as median and IQR. Categorical variables were expressed as frequency and percentage. Univariable and multivariable random effect logistic regression models were carried out to derive unadjusted and adjusted odds ratios and 95% CIs. Random effect was included in the fitted model to minimise bias due to the presence of correlated data (ie, multiple measurements of CRP values from individual patients). One-sample t-test was used to compare the mean GQ scores to the population mean (100.2).¹⁵ For all analyses, a two-tailed $p < 0.05$ was considered statistically significant.

This retrospective study was approved by the institutional ethics committee as a quality assurance activity. All clinical variables and the results of developmental assessments (GMDS-II) collected for this study were retrospective in nature. Strengthening the Reporting of Observational Studies in Epidemiology guidelines were used to report this study.¹⁸

Patient and public involvement

The development of research question and outcome measures for this retrospective study were not informed by patients' priorities, experience and preferences. Patients were not involved in the design, in the recruitment to and conduct of the study. Patients were not invited to comment on the study design and were not consulted to develop patient relevant outcomes or interpret the results. Patients were not invited to contribute to the writing or editing of this document for readability or accuracy. There are no plans to disseminate the results of this study to study participants.

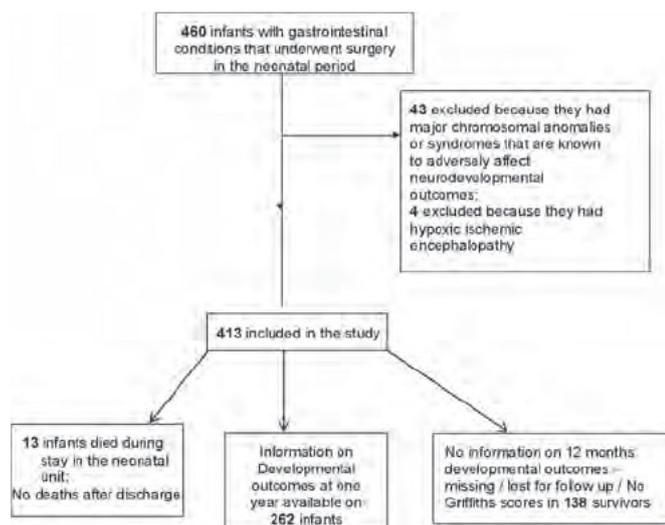


Figure 1 Study flow diagram.

RESULTS

A total of 460 neonates underwent surgery for CGSC during the study period, of which 43 were excluded because of chromosomal anomalies or syndromes that are known to adversely affect neurodevelopmental outcomes. Four infants were excluded because they had moderate to severe hypoxic ischaemic encephalopathy due to perinatal asphyxia. The remaining 413 infants were included in the study. Of them 13 died, and of the 400 surviving infants, full information on developmental outcomes was available for 262/400 (65%) surviving infants. The flow diagram of patient selection process is given in [figure 1](#).

The median gestation was 37.6 weeks (IQR: 36.4–39.1) and median birthweight 3000 grams (IQR: 2590–3405). The median duration of hospital stay was 18 days (IQR: 11–26 days, range: 1–153 days). There were 13 deaths, all of which were during initial hospital stay. There were no deaths after discharge from the hospital. [Table 1](#) summarises the clinical characteristics in detail.

The major surgical conditions were gastroschisis, malrotation, oesophageal atresia with or without tracheo-oesophageal fistula, Hirschsprung disease and congenital diaphragmatic hernia ([table 2](#)).

A total of 43/262 (16.4%) infants had SNDO, with nine infants having a GQ <76 (ie, more than 2 SD below the mean). One infant had deafness, one had cerebral palsy and none had blindness. The mean GQ was 96.3 (SD 10.3), which was significantly lower than the population mean of 100.2; $p < 0.001$. Infants with multiple gut anomalies, oesophageal atresia, Hirschsprung disease, exomphalos and congenital diaphragmatic hernia had highest rates of SNDO among survivors ([table 2](#)).

HABSI occurred in 27 infants (6.5%). A total of 51 (12.4%) infants developed at least one episode of HAI (UTI or HABSI or viral infection or surgical site infection). None of the infants had early-onset sepsis. Coagulase negative *Staphylococcus*, *Klebsiella* spp and *Escherichia Coli* were the most common pathogens isolated ([table 3](#)).

Association between neonatal risk factors and SNDO among survivors

On univariable analysis, lower birthweight z scores, prolonged duration of antibiotic therapy increasing episodes of general anaesthesia and prolonged duration of hospital stay were associated with higher odds of SNDO among survivors ([table 4](#)). On multivariable analysis, lower birthweight z scores and longer duration of hospital stay were associated with increased odds of SNDO among survivors ([table 4](#)).

DISCUSSION

Our study found an overall mortality rate of 3.1% and SNDO in 16.4% of neonates undergoing surgery for CGSC. These findings are similar to a recent study that reported an incidence of 7%–14% in various domains of assessment at 3 years among 124 children who underwent surgery for non-cardiac conditions in the neonatal period.² While the mean GQ of 96.3 in our cohort might not appear too low, it is important to note that the GMDS-II norms are based on population sample more than two decades ago. It is well known that developmental quotients and intelligence quotients in the general population increase by 2–3 points each decade (Flynn effect).¹⁹ If assessed using the GMDS-II tools, healthy 12-month-old infants during the study period of 2005–2014 would probably have scored a mean of 103 rather than 100.

Since the study spanned over 10 years (January 2005 to December 2014), advances in anaesthesia, surgical techniques, intensive care management, and changes to family and societal environment during that period could have influenced the in-hospital clinical outcomes and 1-year developmental outcomes of study infants. Contemporary multicentre studies with adequate sample size are needed to enhance knowledge in this area.

While many variables were found to be associated with increased risk of SNDO on univariable analysis, only lower birthweight z scores and longer duration of hospital stay were found to be having significant association on multivariable analysis. Lower birthweight z scores indicate fetal growth restriction and prolonged hospitalisation is usually related to the complex nature of the underlying surgical condition. Hence their association with adverse neurodevelopmental outcomes is not unexpected. The width of the CI for birthweight z-scores was very wide, ranging between a drop in the odds between 2% and 50%. The probable reason for this wide range could be related to the timing of intrauterine growth restriction (IUGR). For the same degree of IUGR, the one that starts early during pregnancy is known to have worse outcomes compared with late gestation IUGR.

Each additional day of stay in the hospital resulted in a change in the odds of SNDO by 3%. Many surgical infants stay for a protracted period of time in the hospital and hence these odds are likely to be clinically significant.



Table 1 Characteristics of study infants

Clinical characteristic	Median or no (percentage)	IQR	Range	N
Gestation (weeks)	37.6	36.4 to 39.1	34.1 to 41.5	413
Gender (male:female)	57%:43%	NA	NA	413
Birth weight (grams)	3000	2590 to 3405	1664 to 5060	413
Birthweight z scores	-0.23	-0.87 to 0.41	-2.81 to 4.78	413
Birth length (cm)	49	47 to 51	40 to 58	403
Birth length z scores	-0.03	-0.75 to 0.57	-3.38 to 4.23	403
Birth head circumference (cm)	34	32.5 to 35	28.5 to 47	409
Birth head circumference z scores	0.07	-0.62 to 0.78	-3.32 to 3.29	408
APGAR 5 min	9	9 to 9	4 to 10	409
Presurgery C reactive protein (CRP) levels (mg/dL)	7.5	5 to 20	1 to 188	933
CRP levels within 72 hours of initial surgery (mg/dL)	35.5	19 to 69	3 to 346	747
CRP levels after 72 hours of surgery (mg/dL)	15	7 to 29	1 to 325	2912
Healthcare-associated blood stream infection	27 (6.5%)	NA	NA	413
CSF culture positive	1 (0.25%)	NA	NA	413
Lumbar puncture done	14 (3.4%)	NA	NA	413
Viral infections (all respiratory)	17 (4.1%)	NA	NA	413
Urinary tract infections (UTI)	1 (0.24%)	NA	NA	413
Culture positive surgical site infections	14 (3.4%)	NA	NA	413
Any healthcare-associated infection (blood stream or CSF or viral or UTI or wound infection)	51 (12.4%)	NA	NA	413
No of antibiotic courses	2	1 to 2	1 to 14	406
Cumulative duration of antibiotics (days)	6	4 to 8	1 to 56	406
Surgery episodes under GA	1	1 to 2	1 to 5	413
No of episodes of hypoglycaemia (blood glucose <2.6 mmol/L)	0	0 to 0	0 to 15	413
Length of stay (days)	18	11 to 26	1 to 153	413
Post conception age at discharge (weeks)	41	39.4 to 42.4	35.4 to 60.2	413
Death before discharge	13 (3.1%)	NA	NA	413
Death before 1 year	13 (3.1%)	NA	NA	413
Corrected age at Griffiths assessment (months)	12	12 to 12.5	10 to 15.5	270
GQ scores at 12 months	96.5	92 to 102	49 to 131	270
SNDO	43/262 (16.4%)	NA	NA	262

GA, gestational age; GQ, general quotient; NA, not applicable; SGA, small for gestational age; SNDO, Suboptimal developmental outcomes.

The burden of HAI and HABS in neonates with CGSC has not been explored adequately. Donnell *et al* and van Saene *et al* conducted a prospective study of surgical infants <6 months to find infection rates.^{20 21} Thirty-two infants developed blood culture positive sepsis (15%); predominant micro-organisms (86%) were coagulase-negative staphylococci and enterococci. Other pathogens, including aerobic gram-negative bacilli, were responsible for the remainder. They suggested that gut translocation was the main factor behind sepsis in surgical infants rather than central lines and cautioned that prevention is unlikely to be successful if abnormal gut flora is ignored.²¹ Another study by Bishay *et al* reported that 31 out of 112 surgical infants (28%) had a total of 65 episodes of septicemia.²²

In very preterm infants, it is well established that neonatal sepsis is associated with higher risk of adverse neurodevelopmental outcomes. A recent systematic review by Cai *et al*²³ found that preterm infants with neonatal sepsis were at a higher risk of neurodevelopmental impairments such as cerebral palsy and neurosensory deficits, compared with infants without sepsis (OR 3.18; 95% CI 2.29 to 4.41).²³ Hence, we had expected similar findings in our cohort of surgical infants. However, in our study, HAI was not associated with increased risk of SNDO, either on univariable or multivariable analysis. Similarly, higher levels of CRPs were not associated with SNDO irrespective of the timing in relation to the surgeries. This could be related to the resilience of the brain of late preterm and term

**Table 2** Developmental outcomes of neonates with CGISC*

Major gastrointestinal anomaly	No	Mortality	SNDO among infants who were assessed	Median GQ
Gastroschisis	92 (22.3%)	3/92 (3.3%)	8/55 (14.5%)	98.5 (IQR:92.5–103) n=60
Malrotation	48 (11.6%)	3/48 (6.2%)	4/33 (12.1%)	96 (IQR:93–103) n=34
Oesophageal atresia	44 (10.6%)	1/44 (2.3%)	10/27 (37%)	93 (IQR:85–100) n=27
Hirschsprung disease	44 (10.6%)	1/44 (2.3%)	6/32 (18.7%)	98 (IQR:93–102) n=33
Congenital diaphragmatic hernia	42 (10.2%)	1/42 (2.4%)	5/34 (14.7%)	94.5 (IQR:92–105) n=34
Ano-rectal anomalies	39 (9.4%)	0/39 (0%)	3/21 (14.3%)	96 (IQR:90–101) n=22
Gut perforations and stenoses	19 (4.6%)	1/19 (5.3%)	1/12 (8.3%)	101.5 (IQR:94.5–106) n=12
Duodenal atresia	19 (4.6%)	0/19 (0%)	1/13 (7.7%)	99 (IQR:94–110) n=15
Jejuno-ileal atresia	16 (3.9%)	0/16 (0%)	1/9 (11.1%)	97.5 (IQR:95–102) n=10
Exomphalos	13 (3.1%)	0/13 (0%)	1/6 (16.7%)	99 (IQR:89–100) n=7
Meconium ileus	12 (2.9%)	0/12 (0%)	0/5 (0%)	98 (IQR:96–99) n=5
Multiple gut anomalies	10 (2.4%)	1/10 (10%)	3/7 (42.8%)	88 (IQR:84–100) n=7
Short bowel syndrome	5 (1.2%)	2/5 (40%)	0/1 (0%)	95 n=1
Large bowel atresia	5 (1.2%)	0/5 (0%)	0/1 (0%)	104 n=1
Benign abdominal cysts and tumours	4 (0.97%)	0/4 (0%)	0/1 (0%)	103 n=1
Biliary atresia	1 (0.24%)	0/1 (0%)	0/1 (0%)	92 n=1

*For all outcomes, infants who underwent at least one episode of surgery were included; infants who died prior to undergoing any surgery were excluded. The information on neurodevelopmental outcomes was available for 65% of survivors. CGISC, congenital gastrointestinal surgical conditions; GQ, general quotient; SNDO, suboptimal neurodevelopmental outcomes.

infants to the harmful effects of infection and inflammation, unlike the vulnerable extremely preterm infants. However, prolonged duration of antibiotic therapy, which could be a surrogate marker of clinically suspected infection, was associated with SNDO on univariable, but

not multivariable analysis. Further studies with larger sample size and a longer duration of follow-up beyond 1 year of age are needed to explore the role of infection and inflammation in late preterm and term infants undergoing neonatal surgery.

Table 3 Micro-organisms isolated from infants with healthcare-associated infections

Micro-organism	Blood	CSF	Urine	Viral infections	Wound/skin swab
CONS	16	1	–	–	3
<i>Escherichia coli</i>	4	–	–	–	3
<i>Klebsiella</i>	3	–	–	–	–
<i>Pseudomonas</i>	1	–	–	–	2
<i>Streptococcus mitis</i>	1	–	–	–	–
<i>Moraxella</i>	1	–	–	–	–
<i>Enterococcus</i>	1	–	–	–	1
<i>Candida albicans</i>	–	–	1	–	2
<i>Staphylococcus aureus</i>	–	–	–	–	2
<i>Enterobacter cloacae</i>	–	–	–	–	1
Rhino virus	–	–	–	12	–
RSV	–	–	–	2	–
Influenza A	–	–	–	2	–
Parainfluenza	–	–	–	1	–
Total	27	1	1	17	14

CONS, coagulase negative *Staphylococcus*; RSV, respiratory syncytial virus.



Table 4 Risk factors for SNDO

Variable	Unadjusted OR and 95% CI	P value	Adjusted OR and 95% CI	P value
Gestational age at birth (≥ 37 weeks)	1.07 (0.53 to 2.19)	0.840	1.54 (0.65 to 3.63)	0.321
Birthweight z scores	0.64 (0.47 to 0.89)	0.008*	0.69 (0.49 to 0.98)	0.038*
Female gender	0.71 (0.36 to 1.39)	0.313	0.53 (0.24 to 1.16)	0.112
No of episodes of hypoglycaemia (< 2.6 mmol/L)	1.06 (0.75 to 1.49)	0.730	0.98 (0.60 to 1.58)	0.923
General anaesthesia (> 3 episodes)	3.31 (1.29 to 8.50)	0.013*	0.77 (0.17 to 3.59)	0.745
Preoperative CRP levels	0.90 (0.59 to 1.37)	0.627	0.92 (0.60 to 1.40)	0.698
CRP levels within 72 hours of surgery	0.90 (0.63 to 1.28)	0.555	1.06 (0.69 to 1.62)	0.769
CRP levels after 72 hours of surgery	0.64 (0.41 to 1.01)	0.053	0.99 (0.63 to 1.56)	0.985
Any infection	1.20 (0.49 to 2.96)	0.683	0.44 (0.11 to 1.77)	0.247
Cumulative duration of antibiotics	1.05 (1.01 to 1.10)	0.043*	1.00 (0.91 to 1.10)	0.990
Degree of postnatal growth restriction	2.00 (0.59 to 6.81)	0.265	1.56 (0.62 to 3.97)	0.348
Length of stay	1.02 (1.00 to 1.03)	0.003*	1.03 (1.00 to 1.06)	0.034*

*Statistically significant associations
CRP, C reactive protein.

The harmful effect of exposures to general anaesthesia on developing brain is an area of debate and active research.^{24–25} While animal studies have consistently shown general anaesthesia to be toxic to the developing brain,²⁶ one recent large RCT²⁷ and a large prospective cohort study²⁸ found no significant association. Both these studies evaluated a single exposure to general anaesthesia, and hence do not address the issue of repeated exposures. A recent large data linkage study found that children exposed to general anaesthesia before 4 years have poorer development outcomes at school entry and school performance.²⁹ In another cohort study,³⁰ children who had multiple exposure to gestational age (GA) before 3 years of age scored 1.3 points (95% CI -3.8 to 1.2 ; $p=0.32$) less than unexposed children on intelligence tests; children who had one exposure to GA scored 0.5 points (95% CI -2.8 to 1.9 ; $p=0.70$) less than unexposed children. However, the parents of children who had multiple exposure to GA reported increased problems related to executive function, behaviour and reading.³⁰ In our cohort, increasing episodes of general anaesthesia were associated with higher risk of SNDO on univariable analysis, but not on multivariable analysis. Further studies with long duration of follow-up are needed in this area.

While we found lower birthweight z scores and prolonged hospital stay to be associated with increased risk of SNDO, one should not ignore the possibility that the underlying surgical condition in itself could be an important risk factor that drives other morbidities leading to SNDO. In our cohort, multiple gut anomalies and oesophageal atresia had the highest incidence of SNDO (42.8% and 37%, respectively), which is not unexpected because these infants have significant in-hospital and postdischarge morbidities, which puts them at a higher risk of SNDO.

One of the limitations of our study was the shorter duration of follow-up of 1 year and the findings may not track subsequently. In a recent study, Fairbairn *et al* reported that Bayley-III results for all domains at 1 year of age were a weak predictor of outcomes at 3 years of age in infants who had early major cardiac and non-cardiac surgery and healthy infants.³¹ Hence all infants, irrespective of the results of developmental assessments at 1 year should be followed with formal developmental assessments at least until 5 years of age. At the same time, infants identified as high risk based on the 1-year assessments could be provided early developmental interventions to optimise their outcomes. Only recently, we have commenced routine developmental follow-up until 2 years of age with Bayley Scales of Infant Development to all infants undergoing surgery in the neonatal period.

Surgical infants who need prolonged duration of mechanical ventilation are at higher risk of hypoxic episodes and hence worse developmental outcomes. At the same time, prolonged ventilation could be a marker of severity of the underlying anomaly. A limitation of our study was the lack of reliable information on the duration of mechanical ventilation among the study infants.

The other limitations of our study were: (1) retrospective design without healthy controls, (2) the indication for doing CRP levels was at the discretion of clinicians rather than based on a standardised protocol, (3) full information on developmental outcomes was missing from nearly 35% of survivors, (4) lack of information on sociodemographic status of family and (5) missing information about duration of general anaesthesia which can have significant influence on developmental outcomes. The data were from a single centre from a high-income country and hence the findings may not be generalisable. The main strength of the study is the large sample size



of surgical infants and the use of regression analyses to adjust for confounders.

CONCLUSIONS

Late preterm and term infants undergoing surgery for CGSC may be at risk for SNDO at 1 year of age. Studies with long-term follow-up are needed to further evaluate the influence of potentially modifiable risk factors on neurodevelopmental outcomes in such infants.

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Patient consent for publication Not required.

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Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. The deidentified patient data are available from the correspondence author and will be provided on reasonable request.

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Preface to Chapter 4
Gut Dysbiosis in Critical Illness

The previous chapters covered the evidence that neonates with congenital gastrointestinal surgical conditions (CGISCs) are critically ill, have feed intolerance, receive interventions such as parenteral nutrition, antibiotics, and proton pump inhibitors, and are managed in the intensive care environment. The chapters also reported that neonates with CGISCs are at risk of infections, postnatal growth restriction, and long-term adverse neurodevelopmental outcomes.

This chapter provides general evidence from the literature that critical illness and clinical interventions in the intensive care unit can alter gut microbiota, resulting in gut dysbiosis. It also discusses the possibility that dysbiosis can contribute to adverse outcomes in such patients. While much of the evidence is from experimental animals, adult and paediatric patients, and extremely preterm infants, one could hypothesise that similar mechanisms would be operational in neonates with CGISCs. One could also hypothesise that neonates with CGISCs would be at an even higher risk for gut dysbiosis since the gastrointestinal tract is where the majority of the bacteria are located in humans.

CHAPTER 4

Gut Dysbiosis in Critical Illness

ABSTRACT

This chapter provides general evidence from the literature that critical illness and clinical interventions in the ICU can alter gut microbiota, thereby resulting in gut dysbiosis. We also discuss the possibility that dysbiosis can contribute to adverse outcomes in such patients. While much of the evidence is from experimental animals, adult and paediatric patients, and extremely preterm infants, one could hypothesise that similar mechanisms would be operational in neonates with CGISC. One could also hypothesise that neonates with CGISC would be at an even higher risk for gut dysbiosis since the gastrointestinal tract is where the majority of the bacteria are located in humans.

Introduction:

Patients admitted to intensive care units are at risk of developing altered gut microbiota, i.e., ‘dysbiosis’. Dysbiosis can be defined as any change to the composition of resident commensal microbial communities relative to the community found in healthy individuals¹. Interventions such as the use of antibiotics, parenteral nutrition, proton pump inhibitors, withholding of enteral feeds, and the ICU environment can all contribute to the development of dysbiosis. Gut dysbiosis is thought to play a role in the pathogenesis of conditions such as sepsis²⁻⁴, Hirschsprung disease associated enterocolitis⁵, necrotising enterocolitis⁶, feed intolerance⁷, parenteral nutrition-associated cholestasis⁸, and adverse neurodevelopmental outcomes⁹.

Dysbiosis secondary to proton-pump inhibitors (PPI): Use of PPIs is widespread in neonates with CGISC¹⁰⁻¹². Their use could result in small intestinal bacterial overgrowth (SIBO) by altering the intraluminal environment and bacterial microbiota. In a meta-analysis

of 11 studies from the adult population (n=3134), the pooled odds ratio of SIBO in PPI users vs non-users was 2.282 (95% CI, 1.238-4.205)¹³ (Figure 1).

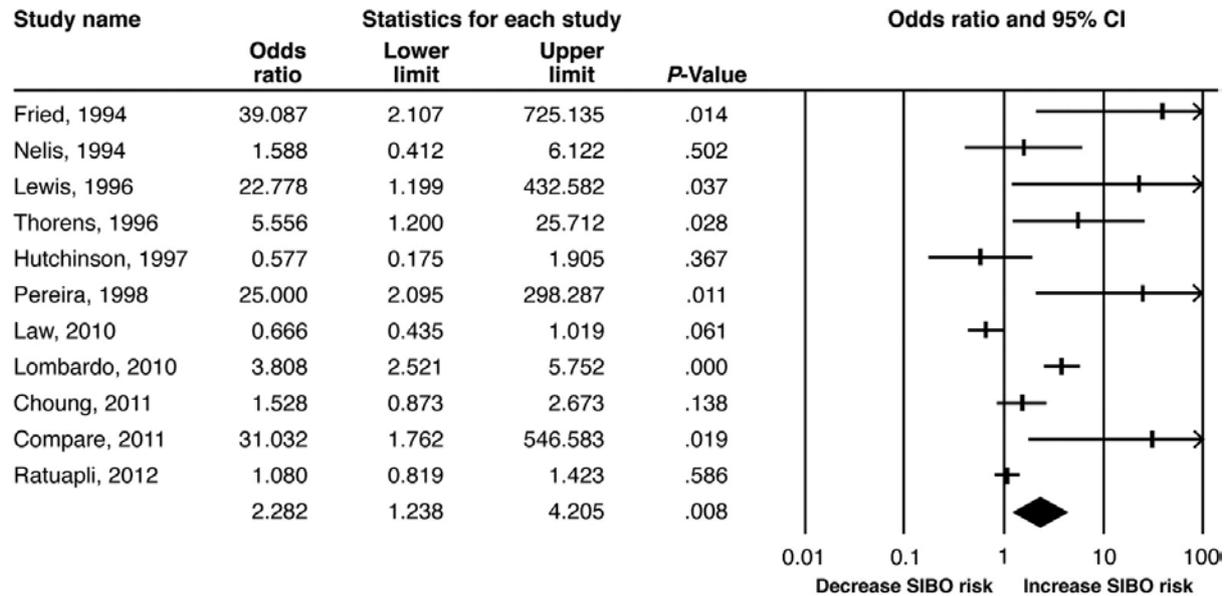


Figure 1. Meta-analysis of SIBO risk with PPI use. Reproduced from Lo, WK and Chan, WW¹³ *Clinical Gastroenterology and Hepatology*, 2013 with permission from the publisher Elsevier.

In a recent (2016) meta-analysis by Imhann et al.¹⁴, the gut microbiome composition of 1815 individuals from three cohort studies on the adult population was assessed by tag sequencing of the 16S rRNA gene. In total, 211 were using PPIs at the time of stool sampling. The use of PPI was associated with a significant decrease in Shannon's diversity (Figure 2) and a significant increase in the abundance of *Enterococcus*, *Streptococcus*, *Staphylococcus*, and the potentially pathogenic *Escherichia coli* in PPI users¹⁴ (Figure 3).

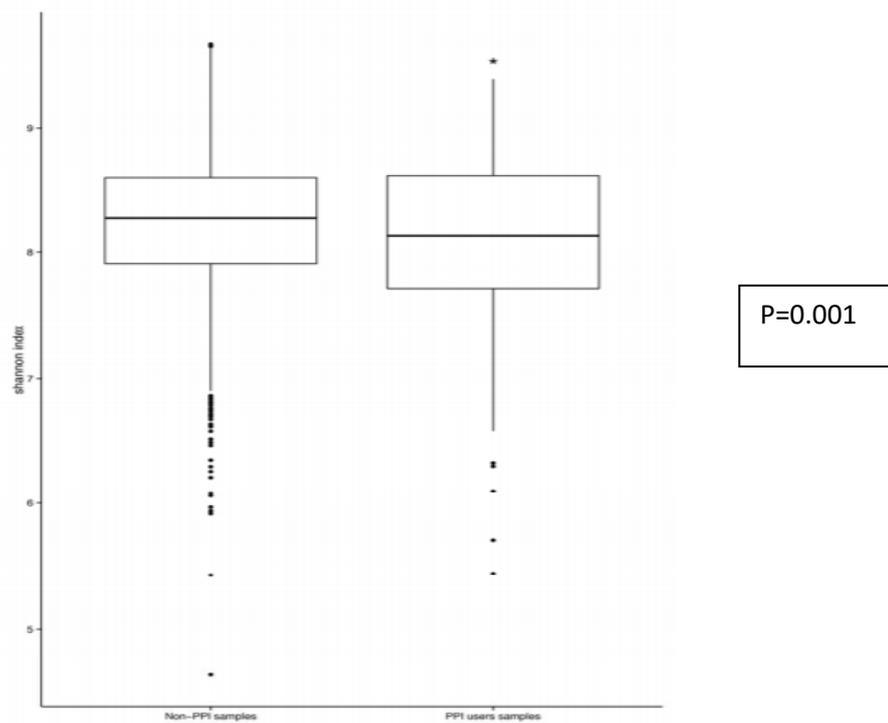


Figure 2: Shannon diversity: In PPI users, gut microbiome diversity was significantly diminished ($p=0.001$). Reproduced from Imhann F et al. *Gut*. 2016 May; 65(5): 740-8 (BMJ Publishing Group; Permission was not required because it was published under the Creative Commons Non-Commercial CC-BY-NC 4.0 license).

The meta-analysis showed statistically significant alterations (increase or decrease) in 92 of the 460 bacterial taxa abundance [False detection rate $FDR < 0.05$]¹⁴.

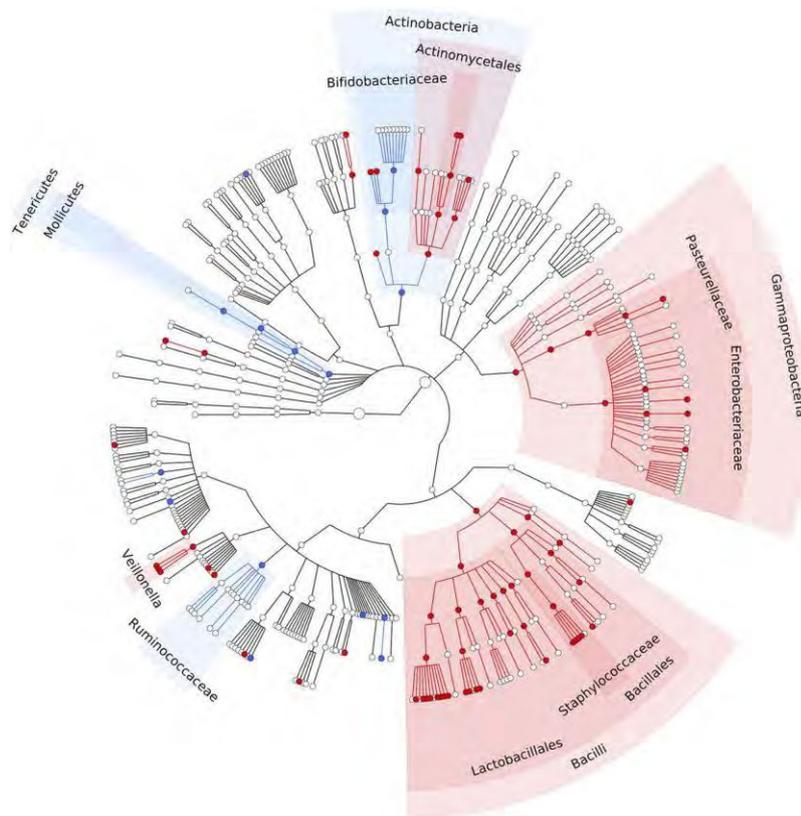


Figure 3: PPI-associated differences in the gut microbiome. Meta-analysis of three independent cohorts comprising 1815 faecal samples, showing a cladogram (circular hierarchical tree) of significantly increased (red dots) or decreased (blue dots) bacterial taxa in the gut microbiome of PPI users compared with non-users ($FDR < 0.05$). Each dot represents a bacterial taxon. The two innermost dots represent the highest level of taxonomy in the data: the kingdoms *Archea* and *Bacteria* (prokaryotes), followed outwards by the lower levels: phylum, class, order, family, genus, and species. FDR, false discovery rate; PPI, proton pump inhibitor. Reproduced from Imhann et al. *Gut*. 2016 May; 65(5): 740-8 (BMJ Publishing Group; Permission was not required because it was published under the Creative Commons Non-Commercial CC-BY-NC 4.0 license).

Dysbiosis secondary to antibiotic therapy: In a prospective study, Hussey et al.¹⁵ investigated the effect of parenteral antibiotics in the newborn period on the evolution of bifidobacterial species. Nine infants (Subjects A to I) were treated with intravenous ampicillin/gentamicin in the first week of life, and nine were control infants (no antibiotic treatment). The composition of *Bifidobacterium* was analysed in stool samples taken at four and eight weeks using denaturing gradient gel electrophoresis [DGGE]. At four weeks,

bifidobacterial species were detected in six (D, E, F, G, H, I) of the nine infants treated with antibiotics. In contrast, it was seen in all nine control infants (J, K, L, M, N, O, P, Q, R) (Figure 4). Moreover, stool samples of control infants showed a greater diversity of *Bifidobacterium* species than antibiotic-treated infants¹⁵.

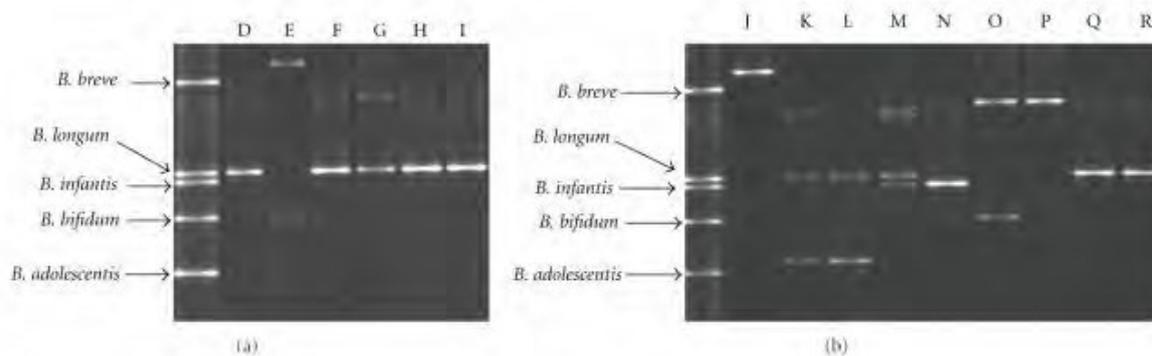


Figure 4: DGGE of bifidobacterial PCR-products (V3-region) from stool samples taken at four weeks of age from infants treated with antibiotics D–I (a) and controls J–R (b). In figure a, infants A, B, and C are not seen because they did not have bifidobacteria. The mobility of the PCR products obtained in DGGE was compared to the PCR pattern of reference strains (first column in figures a and b) obtained with the same primer set, reproduced from Hussey S, *Int J Microbiol.* 2011¹⁵, Hindawi Publishers (Permission was not required because it was published under Creative Commons Attribution License).

At eight weeks of age, 8 out of 9 antibiotic-treated infants (B to I) and all controls (J to R) had detectable levels of bifidobacteria in their stools. The only sample in which bifidobacteria were not detected was from an infant (subject A) that had received antibiotics for nine days (the other infants received antibiotics for 2–5 days). While both groups were similar at eight weeks, samples from antibiotic-treated infants continued to display a less diverse population of bifidobacteria when compared with controls (Figure 5)¹⁵. They concluded that short-term parenteral antibiotic treatment of neonates causes a disturbance in the bifidobacterial colonisation in the first months of life¹⁵.

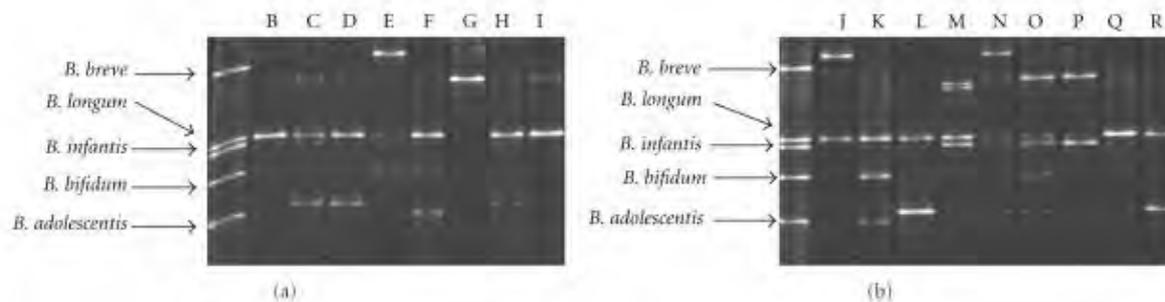


Figure 5: DGGE of bifidobacterial PCR-products (V3-region) from stool samples taken at eight weeks of age from infants treated with antibiotics B–I (a) and controls J–R (b). The mobility of the PCR products obtained in DGGE was compared to the PCR pattern of reference strains obtained with the same primer set (First columns in both figures). In figure a, subjects A is not seen because it did not have bifidobacteria. Reproduced from Hussey S, *Int J Microbiol.* 2011,¹⁵ Hindawi Publishers (Permission was not required because it was published under Creative Commons Attribution License).

In another study, Tanaka et al.¹⁶ assessed the influence of antibiotic exposure on the development of intestinal microbiota in five infants who were treated with a broad-spectrum oral antibiotic for the first four days of life and three caesarean-delivered infants whose mothers were given intravenous antibiotics and compared them to 18 infants who did not receive antibiotics. The faecal bacterial composition was analysed daily for the first five days and monthly for the first two months. Antibiotic-treated subjects showed less bacterial diversity in the first week than antibiotic-free infants. They also had attenuation of *Bifidobacterium* and overgrowth of *Enterococcus* in their stool samples. After one month, the *Enterobacteriaceae* population was markedly higher in the antibiotic-treated group than in the antibiotic-free group. Caesarean delivered infants had similar, although relatively weaker, alterations in their microbiota. The authors concluded that antibiotic exposure in the early neonatal period significantly affects the development of neonatal intestinal microbiota.

Yassour et al.¹⁷ analysed the faecal samples from 39 children (average of 28 samples per child) during the first three years of life. Twenty children received 9 to 15 courses during this period, whereas the remaining 19 never received antibiotics. They performed 16S rRNA gene

and whole-genome shotgun sequencing to analyse microbial diversity at taxonomic levels, including genus, species, and strain. They found decreased microbial diversity and increased short-term changes in the gut microbiomes of antibiotic-treated children. Furthermore, they observed an increased abundance of antibiotic resistance genes following antibiotic therapy, along with concurrent increases in specific bacteria likely harbouring such genes¹⁷.

Dysbiosis secondary to parenteral nutrition: Miyasaka et al.¹⁸ conducted an experiment in which one group of 10–14-week-old mice was kept nil-enterally and given parenteral nutrition. The control mice were given intravenous normal saline infusion in addition to their regular enteral chow diet.

After euthanasia on day 7, one cm segments of the small intestine and colon were collected from each mouse, and mucosa-associated bacteria were isolated. Bacterial DNA was extracted, and 16S rRNA pyrosequencing was performed. Immunofluorescence microscopy, flow cytometry, and epithelial barrier function tests were performed on the isolated intestinal epithelial cells.

They observed that enteral nutrient deprivation with TPN led to significant changes in the microbiota of the intestinal mucosa. The dominant phyla were Proteobacteria and Bacteroidetes in the TPN group and Firmicutes in the control mice. TPN mice had abundant pathogenic genera of Salmonella, Escherichia, Proteus, and Bacteroides at the genus level.

The expressions of pro-inflammatory TLR2, TLR4, and MyD88 were significantly upregulated in the lamina propria of mice who received TPN at the mRNA level. Pro-inflammatory cytokines TNF- α and IFN- γ mRNA levels were markedly higher in TPN mice compared to controls, whereas TGF- β 1 levels significantly were significantly lower in the TPN group¹⁸.

Overall, their results showed a strong association between TPN use and being fed nil-enterally and profound changes in the intestinal microbiota. The investigators speculated that

increased TLR signalling in the lamina propria and loss of the Treg population could have contributed to the mucosal pro-inflammatory response.

Demehri et¹⁹ al in their review article, summarised the effects of lack of enteral nutrition as follows: a. change in luminal microbiota occurs where Gram-negative Proteobacteria dominate; b. Lipopolysaccharide (LPS) derived from these bacteria signals lamina propria (LP) cells via Toll-like receptors (TLR), leading to an increased NF- κ B transcription; c. this creates a pro-inflammatory state with increased TNF- α and IFN- γ , loss of Treg cells, and decreased intraepithelial lymphocyte (IEL)-derived IL-10 and EGF (Figure 6) and d. these changes lead to break down of tight junctions, loss¹⁹ of epithelial barrier function, bacterial translocation, and sepsis¹⁹.

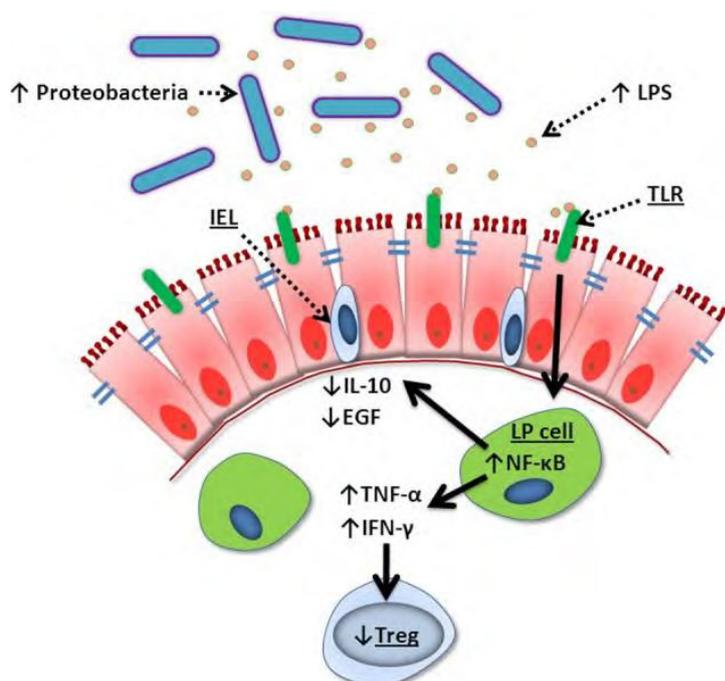


Figure 6: Lack of enteral nutrition leads to a change in luminal microbiota where Gram-negative Proteobacteria dominate. Lipopolysaccharide (LPS) derived from these bacteria signal lamina propria (LP) cells via Toll-like receptors (TLR), leading to increased NF- κ B transcription. This creates a pro-inflammatory state with increased TNF- α and IFN- γ , loss of Treg cells, and decreased intraepithelial lymphocyte (IEL)-derived IL-10 and EGF. These changes lead to the breakdown of

tight junctions, loss of epithelial barrier function, bacterial translocation, and sepsis. Reproduced from Demehri FR et al. Front Cell Infect Microbiol. 2013 Dec 23; 3:105¹⁹(Permission was not required because it was published under the Creative Commons Attribution License (CC BY).

Ralls et al.²⁰ randomised mice into two groups: 1) enterally fed group that received IV normal saline infusion and enteral chow diet; and 2) TPN-fed group that was given TPN infusion without enteral nutrition. Stable isotope-labelled l-leucine was added to TPN as well as normal saline. The study protocol continued for six days, and all animals were euthanised on day 7. The entire small intestine was removed, and its effluent was extracted and analysed using liquid chromatography-mass spectrometry. A significantly higher concentration of isotope-labelled leucine was present in the small bowel effluent in the TPN mice compared with enterally fed mice, suggesting increased permeability of intestinal epithelium in the TPN group compared to the enterally fed group. All amino acids in the TPN were present in the luminal effluent at an almost similar concentration to that of the TPN. These findings suggested a trans-epithelial permeation of amino acids with TPN administration²⁰.

By conducting further in-vitro experiments on the jejunal tissue, they reported that the transepithelial resistance of jejunum from TPN mice was significantly lower than that of enterally fed mice. The relative concentration of [¹³C] leucine applied to the serosal surface progressively rose in the mucosal compartment and was significantly greater by 90 min (Figures 7A and 7B).

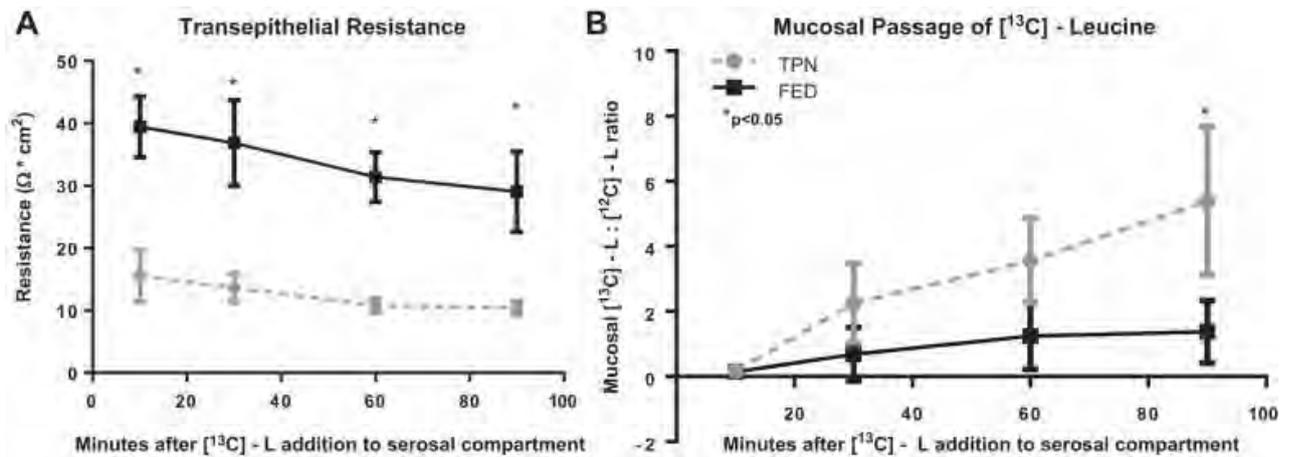


Figure 7: A. The trans-epithelial resistance was lower in TPN mice than in enterally FED mice. B: The relative concentration of [¹³C] leucine applied to the serosal surface progressively rose in the mucosal compartment and was significantly greater by 90 min. Reproduced from Ralls MW et al.²⁰, *Am J Physiol Gastrointest Liver Physiol.* 2016 Oct 1;311(4): G734-G743 with permission from the American Physiological Society.

Microbial and cytokine analysis of the intestinal effluent demonstrated a strong predominance of the *Enterobacteriaceae* and proinflammatory cytokines in the TPN group compared to the enterally-fed group (Figure 8). They concluded that changes in the intestinal environment and microbiota are responsible for the decline in epithelial barrier function (EBF) seen with TPN administration. They hypothesised that therapies directed towards maintaining an EBF-promoting intestinal microbiota might decrease infections in TPN-dependent patients²⁰.

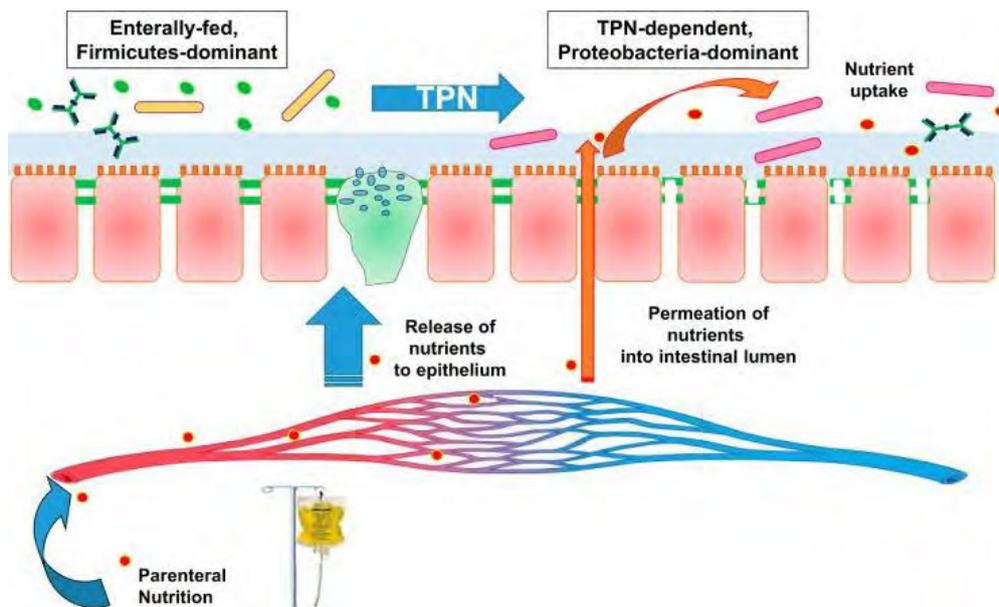


Figure 8: Summary of effects of TPN on the luminal metabolome and microbiome: With TPN dependence, an altered microbiome is associated with a loss of epithelial barrier function. TPN-derived nutrients are supplied to the epithelium while also permeating into the intestinal lumen via a trans-epithelial route, which is utilised predominantly by *Proteobacteria* in the intestinal lumen. Reproduced from Ralls MW et al., *Am J Physiol Gastrointest Liver Physiol.* 2016 Oct 1;311(4):G734-G743²⁰ with permission from the American Physiological Society.

Dysbiosis secondary to being in ICU: McDonald et al. characterised the microbiomes of 115 critically ill adult patients in ICU at two time points²¹. Trained hospital personnel collected faecal, oral, and skin samples from 115 ICU patients >18 years of age who were mechanically ventilated within 72 hours of ICU admission and at discharge or on ICU Day 10, where possible.

For faecal samples, the phylogenetic diversity at discharge was significantly lower than at admission. Faecal samples from ICU patients tended to have a lower relative abundance of Firmicutes and Bacteroidetes and an increased relative abundance of Proteobacteria (Figure 9).

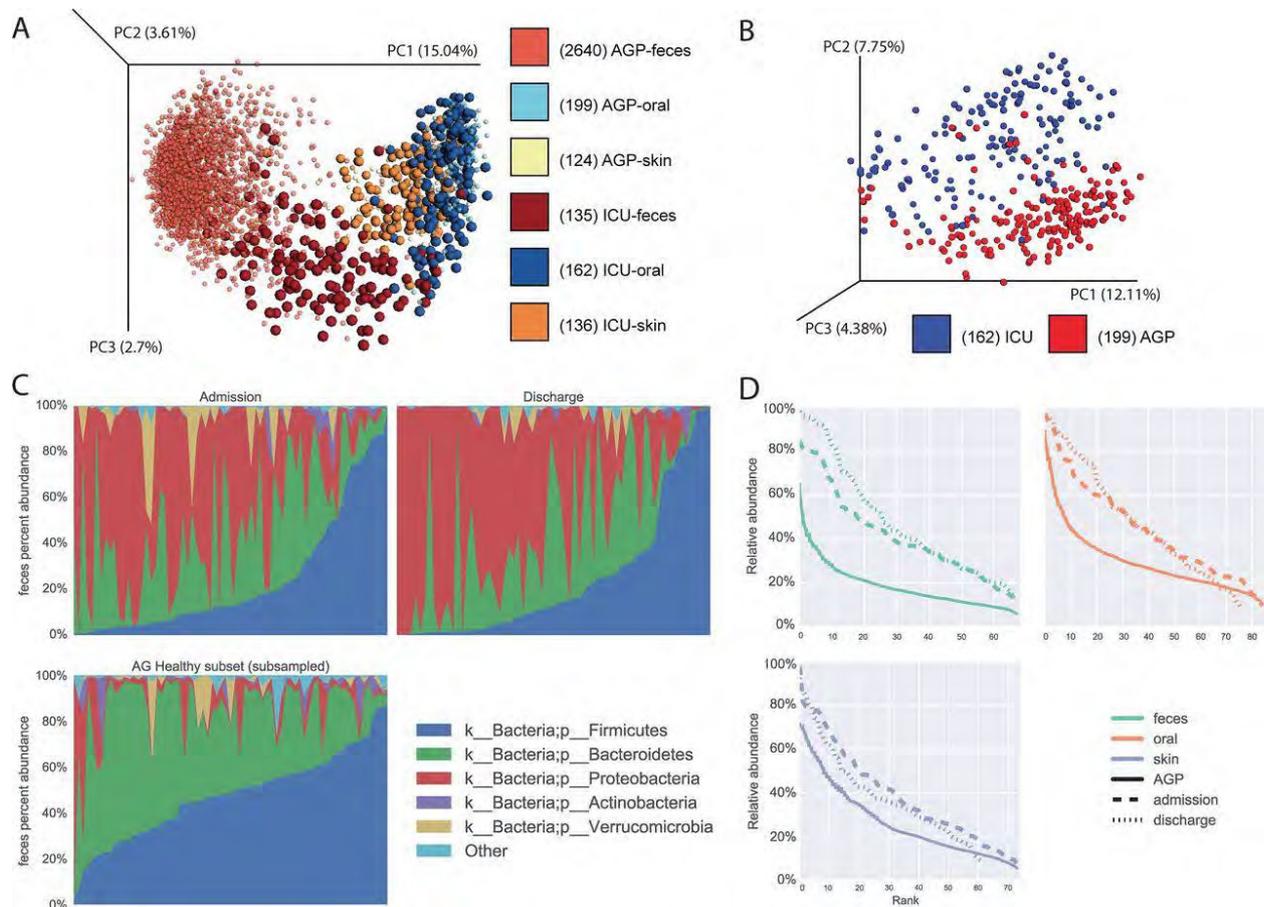


Figure 9: ICU patients differ markedly from healthy American Gut Project (AGP) subjects. (A, B) Principal-coordinate (PC) plots of unweighted UniFrac distances of both ICU patient and healthy AGP subject samples: A, faecal, skin and oral samples with the ICU patient samples enlarged to aid in differentiation; B, oral samples in isolation. (C) Stacked taxonomy bar charts for faecal split by time point, showing a random subsample of healthy AGP subject samples. (D) Rank-abundance curves for all three body sites split by time point, showing random subsamples from the healthy AGP subject subset. Reproduced from McDonald et al. *mSphere*. 2016 Aug 31;1(4). pii: e00199-16²¹ with permission from the corresponding author Prof Paul Wischmeyer (Creative Commons Attribution 4.0 International license).

They concluded that, regardless of the reason for admission, the microbiome of many critical-care patients differs substantially from a healthy population, disruption of the microbial community appears to be greater at a second-time point later in the ICU stay, and a set of

concerning inflammatory taxons co-occur. They advocated the need for studies on interventions to improve the microbiome composition in critical-care patients²¹.

Rogers et al.²² studied the microbial diversity in samples from 37 children in a paediatric ICU (PICU). Bacterial 16S rRNA gene sequences were analysed from 71 tongue swabs, 50 skin swabs, and 77 stool samples or rectal swabs. The mean age of patients was 2.9 years (range 1-9 years). Alpha diversity was decreased in PICU stool samples/rectal swabs and tongue samples compared with adults from healthy microbiome project (HMP) and healthy children. Taxonomic alterations in the PICU patients included enrichment of gut pathogens such as *Enterococcus* and *Staphylococcus* at multiple body sites and depletion of commensals such as *Faecalibacterium* and *Ruminococcus* from GI samples. They concluded that the microbiota of critically ill children is significantly different from that of healthy children and adults. They suggested that precise modulation of the microbiota could improve patient outcomes²⁰

Yeh et al.²³ have demonstrated that the microbiome of critically ill surgical patients undergoes a loss of diversity, site-specificity, and a shift towards dominant pathogens²³.

Moles et al.²⁴ evaluated gut microbiota of 26 preterm infants (gestation ≤ 32 weeks) admitted to the NICU using culture methods. The study had low risk of bias. Meconium and faecal samples were collected weekly during the first month of life and subsequently every 15 days until discharge. An additional faecal sample was collected from these infants at two years. A high proportion of antibiotic-resistant clones was detected in faecal samples during the NICU stay. Almost all infants were colonised by *Enterococcus faecalis* and *Enterococcus faecium* clones, while a more comprehensive genetic diversity was observed for the Gram-negative isolates. Multidrug-resistant high-risk clones were not recovered from the faecal samples of the 2-year-olds. They concluded that the gut of preterm infants admitted to the NICU might

be colonised by antibiotic-resistant and virulent high-risk lineages, which are subsequently replaced by antibiotic-susceptible community ones²⁴.

Dysbiosis secondary to surgery and anaesthesia: Liufu et al.²⁵ assessed the gut microbiota of mice aged 9 and 18 months before and after abdominal surgery under 1.4% isoflurane anaesthesia for two hours. They found that anaesthesia /surgery induced a significant change rate of reduction in the relative abundance of *Lactobacillus* as compared to the control condition at 9 hours and 11 days post-anaesthesia /surgery in the 18, but not 9, month old mice (Figure 10).

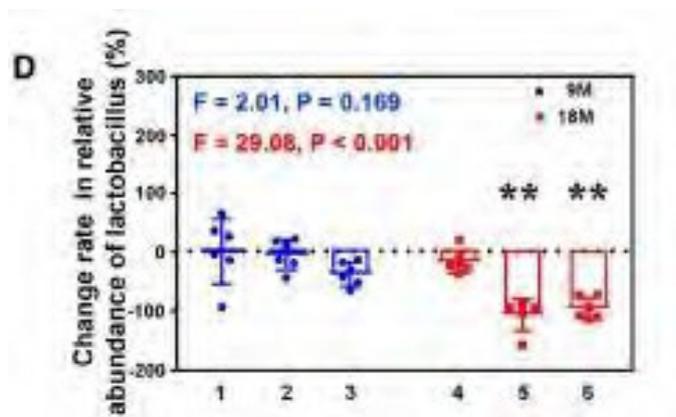


Figure 10: Anesthesia/surgery-induced significant change rate of reduction in the relative abundance of *Lactobacillus* as compared to the control condition at 9 hours and 11 days post-anesthesia/surgery in the 18, but not 9, months old mice. Blue font: 9-month-old mice. Red font: 18-month-old mice. Adapted from Figure 4D of Liufu N et al. *Aging (Albany NY)*. 2020 Jan 24; 12(2):1965-1986 (Permission was not required because it was published under the Creative Commons Attribution 3.0 License CC BY 3.0).

Aardema et al.²⁶ studied the gut microbiota of 97 adult patients undergoing cardiac surgery using 16S rRNA gene sequencing at three-time points: T1: Before admission; T2: During admission; T3: Post-discharge. During the hospital stay, a significant change in microbial composition occurred in most patients (Figure 11 below), with a considerable increase in pathogenic organisms combined with a decrease in strictly anaerobic gut bacteria. A lower

bacterial diversity during admission was associated with a more extended hospital stay. The microbiota reverted to the original composition post-discharge²⁶.

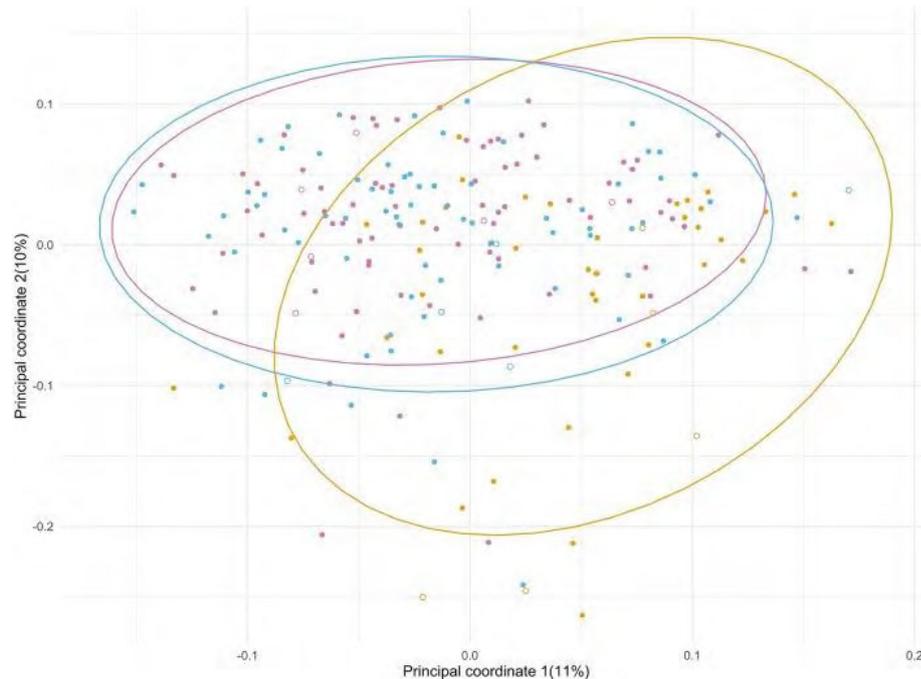


Figure 11: Principal coordinate analysis of the microbial composition of the samples from ICU patients undergoing cardiac surgery significantly changed over the course of the study period ($p < 0.001$). The different time points are depicted in purple (T1), yellow (T2), and blue (T3). Reproduced from Aardema H et al. *Front Cell Infect Microbiol.* 2020 Jan 15; 9:467²⁶ (Permission not required since it was an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

Overall, the above evidence, which comes predominantly from animal studies, adult human, and paediatric studies, indicates that being in the ICU, undergoing surgery and anaesthesia, and exposure to antibiotics and proton pump inhibitors results in gut dysbiosis. Evidence is also accumulating that dysbiosis is associated with adverse clinical outcomes.

The following discussion covers the evidence supporting the harmful effects of dysbiosis in early life.

Dysbiosis as a predisposing risk factor for parenteral nutrition-associated cholestasis: In a prospective study, Hourigan et al.⁸ investigated the composition of intestinal microbiota from serial stool samples of preterm twins *discordant* for parenteral nutrition-associated cholestasis (PNAC), i.e., twin pairs simultaneously receiving PN but only one twin developed PNAC. Twin sets were chosen as a strategy to reduce potential confounding variables such as intrauterine environment, genetics, and age. Eighty-four serial stool samples were included from four such twin sets. Beta diversity of the PNAC samples cluster was different from the non-PNAC samples cluster ($p < 0.001$). PNAC was associated with increased levels of Gram-negative bacteria such as *Klebsiella*, *Veillonella*, and *Enterobacter* and Gram-positive *Enterococcus*. None of the infants had blood culture-positive sepsis at any stage during the course of the study. The investigators contemplated whether increased intestinal colonisation alone, even without sepsis with these microbes, was sufficient to provoke cholestasis. They advocated for similar studies in a large population cohort and discussed the potential for probiotic supplementation to prevent or treat PNAC⁸.

Dysbiosis as a predisposing risk factor for sepsis: In a prospective study, Graspeuntner et al.⁴ assessed specific gut microbiome signatures in preterm infants <34 weeks of gestation preceding late-onset sepsis (LOS). Stool samples from study infants were collected on days 7, 14, and 21 of life. They selected 71 preterm infants who developed LOS and 164 control infants (no LOS/necrotising enterocolitis) and compared their faecal microbiota. A total of 607 faecal samples were analysed. In most cases, the bacteria isolated in blood cultures corresponded to the genera in the gut microbiome. LOS cases had a decelerated development of microbial diversity. Before the disease onset, LOS cases had specific gut microbiome signatures with a higher abundance of coagulase-negative *Staphylococci* and a deficiency of anaerobic bacteria⁴.

In another prospective study, Stewart et al.²⁷ compared stool bacterial profiles (n = 613) from 7 infants with LOS to 28 matched healthy (no LOS or NEC) controls²⁷. In all cases, the genera of the bacteria isolated in blood culture were present in the stool samples before LOS diagnosis. On the other hand, control infants had a predominance of *Bifidobacterium*. They speculated that *Bifidobacterium* is a marker of protection or may directly protect against gut epithelial translocation²⁷.

Other recent studies in very preterm infants have similarly concluded that significant preclinical microbial alterations occur in the preterm gut before the onset of LOS^{28,29}.

In an animal study by Singer et al.³⁰, 5-day-old pups were administered virulent *K. pneumoniae* (ATCC, 43816; Kp-43816) through a feeding tube into the stomach. About 24h later, intestinal translocation was confirmed by bioluminescence imaging in the livers of pups that developed sepsis, but not in those without sepsis. They concluded that *K. pneumoniae* dysbiosis and intestinal translocation led to LOS³⁰.

Dysbiosis as a predisposing risk factor for Hirschsprung-associated enterocolitis: Li et al.⁵ investigated the characteristics of the intestinal microbiome of HD children aged between 0.3-48 months with or without enterocolitis⁵. During surgery, they collected 35 intestinal content samples from five patients with HAEC, five HD patients without HAEC, and three patients in HAEC remission (HAEC-R). The microbiota differed significantly between the HD patients (characterised by the prevalence of Bacteroidetes) and HAEC patients (characterised by the prevalence of Proteobacteria). In contrast, the microbiota of the HAEC-R patients was more similar to that of the HAEC patients (Figure 12).

The intestinal microbiota of the HD patients was characterised by high levels of Bacteroidetes (45%), Firmicutes (24%), and Proteobacteria (16%). In contrast, the most abundant phylum detected in the HAEC patients was Proteobacteria (60%), followed by Firmicutes (30%). The microbiota of the HAEC-R patients was similar to HAEC patients and

was characterised by an increased abundance of Proteobacteria (70%) and Firmicutes (18%) and a reduced abundance of Bacteroidetes (Figure 12).

They concluded that HD patients had a relatively distinct, more stable community than the HAEC and HAEC-R patients, suggesting that enterocolitis may either be caused by or result in a disruption of the patient's intestinal microbiota⁵. Similar findings of altered gut microbiota in children with HAEC have been reported by other investigators³¹⁻³³

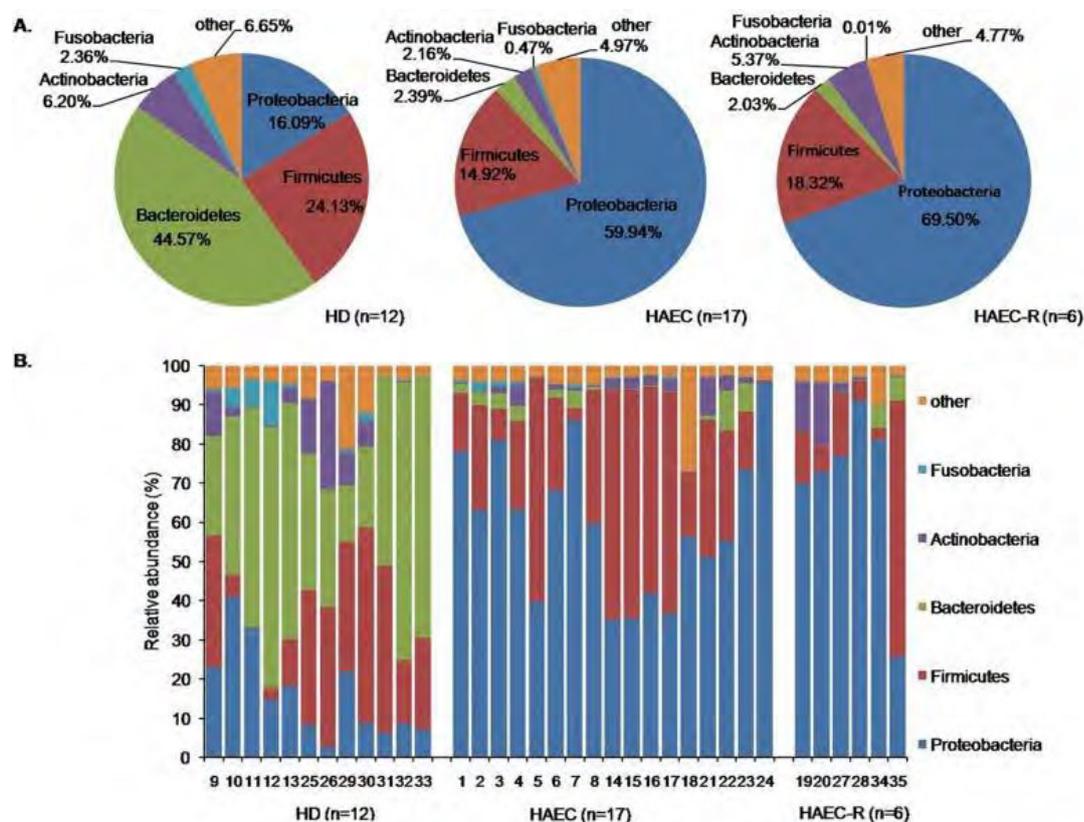


Figure 12: Relative abundance of phyla in the intestinal microbiomes of the HD, HAEC, and HAEC-R patients. (A) The pie charts show the average relative abundance of five major phyla. (B) The histograms demonstrate the bacterial composition of the stool samples at the phylum level. The X-axis shows the sample numbers. The colours assigned to each of the five detected phyla are shown on the right side. Reproduced from Li et al. *PLoS One*. 2016; 11(9): e0162079 (Permission not required since it was published under the terms of the Creative Commons Attribution License (CC BY)).

Dysbiosis as a predisposing risk factor for NEC in preterm infants: Pammi et al.⁶ performed a systematic review and meta-analyses of stool microbiome profiles in preterm

infants to describe microbial dysbiosis before the onset of NEC⁶. They included 14 studies in the review, of which eight were available for meta-analysis (106 NEC cases, 278 controls, 2944 stool samples). Preterm infants with NEC had increased relative abundances of proteobacteria and decreased relative abundances of Firmicutes and Bacteroidetes in their faecal samples before the onset of NEC (Figure 13).

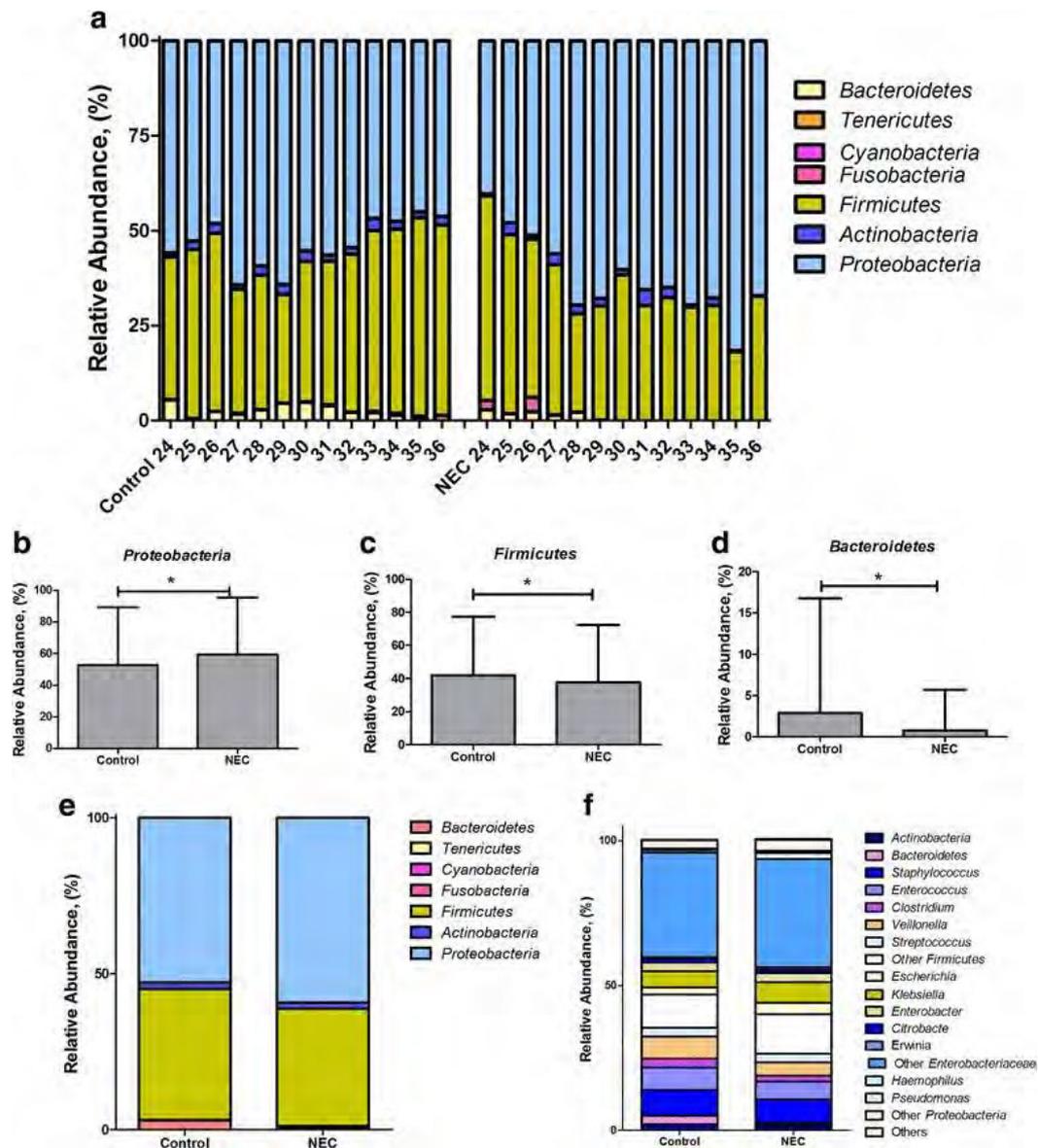


Figure 13: Comparison of taxonomic profiles between infants with necrotising enterocolitis (NEC) and controls. *a.* NEC infants had trends of increased relative abundance in Proteobacteria from 24 to 36 weeks corrected gestational age (CGA) accompanied by decreased abundances in Firmicutes and Bacteroidetes, relative to controls. In control infants, the relative abundance of Proteobacteria decreased after 27 weeks and coincided with an increase in Firmicutes and Bacteroidetes. *b–d*

*Phylum level differences between NEC cases and controls across CGA showed significant differences in Proteobacteria, Firmicutes, and Bacteroidetes (*p < 0.05). e, f Mean relative abundance distributions between NEC cases and controls at the phylum level (e) and genus level (f) when data from all CGAs are included. Reproduced from Pammi M, Microbiome. 2017 Mar 9; 5(1):31⁶ (Permission not required since it was published under the terms of the Creative Commons Attribution 4.0 International License (CC BY).*

In summary, evidence is emerging that exposure to parenteral nutrition^{20,34}, proton pump inhibitors³⁵, multiple antibiotics^{17,36-39}, ICU environment^{21,22,40}, and gut surgery and anaesthesia⁴¹ can increase the risk of dysbiosis. Evidence is also emerging that dysbiosis is associated with adverse clinical outcomes in ICU patients. Neonates with CGISC are probably more vulnerable to dysbiosis because, besides being exposed to the above risk factors, they undergo surgery of the gastrointestinal tract, where most of the bacteria are located. It is possible that when the lumen of the neonatal gut is exposed to the environment due to surgical incision, the hypoxic intraluminal environment will be replaced by the high oxygen content of the atmosphere. Since oxygen is a driver of dysbiosis⁴², such relative intraluminal hyperoxia can restrict the growth of beneficial anaerobes such as bacteroides and bifidobacteria and encourage the growth of pathogenic anaerobes such as proteobacteria.

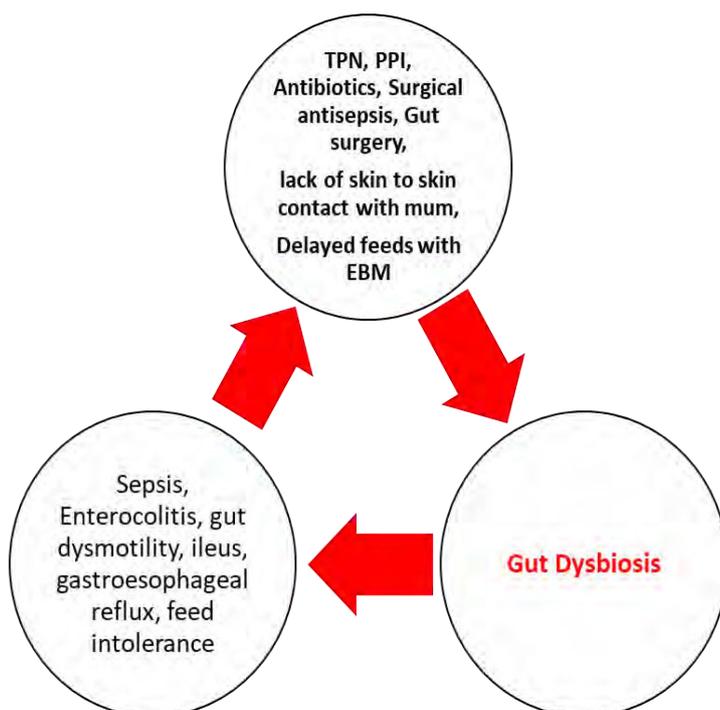


Figure 14: A vicious cycle wherein dysbiosis perpetuates in neonates with CGISC leading to worse clinical outcomes (PPI: Proton pump inhibitors; EBM: Expressed breastmilk).

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Preface to Chapter 5

Short-Chain Fatty Acids in Health and Disease

This chapter covers the biological functions of short-chain fatty acids (SCFAs) in humans. The main SCFAs are acetic acid, butyric acid, and propionic acid. They are synthesised in the colon by fermentation of non-digestible carbohydrates such as the human milk oligosaccharides by gut bacteria, especially bifidobacteria and bacteroides. We summarise the evidence from the literature that SCFAs have critical biological functions in humans, and sick patients have low faecal SCFA levels. Considering that neonates with congenital gastrointestinal surgical conditions (CGISCs) are usually critically ill and have delayed commencement of feeds, we speculate that they would be at risk of SCFA deficiency in their gut.

CHAPTER 5

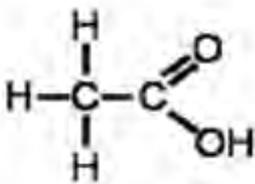
Short-Chain Fatty Acids in Health and Disease

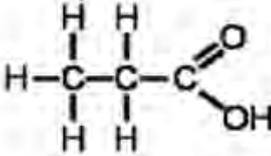
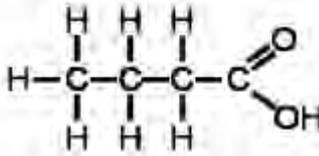
ABSTRACT

Short-chain fatty acids (SCFAs) are volatile saturated fatty acids with two to six carbon atoms in their structure. The main SCFAs include acetic, propionic, and butyric acid. They are synthesised in the colon by fermentation of non-digestible carbohydrates (NDC) such as the human milk oligosaccharides (HMOs) by gut bacteria, including bifidobacteria and bacteroides. Studies have shown that SCFAs have various biological functions in humans and that sick patients have low SCFA levels in their stools. Neonates with congenital gastrointestinal conditions (CGISCs) are usually critically ill and have delayed commencement of breast milk feeds and hence could be at risk of SCFA deficiency in their gut. Such deficiency of SCFAs could worsen the clinical outcomes of these vulnerable infants.

Introduction:

Short-chain fatty acids (SCFAs) are volatile acids with two to six carbon atoms in their structure. The main SCFAs include acetic (C2), propionic (C3), and butyric (C4) acids^{1,2}. Their chemical formulae and structure are given below.

Name of SCFA	Chemical formula	Chemical Structure
Acetic Acid (C2)	CH ₃ COOH	

Propionic Acid (C3)	$\text{CH}_3\text{CH}_2\text{COOH}$	
Butyric Acid (C4)	$\text{CH}_3(\text{CH}_2)_2\text{COOH}$	

SCFA Synthesis: SCFAs are synthesised mainly in the colon by fermentation of non-digestible carbohydrates (NDC) such as resistant starch (RS) and non-starch polysaccharides (NSP) present in plant-based fibre diets³, human milk oligosaccharides (HMO) in breastmilk²⁻⁶ and prebiotics including galactose-oligosaccharide (GOS) and fructose-oligosaccharide (FOS) present in some fortified formula milk⁷⁻⁹. Many of the health benefits of plant-based fibre diets, HMOs, and prebiotics are thought to be due to their effect on enhancing the composition and diversity of gut microbiota and the production of SCFA^{4,10-17}. In adults, plant-based dietary fibres exert beneficial effects on body weight, food intake, glucose homeostasis, insulin sensitivity and other aspects of human health. Epidemiological studies have found an association between higher fibre intake and reduced risk of inflammatory bowel disease, cardiac disease, irritable bowel syndrome, diabetes, and colonic cancer^{18,19}.

In infants, bifidobacteria and bacteroides have the necessary enzymes to utilise HMOs and hence breastfeeding facilitates the establishment of a gut microbiota dominated by bifidobacteria and bacteroides²⁰. In turn, these healthy bacteria inhibit the growth of pathogenic bacteria such as *Escherichia coli*, *Salmonella*, or *Campylobacter* by competing for colonisation sites^{21,22}. Since such nondigestible oligosaccharides (NDOSs) are virtually absent from cow milk, formula-fed infants usually have a deficiency of bifidobacteria and

bacteroides. To overcome this problem, fortifying cow's milk-based formula with prebiotics such as galactose-oligosaccharide (GOS) and fructose-oligosaccharide (FOS) has become common in recent times. FOS are the soluble fibres that occur naturally in vegetables such as onion, chicory, garlic, asparagus, banana and artichokes²³. They can be commercially produced from sucrose or through hydrolysis of chicory roots²⁴. GOS is a collective term for carbohydrates composed of oligo-galactose with some lactose and glucose. They are made commercially from lactose by β -galactosidase. The GOS and FOS are not digestible by the infant's intestine but are utilised by the gut microbiota, especially bifidobacteria and lactobacilli²⁵. Oligosaccharides resembling GOS occur naturally in human milk.²⁴ Infants fed breastmilk or GOS-FOS enriched-formula have high acetate levels and low amounts of butyrate and propionate in their colons, similar to breastfed infants²⁶.

In effect, since humans do not have the necessary enzymes to NDCs, they reach the colon intact, where the commensal bacteria utilise them as nutrition^{4,27-31}. The primary end products of this microbial fermentative metabolism of NDCs are SCFAs, H₂, and CO₂. Acetate, propionate and butyrate constitute 85%–95% of total SCFAs in the colon² and are present in an approximate molar ratio of 60:20:20, respectively.

The critical pathways of biosynthesis of SCFA and the microorganisms involved are given in the table below³².

Table 1: Biosynthesis of SCFAs in the intestines

SCFA	Biosynthesis	Microorganism
Acetate	From pyruvate in acetyl-CoA pathway	<i>Akkermansia muciniphila</i> , <i>Bacteroides</i> sp., <i>Bifidobacterium</i> spp., <i>Prevotella</i> sp., <i>Ruminococcus</i> sp.

	Reductive acetyl-CoA pathway (Wood-Ljungdahl pathway)	<i>Blautia hydrogenotrophica</i> , <i>Clostridium</i> spp., <i>Streptococcus</i> sp.
Propionate	Succinate pathway	<i>Bacteroidetes</i> sp, <i>Roseburia</i> sp., <i>Firmicutes</i> , <i>Roseburia inulinivorans</i> , <i>Ruminococcus</i> spp., <i>Clostridium</i> sp., <i>Clostridiales bacterium</i> , <i>Eubacterium</i> sp, <i>Coprococcus</i> sp., <i>Dialister succinatiphilus</i> , <i>Phascolarctobacterium succinatutens</i> , <i>Akkermansia muciniphila</i>
	Acrylate pathway	<i>Clostridium</i> sp., <i>Clostridiales bacterium</i> , <i>Coprococcus catus</i> , <i>Clostridium</i> sp.,
	Propanediol pathway	<i>Roseburia insulinivorans</i> , <i>Ruminococcus</i> sp., <i>Eubacterium halli</i> , <i>Clostridium</i> sp
Butyrate	Butyryl-CoA transferase: acetate Co-A pathway	<i>Roseburia intestinalis</i> , <i>Eubacterium rectale</i> , <i>Ruminococcus</i> , <i>Roseburia insulinivorans</i> , <i>Clostridiales bacterium</i> , <i>Anaerostripes hadrus</i> , <i>Coprococcus</i> spp, <i>Clostridium symbiosum</i> , <i>Faecalibacterium prasnitzii</i>
	Butyrate kinase pathway	<i>Bacteroidetes</i> spp., <i>Coprococcus</i> sp

Adapted from Ratajczak W et al *Acta Biochim Pol.* 2019 Mar³². Permission to reproduce the table was not required as it was published under the Creative Commons Attribution-ShareAlike 4.0 International (CC BY-SA 4.0) licence.

SCFA Pathway after production in the colon:

The concentration of SCFAs is highest in the caecum and proximal colon and diminishes downstream in the distal colon (Figure 1).

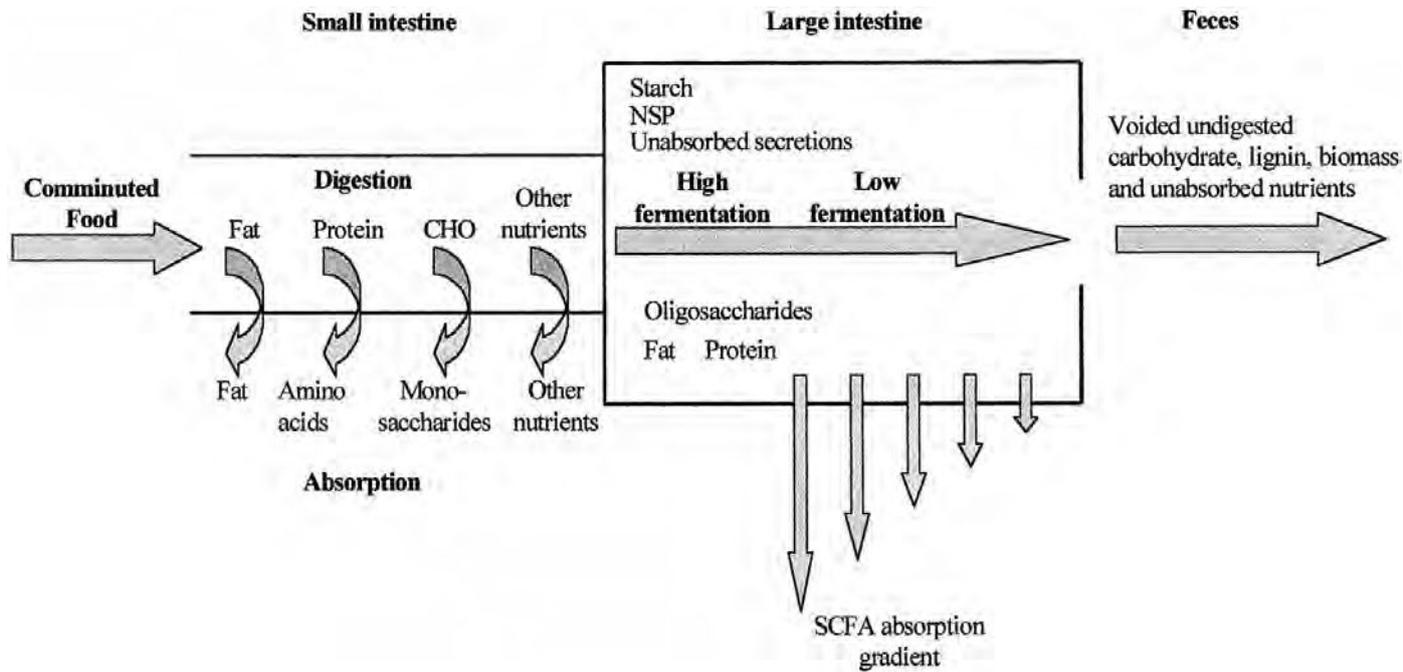


Figure 1: An overview of the relationship between the transit of food through the gut and the digestion of nutrients in the small intestine and fermentation in the cecum and colon. Food is digested in the upper GIT and absorbed in the small intestine. Unabsorbed food passes through the ileocecal valve and is fermented, leading to SCFA production. SCFA production and absorption is high in the proximal large intestine. Downstream, fermentation declines due to substrate depletion, so SCFA concentration falls. The distal colon and rectum are the regions of the large bowel with a limited supply of SCFA and the sites of most pathology. Bacteria and unfermented components are finally excreted in the faeces. Reproduced from Topping et al. ³³ *Physiol Rev.* 2001 Jul;81(3):1031-64 with permission from The American Physiological Society.

Nearly 95% of the SCFAs produced in the colon are absorbed by colon epithelial cells, with 5% being excreted in the stools. Once within the colonic cells, the SCFAs are partly oxidised to CO₂, producing energy (ATP) for the colonic epithelium. SCFAs not metabolised by colonic cells enter the portal circulation to reach the liver, providing an energy substrate for hepatocytes via oxidation. SCFAs are also incorporated in hepatocytes during the biosynthesis of glucose, cholesterol, and fatty acids³⁴ (Figure 2). Only a fraction of the colon-derived acetate, propionate, and butyrate (36%, 9% and 2%, respectively) reaches the systemic circulation and peripheral tissues.

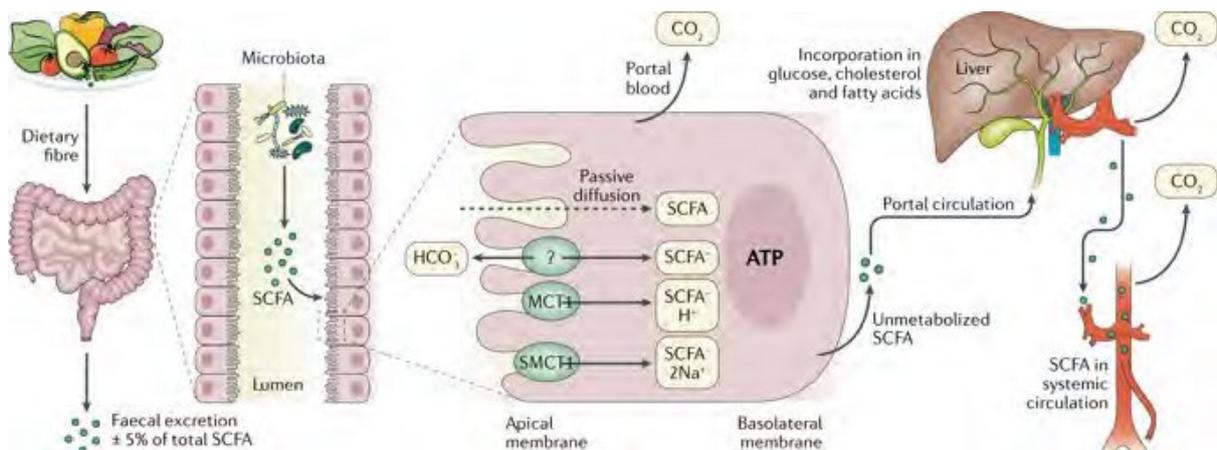


Figure 2: Fermentation of dietary fibre by commensal bacteria in the colon leads to the production of SCFAs, which are rapidly absorbed by colonic cells via monocarboxylate transporters, passive diffusion, or exchange with bicarbonate (HCO_3^-) via an exchanger and then oxidised to CO_2 , producing energy for the cells in the form of ATP. SCFAs that are not metabolised by colonic cells travel via the basolateral membrane into the portal circulation to the liver, providing an energy substrate for hepatocytes via oxidation. SCFAs are also incorporated in hepatocytes during the biosynthesis of glucose, cholesterol, and fatty acids. Thus, only small amounts of the SCFAs reach the systemic circulation. MCT1, monocarboxylate transporter 1; SMCT1, sodium-dependent monocarboxylate transporter 1. Reproduced from Dalile et al. ³⁴Nature Reviews, August 2019 with permission from Springer Nature.

Biological Functions of SCFAs:

Provision of energy to intestinal epithelial cells: SCFAs provide energy to the intestinal epithelial cells (IECs), with nearly 70% of the energy used by IECs being supplied by butyric acid produced by commensal bacteria, especially ruminococcus and faecalibacterium².

Maintenance of acidic PH: Acetate helps maintain the colon's acidic PH, which in turn suppresses the proliferation of pathogenic bacteria.

Intestinal mucous production: SCFAs help mucous production in the gastrointestinal tract via the synthesis of mucin-MUC2 protein. Mucus acts as a natural lubricant, decreasing the interaction between the epithelial cells and luminal microorganisms and toxic agents and protecting these cells from the fluctuating acidity^{35,36}. Bacteria-derived butyrate affects

epithelial O₂ consumption and stabilises hypoxia-inducible factor (HIF), a transcription factor coordinating barrier protection³⁷.

Immune modulation: SCFAs induce metabolic alterations in T cells by enhancing the activity of the mTOR complex and by regulating their glucose metabolism. Once taken up into T lymphocytes, SCFA-derived acetyl groups contribute to the cellular acetyl-CoA pool, influencing histone acetylation and cytokine gene expression³⁸. SCFA enhance the immune system through G-protein-coupled receptors (GPR41, GPR43, GPR109A) and Olfr78 receptor signalling. They regulate the histone deacetylase (HDAC) activity, which inhibits nuclear factors (nuclear factor- κ B; NF- κ B). SCFAs affect the differentiation of regulatory T (Treg) cells and the production of interleukin-10 (IL-10) with the participation of GPR43. They also regulate dendritic cell function. In addition, SCFAs influence AIM2 and NLRP3 inflammasomes activation, affecting the production of interleukin-18 (IL-18) and enhanced epithelial barrier function. The NLRP6 inflammasome activation and secretion of IL-18 regulate the production of intestinal antimicrobial peptides (AMPs) (Figure 3).

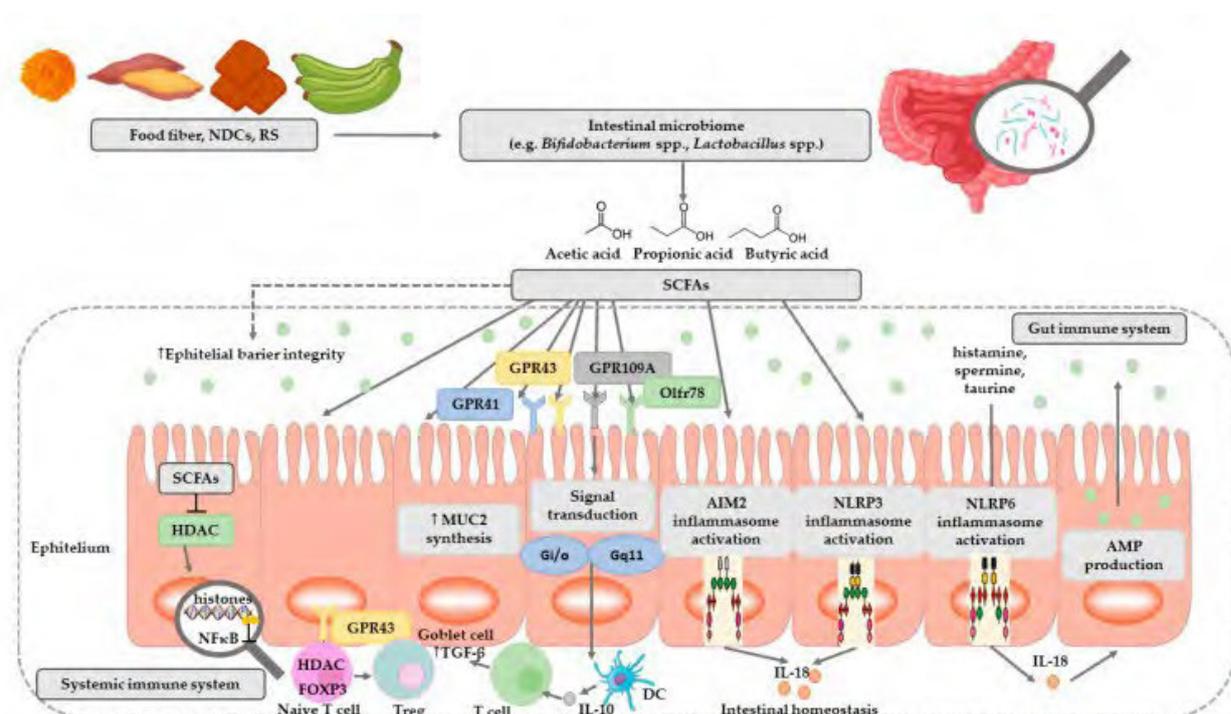


Figure 3: *The role of SCFAs in the regulation of intestinal homeostasis. SCFAs are energy substrates for colonocytes and regulate intestinal barrier function (synthesis of mucin-MUC2) and immune system through G-protein-coupled receptors (GPR41, GPR43, GPR109A) and Olfr78 receptor signalling. SCFAs regulate the histone deacetylase (HDAC) activity which affects inhibition of nuclear factors (nuclear factor- κ B; NF- κ B). SCFAs affect the differentiation of regulatory T (Treg) cells and the production of interleukin-10 (IL-10) with the participation of GPR43. SCFAs also regulate dendritic cell (DC) function. In addition, SCFAs influence AIM2 and NLRP3 inflammasomes activation, affecting the production of interleukin-18 (IL-18) and enhanced epithelial barrier function. Moreover, NLRP6 inflammasome activation and secretion of IL-18 regulate the production of intestinal antimicrobial peptides (AMPs). Abbreviations: FOXP3-forkhead box P3; TGF- β -transforming growth factor β . Reproduced from Markowiak-Kopeć P et al. *Nutrients*. 2020 Apr16;12(4):1107². Permission to reproduce the figure was not required since it was published under the Creative Commons Attribution 4.0 International (CC BY 4.0) licence.*

Protection against respiratory infections: In an animal study, Antunes et al. demonstrated that acetate protected against RSV-induced disease by improving type 1 interferon responses and increasing interferon-stimulated gene expression in lung epithelial cells.³⁹

Effect on the microbiota-gut-brain axis: SCFAs are thought to play a vital role in microbiota-gut-brain crosstalk^{34,40}. The gut-brain axis refers to the bidirectional signalling mechanisms between the gastrointestinal tract and the central nervous system (CNS)⁴¹ (Figure 4).

and its putative effects on human brain development, behaviour, cognition, and mood are an area of intense research.

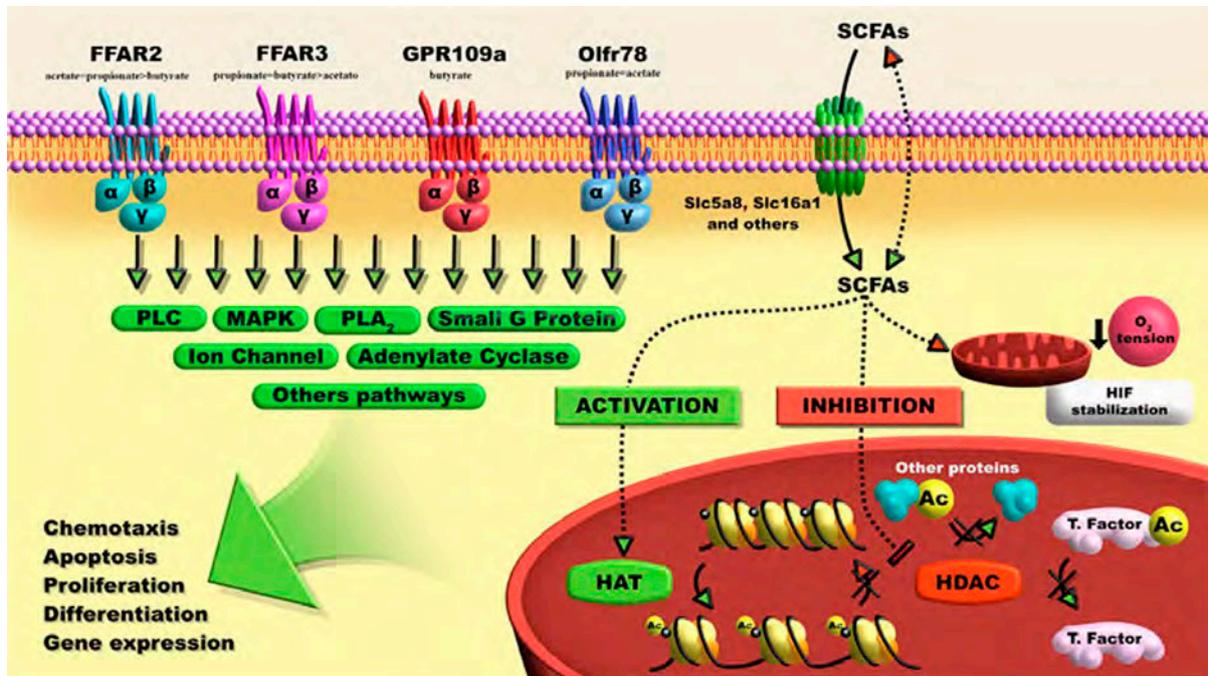


Figure 5: Cellular signaling pathways activated by the short-chain fatty acids. SCFAs activate membrane receptors called GPCRs (FFAR2, FFAR3, GPR109a and Olfr78). They can also reach the cytoplasm of the cells through transporters (Slc16a1 and Slc5a8) or passive diffusion across the plasma membrane. They modulate the activity of several enzymes and transcription factors, including the HIF, HDACs and histone acetyltransferase (HAT). SCFAs modify several cellular processes, including gene expression, chemotaxis, differentiation, proliferation, and apoptosis, through these mechanisms. Reproduced from Corrêa-Oliveira R et al., *Clinical and Translational Immunology*⁴³, Publisher: John Wiley and Sons. Permission to reproduce the figure was not required since it was published under the Creative Commons CC BY license.

Intestinal motility: SCFAs have both excitatory⁴⁴ and inhibitory function⁴⁵ on gut motility. In vitro studies have reported that different SCFAs have different effects on colonic segments, and that the net effect of SCFAs on colonic motility would depend on the balance of SCFAs⁴⁶. A recent study⁴⁷ demonstrated that the SCFAs acetate, propionate, and butyrate

could stimulate Ca²⁺ signalling in the intestinal mucosal epithelium and in submucosal and myenteric neurons.

Anticancer effects: Butyrate is known to exhibit inhibitory effects on tumorigenesis and might reduce the risk of colorectal cancer.⁴⁸

Metabolic effects: Propionic acid produced mainly by Bacteroidetes and Firmicutes⁴⁹ is an inhibitor of gluconeogenesis and cholesterol synthesis in the liver.

Table 2: Summary of Biological Functions of SCFA

1	Provide Nutrition to the intestinal epithelial cells
2.	Facilitate glucose homeostasis, lipid metabolism
3.	Immune modulation
4.	Anti-Inflammatory
5.	Anti-Tumorigenic
6.	Enhancement of gut integrity and improved gut motility
7.	Antimicrobial effect
8.	Appetite regulation
9.	Stimulate colonic blood flow and fluid and electrolyte uptake
10.	Lower the colonic pH, which can promote the growth of beneficial bacteria, such as Lactobacillus and Bifidobacterium
11	Mediators in the Gut-Brain Axis

SCFA levels in critically ill patients: Many studies have evaluated stool SCFA levels in critically ill patients. In a multicentre observational study, Valdés-Duque et al.¹ measured the stool SCFA levels in 44 critically ill patients with sepsis admitted to ICUs and 45 controls. Propionic acid, acetic acid, butyric acid, and isobutyric acid concentrations were significantly

lower in critically ill patients than controls. However, SCFA levels were not associated with clinical outcomes was observed.

In another study, Yamada et al.⁵⁰ measured stool SCFA levels in 140 ICU patients with systemic inflammatory response syndrome (SIRS) every week and compared them to SCFA from healthy volunteers. The aetiology of SIRS was an infection in 78 patients, trauma in 30, burns in 12, and others in 20. Faecal butyrate, propionate, and acetate levels were significantly lower than in healthy volunteers and remained low throughout the six weeks of ICU stay. They suggested that maintaining adequate SCFA levels may improve the outcomes of critically ill patients⁵⁰.

In a retrospective study, Nakahori et al.⁵¹ evaluated the relationship between faecal organic acids and mortality in 128 critically ill patients, of which 90 survived, and 38 died. Low propionate and acetate levels were associated with higher odds of mortality.

SCFA levels in neonates: Siigur et al reported that total SCFA levels were 45.9 micromol/gram on day 3 of life, 59.9 by day 6, and 58-74 by two months⁵². Bridgman reported that by 3-5 months of age, the levels were 142.0 (101.6–203.8) micromol/gram⁵³. Such gradual increase in SCFA levels is due to increased microbial diversity over the same period.

Summary and conclusions:

SCFAs have various biological functions in humans, and sick patients in ICU have low levels of SCFA in stools. Neonates with CGISCs are usually critically ill and hence theoretically at risk of SCFA deficiency. The probable reasons for SCFA deficiency are delayed commencement of breast milk feeds, leading to decreased intake of HMOs and reduced utilisation of HMOs due to the deficiency of bifidobacteria and other anaerobes. Such deficiency of SCFAs could worsen the clinical outcomes of these vulnerable infants.

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Preface to Chapter 6

Gut Microbiota in Neonates with Congenital Gastrointestinal Surgical Conditions: A Prospective Study

The previous two chapters presented evidence about the importance of gut microbiota and short-chain fatty acids (SCFA) for health and wellbeing and that sick patients in intensive care units (ICUs) have gut dysbiosis and deficiency of SCFAs. This chapter provides evidence from our prospective study that neonates with congenital gastrointestinal surgical conditions develop gut dysbiosis and deficiency of SCFA during their stay in neonatal intensive care units.

The results of this study were published in *Pediatric Research* (March 2020)

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CLINICAL RESEARCH ARTICLE

Gut microbiota in neonates with congenital gastrointestinal surgical conditions: a prospective study

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BACKGROUND: There is limited information on gut microbiota of neonates with congenital gastrointestinal surgical conditions (CGISCs) available.

METHODS: This study compared stool microbiota and short-chain fatty acids (SCFAs) of 37 term infants with CGISCs with 36 term healthy infants (HIs). Two stool samples were collected from each infant: as soon as possible after birth (week 1) and 10–14 days of life (week 2).

RESULTS: Bacterial richness and alpha diversity were comparable between CGISCs and HIs at week 1 and week 2 (all $p > 0.05$). Beta diversity analysis revealed that at week 1, CGISCs had similar community structures to HIs ($p = 0.415$). However, by week 2, community structures of CGISCs were significantly different from HIs ($p = 0.003$). At week 1, there were no significant differences in the relative abundances of genera *Bifidobacterium* and *Bacteroides* between CGISCs and HIs. At week 2, the relative abundance of *Bifidobacterium* was significantly lower in CGISCs (mean percentage 7.21 ± 13.49 vs. 28.96 ± 19.6 ; $p = 0.002$). *Bacteroides* were also less abundant in the CGISC group (mean percentage 0.12 ± 0.49 vs. 6.59 ± 8.62 ; $p = 0.039$). Relative abundance of genera *Pseudomonas* and *Escherichia-Shigella* were higher in CGISCs. At week 2, stool concentrations of all SCFAs were lower in CGISCs (all $p < 0.001$).

CONCLUSIONS: During hospitalization, neonates with CGISCs develop gut dysbiosis and deficiency of SCFAs.

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IMPACT:

- During hospitalisation, neonates with congenital gastrointestinal surgical conditions develop gut dysbiosis with deficiency of Bifidobacteria and Bacteroides and increased abundance of Escherichia-Shigella and Pseudomonas. They also have low levels of short chain fatty acids in their stools compared to healthy infants.
- This is the first study evaluating the gut microbiota using 16S ribosomal RNA sequencing methods and stool short chain fatty acids in neonates with congenital gastrointestinal surgical conditions and comparing them to healthy infants.
- The findings of this study will pave the way for randomised trials of bifidobacterial supplementation in neonates with congenital gastrointestinal surgical conditions.

INTRODUCTION

The major congenital gastrointestinal surgical conditions (CGISCs) are gastroschisis, exomphalos, duodenal atresia, small and large intestinal atresia, oesophageal atresia, congenital short bowel syndrome (SBS), malrotation and volvulus, meconium ileus, hypoplastic left colon, Hirschsprung disease (HD), anorectal malformations and others.

Common morbidities in these conditions are feed intolerance and increased risk of infections. Infants with CGISCs are cared for in intensive care units and do not receive breastmilk in the first few days of life. They undergo invasive procedures and do not receive adequate skin-to-skin contact with their mothers. They get exposed to gastric acid suppressants, parenteral nutrition and

multiple courses of antibiotics. All these factors have the potential to increase the risk of gut dysbiosis.^{1–3}

While the gut microbiota of extremely preterm non-surgical infants has been well studied using culture-independent genomic approaches,⁴ there is very limited information on gut microbiota of term infants with CGISCs. The studies that evaluated gut flora in neonates with surgical conditions in the past were based on the conventional culture-dependent techniques.⁵ However, a growing body of evidence in the recent decade has shown the importance of culture-independent, genomic approaches in understanding the role of the human microbiota in health and disease.⁶ Hence, we conducted this prospective study to investigate the gut microbiota in term neonates with CGISCs using culture-independent techniques.

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Many of the biological functions of healthy gut microbiota are mediated via short-chain fatty acids (SCFAs), such as acetic acid, butyric acid and propionic acid. SCFAs have important biological functions in humans, such as immune modulation, anti-inflammatory, anti-tumorigenic and antimicrobial effects and enhancement of gut integrity.⁷ They are thought to play a key role in microbiota–gut–brain crosstalk.⁸ In infants, SCFAs are the by-products of fermentation of human milk oligosaccharides (HMOs) by the anaerobic bacteria in the colon. HMOs are not digested by the small intestine of infants and hence they reach the colon intact, where they are utilized as nutrition by bacteria such as *Bifidobacteria* and *Bacteroides*. Since not all bacteria have the necessary enzymes to utilize HMOs, these milk glycans facilitate the establishment of a highly specialized microbial ecosystem dominated by *Bifidobacteria* and *Bacteroides* among others, while indirectly limiting growth of other bacteria.⁹ It is possible that neonates with CGISCs have insufficient amounts of anaerobes such as *Bifidobacteria* and *Bacteroides*, which in turn could result in decreased utilization of HMOs, and hence lower amounts of SCFAs in the colon. Given the importance of SCFAs in human health and the interplay between gut microbiota and SCFAs,⁷ we investigated stool SCFA levels in these infants.

METHODS

This was a prospective cohort study in which neonates with CGISCs were recruited from the neonatal intensive care unit of Perth Children's Hospital (PCH) and healthy infants (HIs) were recruited from the postnatal ward of King Edward Memorial Hospital (KEMH), Perth, Western Australia.

The study was approved by the institutional human research ethics committees of both hospitals. Informed parental consent was obtained for all studied infants.

Eligibility criteria

Neonates (≥ 36 weeks) with gastroschisis, exomphalos, Hirschsprung disease, duodenal atresia, other intestinal atresia, congenital diaphragmatic hernia, oesophageal atresia, congenital SBS and conditions needing enterostomy. Controls were healthy term newborn infants (≥ 36 weeks). We chose to include infants born at 36 weeks also instead of the conventional definition of 37 weeks because many infants with surgical conditions are born at 36 weeks and would not have been eligible for inclusion, thereby resulting in difficulty in achieving the sample size. Preterm infants <36 weeks were excluded to avoid the confounding effect of prematurity, which itself is a significant risk factor for gut dysbiosis. Even though congenital diaphragmatic hernia is not truly a gastrointestinal (GI) condition, we decided to include it because the intestines are not in the abdominal cavity throughout pregnancy in this condition.

Outcomes

Stool microbiota using 16S ribosomal RNA gene sequencing, and SCFAs using modified gas chromatography-mass spectrometry (GC-MS) were measured on samples collected as soon as possible after birth/admission (week 1) and 10–14 days of life (week 2).

Stool sample collection

Two stool samples were collected in sterile containers from each consented infant. The first sample was collected as soon as possible after birth/admission (week 1) and the second sample was taken between 10 and 14 days of life (week 2). All neonates with CGISCs were inpatients at the time of week 1 as well as week 2 sample collection. The week 1 samples of healthy term infants were collected while in hospital, whereas the week 2 samples were collected in sterile containers using sterile spatula at home by parents and kept in the refrigerator at home. Parents were advised to do thorough hand washing before collecting the

samples and to screw the lid tightly immediately after collection to prevent contamination. Accredited couriers retrieved the samples from infants' homes in cooler bags with ice packs within 48 h of collection by parents. Subsequently, all stool samples were initially stored at 20 °C for 3–5 days and subsequently at 80 °C. At the completion of recruitment of all study participants, samples were shipped on dry ice (carbon dioxide) to the University of New South Wales (Sydney, Australia), where microbial analysis was undertaken. Acidified samples were shipped on dry ice to the School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore, where SCFA analysis was carried out.

DNA extraction

DNA was extracted from stool samples using the method of Matsuki et al.¹⁰ Briefly, stool samples were thawed on ice, diluted 10-fold with sterile water and bacterial cells harvested by centrifugation. DNA was subsequently extracted from the bacterial cells using chemical and physical lysis methods. DNA was stored at 80 °C.

PCR amplification and 16S rRNA sequencing

Polymerase chain reaction (PCR) amplification of the V3–V4 region of the 16S ribosomal RNA (rRNA) gene was conducted using the 341 F' (5'-CCTACGGGNGGCWGCAG-3') and 785 R' (5'-GACTAC HVGGGTATCTAATCC-3') primers with the Nextera indexes. Purified PCR products were submitted to the Ramaciotti Centre for Genomics (UNSW, Sydney, Australia) for library preparation and sequencing on the Illumina MiSeq platform using the MiSeq Kit v3 (2 × 300 cycles).

rRNA sequence analysis

16S rRNA sequence data were initially quality filtered and trimmed using TRIMMOMATIC VERSION 0.36 truncating reads if the quality was found to be below 12 in a sliding window of 4 bp. Reads shorter than 100 bp were discarded after quality trimming. USEARCH version 11.0.667 was used to merge forward and reverse reads between 350 and 550 nucleotides. Primer sequences were truncated with cutadapt version 2.5.¹¹ Reads with no detectable primers were removed from the further analysis. Afterwards, reads were quality filtered using USEARCH. All reads with an expected error of more than 2 and more than 1 ambiguous base were removed. All sequences of all samples were concatenated in a single file and subsequently dereplicated to form unique sequences. Unique sequences were clustered into zero-radius operational taxonomic units (zOTUs, also called ASVs) using the UNOISE3 algorithm implemented in USEARCH. Chimeras were removed de novo during clustering and in reference mode using the UCHIME2 algorithm together with the SILVA SSURef NR99 v132. Processed, concatenated sequences were mapped on the final set of zOTUs to determine their occurrence and abundance in each sample using the `otu` tab command with an identity cut-off of 97% and termination options disabled, which means that every sequence is searched against every zOTU to find the best hit. Taxonomy was assigned to each zOTU using the SINA aligner version 1.6¹² and the SILVA SSURef NR99 v132 database.

For alpha diversity measures, each sample was subsampled 100 times to a count of 25,400 counts per sample and the average was taken. OTU richness and diversity indices, Shannon, ACE and Chao1, were calculated in R (version 3.5.1) using the *vegan* package. Relative abundance analysis at the Phylum, Family and Genus levels were carried out using *phyloseq* package in R. Data were visualized using *ggplot2* and *ggpubr* packages.

For beta diversity analysis, data were square root transformed. To generate a phylogenetic tree for diversity computations, zOTUs were aligned with *muscle* (version 3.8.31)¹³ and the tree was calculated with *RaxML* (version 8.2.10)¹⁴ using the GTRGAMMA model. Weighted unifracs distances were calculated and visualized on a principal coordinate analysis plot.

Statistical considerations

At the time of commencing this study, to our knowledge, there were no studies that had evaluated gut microbiota in neonates with CGISCs using culture-independent techniques. In the absence of baseline data, we aimed to study 35 neonates with CGISCs and 35 healthy term infants. Since the gut microbiota changes rapidly in the first few months of life, we attempted to collect the stool samples from cases and respective controls within ± 2 days of each other.

Statistical analysis of clinical data

Summary data for continuous variables with normal distribution were reported using mean \pm SD. Median and range were used to report data with skewed distribution. Continuous variables were compared using the *t* test for normally distributed data and Wilcoxon' rank-sum test for skewed data. Binary outcomes were compared using the Fisher's exact test. A *p* value of <0.05 was considered statistically significant.

Statistical analysis of microbiota data

All data analyses were conducted with R version 3.5.1. For microbial richness, linear mixed model effects (LME) test (*MASS*, *lme4* and *lmerTest* packages) was used to identify if there were statistical differences between the groups over time as well as between the groups at the two timepoints. In our model, Patient ID was a random factor, while time and treatment were used as fixed factors. Post hoc pairwise comparisons between the groups were performed using Tukey's HSD (honestly significant difference) method to adjust for multiple comparisons.

Differential abundance of phyla and genera were examined using the Wilcoxon's rank-sum test.

For beta diversity, PERMANOVA (permutational multivariate analysis of variance) was used to check if community structures differed between the groups at the two timepoints followed by pairwise.adonis test (<https://github.com/bwemheu/pairwise.adonis>) for pairwise comparisons between the groups. *P* values were adjusted for multiple testing using the Benjamini–Hochberg correction.

For all analyses, a *p* value of <0.05 was considered statistically significant.

Quantification of SCFAs in faecal samples of study infants

Faecal SCFA analyses were performed using a modified GC-MS method.¹⁵ Acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid and 4-methyl valeric acid were purchased from Sigma-Aldrich (Merck, Singapore). First, 1 g of the faecal sample was suspended in 5 mL of 1% phosphoric acid and frozen at -20°C immediately after collection. Once thawed, the faecal suspensions were homogenized with vortex. Then, 100 μL of 10% meta-phosphoric acid solution was added to 0.5 mL baby faecal sample to adjust the pH to about 2.0. Samples were vortexed for about 10 min and centrifuged for 30 min at $20,817 \times g$, 4°C to solidify the precipitate. After that, 0.5 mL of aqueous supernatant was transferred into a new tube; 4-methyl valeric acid was added as internal standard (IS) to a final concentration of 500 μM . Then, 250 μL of ethyl acetate was added to extract SCFAs with a vortex for about 30 min and centrifuged for 20 min at $20,817 \times g$; lastly, 50 μL volume of organic extracts were transferred into GC glass vial for GC-MS analysis. One microliter of organic extracts was injected into GC-MS system (Agilent Technologies 7890B-5977B) equipped with a HP-FFAP capillary column (30 m \times 0.250 mm \times 0.25 μm ; Agilent). Helium was used as the carrier gas at 1 mL/min. Split ratio is 30:1. The oven temperature was initially held for 1 min at 80°C , then increased to 120°C at $20^{\circ}\text{C}/\text{min}$, and finally to 210°C at $6.13^{\circ}\text{C}/\text{min}$ and held for 2 min. The temperature of the injector, ion source, quadrupole, and interface were 250, 230, 150, and 280°C respectively. The SCFA data analyses were performed in duplicate. Identification of the SCFAs was based on the retention time of standard compounds and with the assistance

of the NIST 17 library. Quantifications were carried out in selected ion monitoring acquisition mode in MassHunter Acquisition software with base peak ion selected as quantifier for each compound. The calibration graphs were constructed in MassHunter Quantitative software (version B.09.00) by plotting the relative response (ratio of peak area SCFAs/peak area IS) vs. relative concentration for each individual SCFAs. The final SCFA concentrations were expressed as microgram per gram wet weight faecal sample. Since the data were not of normal distribution, Wilcoxon's rank analysis was performed to compare SCFA concentrations between the two groups (CGISCs and HIs).

Linearity and sensitivity

A stock solution containing the mixture of standards (20 mM final concentration each) in ethyl acetate was diluted to obtain a calibration curve ranging from 2 to 15,000 μM . IS was added to each diluted standards mixture (500 μM final concentration).

The calibration graphs were constructed by plotting the ratio peak area SCFAs/peak area IS vs. concentration for each individual SCFAs. By normalizing the peak area to that of the IS, the variability in the instrument response was corrected (in particular, the injection volume variability and the MS response). Each point of the calibration graph corresponds to the mean value from independent replicate injections.

The limits of detection (LOD) and limits of quantification (LOQ) of the individual analytes were obtained by injecting successively more diluted standard solutions and were calculated according to the International Union of Pure and Applied Chemistry¹⁶ method based on a signal-to-noise ratio of 3 for the LOD and of 10 for the LOQ.

Reporting

STROBE checklist was followed for reporting the results of this observational study.¹⁷

RESULTS

In total, 37 CGISCs and 36 HIs were recruited into the study. The surgical conditions in the CGISC group were oesophageal atresia: 4; gastroschisis: 9; malrotation: 4; duodenal atresia: 4; small intestinal atresia: 2; colonic atresia: 1; imperforate anus: 2; HD: 3; meconium ileus needing enterostomy: 2; and congenital diaphragmatic hernia: 6. The relevant clinical details are given in Table 1.

For microbial analysis at week 1, 36 stool samples from CGISCs and 25 samples from HIs were available; at week 2, 32 stool samples from CGISCs and 17 samples from HIs were available.

For SCFA analysis at week 1, 35 stool samples from CGISCs and 23 samples from HIs were available; at week 2, 30 stool samples from CGISCs and 17 samples from HIs were available.

Microbial analysis

For CGISCs, the total number of reads were 2,904,691 (median 76,125; range: 34,601–116,020) at week 1 and 2,313,892 (median 73,228; range: 25,460–126,106) at week 2. For HIs, total reads were 2,424,727 (median 80,631, range: 49,494–131,223) at week 1 and 1,247,024 (median 74,409, range: 54,056–104,347) at week 2.

Richness

Week 1 samples (CGISC vs. HI): There were no statistically significant differences in the number of OTUs between neonates with CGISCs and HIs at week 1 (mean OTU: 121 vs. 100; $p = 0.07$) (Fig. 1a).

Week 2 samples (CGISCs vs. HIs): There were no statistically significant differences in the number of OTUs between neonates with CGISCs and HIs (mean OTU: 83 vs. 73; $p = 0.82$) (Fig. 1a).

Bacterial richness decreased significantly in both CGISC and HI groups from week 1 to week 2 ($p < 0.001$ and $p = 0.048$, respectively) (Fig. 1a).

Table 1. Baseline clinical data of surgical and healthy infants.

	Neonates with CGISCs (N 37)	Healthy term infants (N 36)	P value
Gestational age (weeks)	37.2 ± 1.2	38.9 ± 1.3	<0.0001
Birth weight (weeks)	2946 ± 489.9	3344.9 ± 399.8	0.0002
Female, N (%)	13 (35%)	15 (42%)	0.634
Maternal pregnancy induced hypertension	3 (8%)	0 (0%)	0.240
Chorioamnionitis	2 (5.4%)	0 (0%)	0.493
Antepartum haemorrhage	1 (2.7%)	0 (0%)	1.000
Caesarean section, N (%)	16 (43.2%)	12 (33%)	0.472
Apgar at 5 min	9 (IQR: 9 9; range: 5 10)	9 (IQR: 9 9; range: 8 10)	0.026
Age at admission (days)	1 (IQR: 1 2; range: 1 9)	1 (IQR: 1 1; range: 1 1)	<0.0001
Age at initial surgery (days)	4 (IQR 2 7; range: 1 15)	NA	NA
Day of life enteral feeds commenced	6 (IQR: 3 9; range: 1 18)	1 (IQR: 1 1; range: 1 1)	<0.0001
Time to full enteral feeds (days)	15 (IQR: 9 25; Range: 4 65)	1 (IQR: 1 1; range: 1 1)	<0.0001
Duration of parenteral nutrition (days)	13 (IQR: 7 24; range: 1 62)	0	<0.0001
Duration of antibiotic therapy (days)	10 (IQR: 6 21; range: 2 64)	0	<0.0001
Duration of ventilator support (h)	55 (IQR: 38 137; range: 0 616)	0	<0.0001
Duration of hospital stay (days)	22 (IQR: 16 38; range: 6 167)	3 (IQR: 2 4; range: 1 7)	<0.0001
Use of proton pump inhibitors	15 (40.5%)	0	<0.0001
Use of H2 receptor blockers	0	0	NA
Number of surgeries during NICU stay	1 (IQR: 1 2; range: 1 5)	NA	NA
Mortality	0	0	NA
Early onset sepsis	0	0	NA
Hospital acquired blood stream infection (HABSI) ^a	8 (21.6%)	0	0.005
Organisms causing HABSI	CONS: 4, <i>E. cloacae</i> : 1, <i>E. coli</i> and <i>E. fecalis</i> : 1; CONS and <i>E. fecalis</i> : 1, <i>E. coli</i> : 1	NA	NA
Use of breastmilk	32 (89%)	26 (72%)	0.135
Day of life at collection of first stool sample	4 (IQR: 2 6; range: 1 10)	2 (IQR: 2 3; range: 1 6)	<0.0001
Day of life at collection of second stool sample	13 (IQR: 12 15; range: 12 19)	13 (IQR: 12 15; range: 10 17)	0.621

Data are given as mean ± SD or median (IQR; range) or number (%).
^aPositive blood culture on a sample collected 48 h after admission to the NICU.

Alpha diversity. Alpha diversity in the study samples was measured using three different measures: Shannon, Chao1 and abundance-based coverage estimators (ACE) (Fig. 1b–d).

Week 1 samples (CGISCs vs. HIs): All alpha diversity measures showed no statistically significant differences between neonates with CGISCs and HIs at week 1 Shannon index (2.33 vs. 1.96; $p = 0.14$).

Week 2 samples (CGISCs vs. HIs): All alpha diversity indices showed no statistically significant differences between neonates with CGISCs and HIs at week 2 (Shannon index: 2.91 vs. 2.00; $p = 0.94$).

Alpha diversity decreased significantly in CGISCs from week 1 to week 2 ($p = 0.014$), but not in HIs ($p = 0.998$).

Beta diversity. Weighted Unifrac and Bray–Curtis distances were used to assess the beta diversity between groups.

Week 1 samples (CGISCs vs. HIs): The microbial community structures of neonates with CGISCs were comparable to HI on both weighted Unifrac and Bray–Curtis measures ($p = 0.415$ and 0.241, respectively) (Fig. 2a, c).

Week 2 samples (CGISCs vs. HIs): The microbial community structures of neonates with CGISCs were significantly different

from HI on both weighted Unifrac and Bray–Curtis measures (both $p = 0.003$) (Fig. 2b, d)

Relative abundance of bacteria at the phylum level. In both neonates with CGISCs and HIs, bacteria belonging to the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria accounted for more than 99.5% of all bacteria. Cyanobacteria, Deinococcus-Thermus, Fusobacteria, Planctomycetes and Verrucomicrobia accounted for the remaining 0.5%.

Comparison of relative abundance of major phyla on week 1 samples (CGISCs vs. HI): The levels of the four major phyla were comparable between CGISC and HI groups at week 1 (all $p > 0.05$; Fig. 3a).

Comparison of relative abundance of phyla on week 2 samples (CGISCs vs. HIs): CGISC group had significantly less Actinobacteria and Bacteroidetes ($p < 0.0001$ and < 0.001 , respectively) than HIs. CGISCs and HIs had comparable levels of Firmicutes. The stools of neonates with CGISCs had significantly more Proteobacteria compared to HIs ($p = 0.003$) (Fig. 3b).

Relative abundance at the genus level

Comparison of relative abundance of major genera on week 1 samples (CGISCs vs. HIs): CGISC infants had significantly more

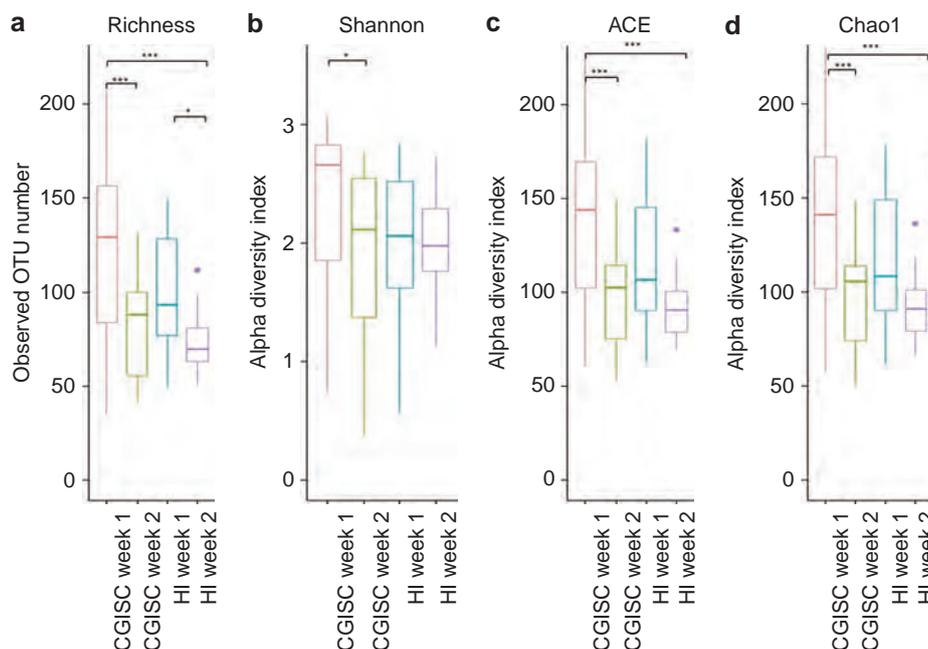


Fig. 1 Richness and alpha diversity measures of faecal microbiota in the study infants. The faecal microbiota of CGISC infants demonstrated significant decrease in bacterial richness and alpha diversity shown by Shannon index, ACE and Chao1 from week 1 to week 2 ($p < 0.05$), while the HI infants exhibited significant decrease in only bacterial richness ($p < 0.05$) (* $p < 0.05$; *** $p < 0.001$).

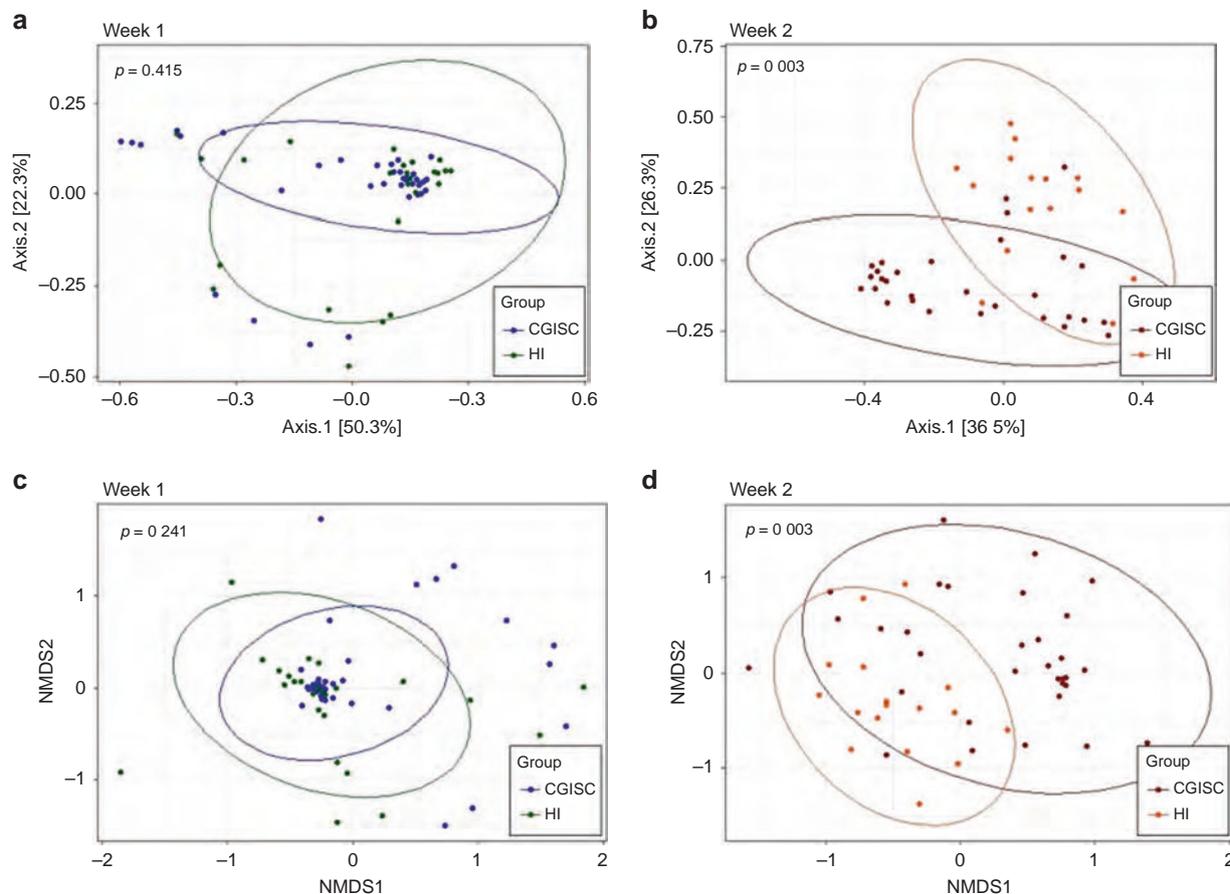


Fig. 2 Beta diversity measures in the study infants. Principal coordinate analysis plots of weighted Unifrac distance of the infants at week 1 (a) and week 2 (b). NMDS plots on Bray-Curtis dissimilarity at week 1 (c) and week 2 (d) of the infants. At week 1, HI and CGISC infants had similar community structures (a, c). However, at week 2, HI had significantly different community structure compared to CGISC infants (b, d).

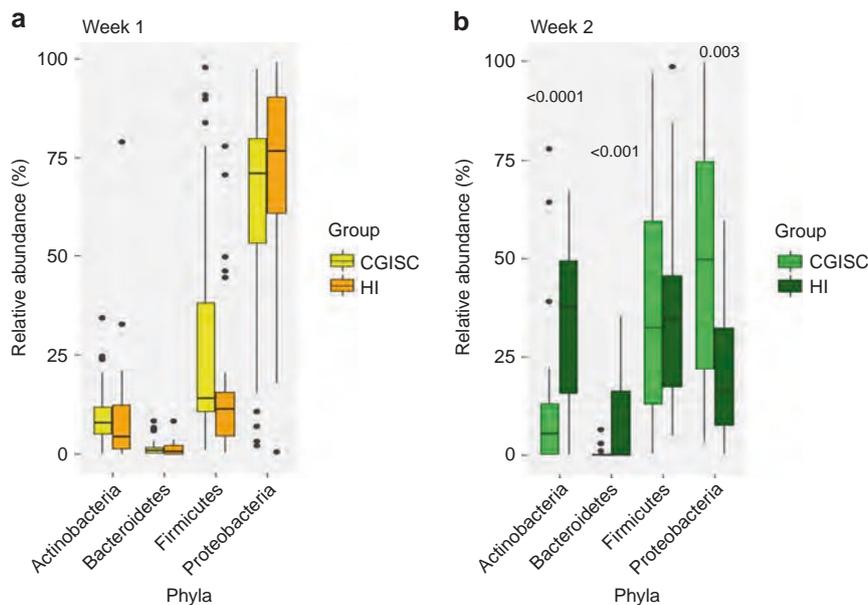


Fig. 3 Relative abundance of the top four phyla in the study infants. Both CGISC and HI infants have similar levels of the four phyla at week 1. However, at week 2, CGISC infants are significantly enriched for Proteobacteria and lower in abundance for Actinobacteria and Bacteroides.

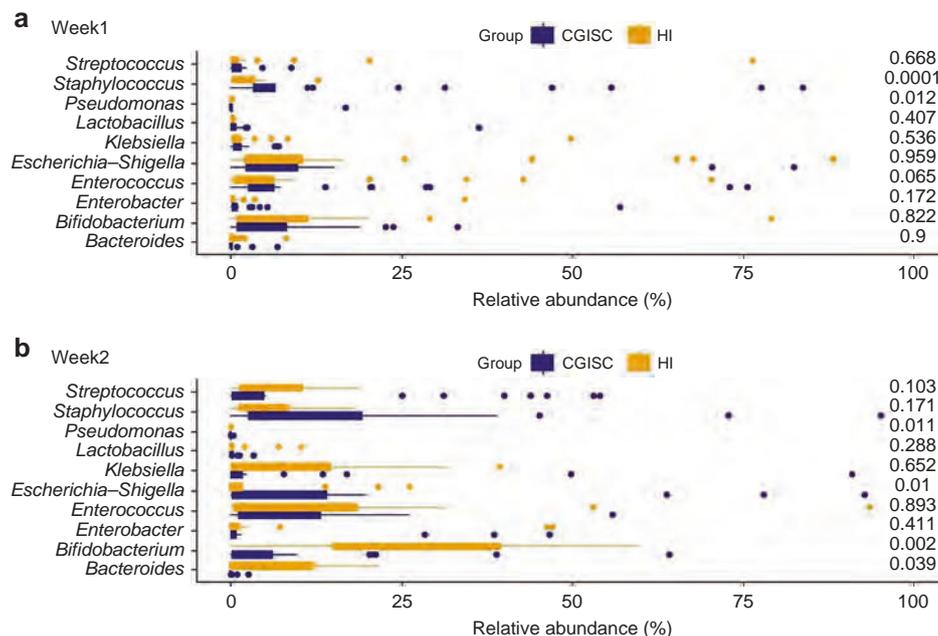


Fig. 4 Comparison of various genera in study infants. CGISC infants have significantly increased levels of *Staphylococcus* and *Pseudomonas* in week 1 compared to HI infants. At week 2, CGISC infants have significantly increased *Pseudomonas* and *Escherichia Shigella*, while HI infants are significantly enriched for *Bifidobacterium* and *Bacteroides*.

Staphylococcus ($p = 0.001$) and *Pseudomonas* ($p = 0.012$) than HI infants at week 1 (Fig. 4 and Supplementary Table S1). There were no significant differences between the groups for the other important bacterial genera (Fig. 4 and Supplementary Table S1).

Comparison of relative abundance of major genera on week 2 samples (CGISCs vs. HI): CGISC infants had significantly lower abundance of *Bifidobacterium* ($p = 0.002$) and *Bacteroides* ($p = 0.039$) and significantly higher abundance of *Escherichia–Shigella* ($p = 0.01$) and *Pseudomonas* ($p = 0.011$) than HIs (Fig. 4 and Supplementary Table S1). There were no significant differences

between the two groups for other genera such as *Staphylococcus*, *Enterococcus*, *Enterobacter*, *Klebsiella* and *Streptococcus* (Fig. 4 and Supplementary Table S1).

Results of SCFA analysis

The total SCFA levels were significantly lower in the CGISC group at week 1 (median 407.7, range: 302.2–696.1 $\mu\text{g/g}$ of wet faeces vs. 1208.2, range: 1036.5–6846.9, $p < 0.0001$) as well as at week 2 (median 410.2, range: 300.1–664.1 vs. 1750.6, range: 1046.8–7781.7, $p < 0.0001$) (Fig. 5). Analysis of individual SCFAs found that there were lower levels of acetic acid, isobutyric acid,

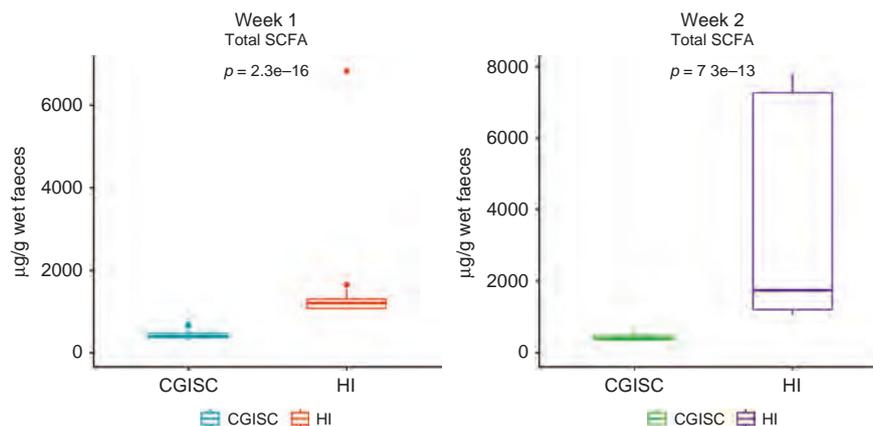


Fig. 5 Stool SCFA levels in study infants. At week 1, CGISC infants have significantly lower amounts of total short-chain fatty acid levels and remain so at week 2.

isovaleric acid, valeric acid, hexanoic acid, and heptanoic acid in the CGISC group as compared to HI at week 1 (Supplementary Table S2). There were no significant differences in concentrations of propionic acid and butyric acid between the groups at week 1 (Supplementary Table S2). At week 2, the concentrations of acetic acid, butyric acid, propionic acid and all other SCFAs were lower in the CGISC group (Supplementary Table S2). Among the CGISC group, there were no significant differences in SCFA levels between week 1 and week 2 samples (all $p > 0.05$). On the other hand, HI group showed significant increases in SCFA levels at week 2 compared to week 1 (all $p < 0.01$ except valeric acid and heptanoic acid where $p > 0.05$).

DISCUSSION

This prospective observational study found that by 10–14 days of life, term neonates with CGISCs developed significant dysbiosis with deficiency of the genera *Bacteroides* and *Bifidobacterium* and increased abundance of the genus *Escherichia-Shigella* and *Pseudomonas*. It also found significant deficiency of SCFAs in the stools of neonates with CGISCs.

Bifidobacterium was the predominant genus during the second week in HIs because they were predominantly breastfed and at home compared to CGISCs, who were still in hospital and had issues, such as feed intolerance, parenteral nutrition and intravenous antibiotics, all of which would have interfered with colonization by *Bifidobacteria*.^{18,19} Normally, HMOs are utilized by *Bifidobacteria*, which enables them to grow and enrich the gut.¹⁸ In the absence of adequate intake of breastmilk, there can be delay in the gut colonization by *Bifidobacteria*. In our study, the median age at the collection of second sample was 13 days, whereas the median age at full feeds was 15.5 days in infants with CGISCs. Many infants with CGISCs were still on parenteral nutrition and were being graded up on milk feeds when the second stool sample was collected.

Bifidobacteria are among the first microbial colonizers of the intestines of newborn infants and play key roles in the development of their physiology, including maturation of the immune system.^{20,21} Hence, their deficiency in surgical infants is of concern and may contribute to the morbidities faced by these vulnerable infants. A vicious cycle may arise wherein clinical morbidities lead to gut dysbiosis, which in turn worsens clinical morbidities.

In our cohort of HIs, richness was significantly lower at week 2 compared to week 1 in HIs. While the drop in alpha diversity was not discernible on Shannon index ($p = 0.998$), ACE and Chao1 indices suggested a trend towards lower alpha diversity at week 2 compared to week 1 even in HIs (ACE, $p = 0.075$; Chao1, $p = 0.084$, Fig. 1). These findings are similar to that of Chi et al.,²² who found

that alpha diversity drops significantly from week 1 to week 2 of birth in low birth weight as well as healthy term newborn infants, before stabilizing.

The richness and alpha diversity decreased markedly from week 1 to week 2 in surgical infants; in addition to the normal drop that occurs in HIs as observed in our study and the study by Chi et al.,²² the other contributing factor could be the use of antibiotics during that period. Bokulich et al.²³ reported that antibiotic use significantly diminishes the phylogenetic diversity and richness in the early newborn period.

The genera *Pseudomonas* and *Escherichia-Shigella* were significantly higher in CGISCs at week 2, compared to HIs. There were no significant differences in the relative abundances of other clinically important genera such as *Enterococcus*, *Enterobacter*, *Klebsiella*, *Staphylococcus* and *Streptococcus* between the surgical and HIs. Probable reason could be the extensive use of antibiotics in surgical infants, which might have suppressed these bacteria, and the short duration of follow-up; it takes some weeks before *Bifidobacteria* become well established at higher levels in healthy breastfed infants, replacing the facultative anaerobes.^{24,25} Newborn infants have an aerobic intestine at birth.²⁶ The high level of oxygen in the newborn GI tract favours the appearance of facultative anaerobes (e.g. *Enterobacter*, *Enterococcus*, *Streptococcus*, *Staphylococci*, *Escherichia coli* and *Klebsiella*). These early colonizers gradually create a reduced, anaerobic environment within the GI tract by consuming the available oxygen, consequently facilitating the establishment of obligate anaerobes such as *Bifidobacterium* and *Bacteroides*.

Evidence is mounting that gut microbial metabolites have a major influence on host physiology. SCFAs are volatile fatty acids produced by the gut microbiota in the large bowel as fermentation products from food components that are unabsorbed/undigested in the small intestine.²⁷ Acetic acid, propionic acid and butyric acid are the most abundant, representing 90–95% of the SCFAs present in the colon. SCFAs result in reduction in the luminal pH in the gut, which inhibits pathogenic microorganisms.²⁸ Acetate produced by *Bifidobacteria* and other commensals improves intestinal defence mediated by epithelial cells and thereby protects the host against lethal infections by enteropathogens. Butyrate is an important fuel of intestinal epithelial cells and stimulates the mitogen-activated protein kinase signalling pathway in intestinal cells, which is positively correlated with gut defences.²⁹ Butyrate also enhances the intestinal barrier by regulating the assembly of tight junctions. In addition, SCFAs are known to have anti-inflammatory properties.⁷

Bacteroides are known to increase the intestinal concentrations of acetate as well as propionate,³⁰ whereas Firmicutes are predominant contributors of butyrate. While *Bifidobacteria* are not butyrogenic by themselves, acetate and other organic acids

produced by them are converted to butyrate by other colonic bacteria via cross-feeding interactions.³¹

It is concerning that SCFAs were lower in CGISCs compared to HIs. The deficiency of SCFAs in neonates with CGISCs has the potential to increase the risk of sepsis due to weakened gut barrier function and other adverse outcomes.

Given that neonates with CGISCs suffer from gut dysbiosis, probiotic supplementation may improve the dysbiosis, SCFA levels and clinical outcomes of these infants. Probiotics are known to inhibit gut colonization with pathogenic bacteria enhance gut barrier function, facilitate colonization with healthy commensals, protect from enteropathogenic infection through production of acetate, reduce antimicrobial resistance, enhance innate immunity and increase maturation of the enteric nervous system and promote gut peristalsis.³² Through these mechanisms, probiotics have the potential to decrease the risk of sepsis, improve feed tolerance and minimize parenteral nutrition-associated cholestasis in infants with CGISCs.³²

Meta-analyses of RCTs in preterm infants (non-surgical) have shown probiotic supplementation to be beneficial in decreasing mortality, necrotizing enterocolitis (NEC), late-onset sepsis and improving feed tolerance.³³ Majority of the RCTs included in those meta-analyses used *Bifidobacteria* as the sole or one of the components of probiotic supplements. Recent meta-analyses that focussed on bifidobacterial supplementation found a significant reduction in the incidence of mortality and NEC in preterm infants.³⁴ In a RCT that included 24 neonates with gastroschisis (probiotics: 12; placebo: 12),³⁵ significant dysbiosis was noted, and it was partially attenuated by the administration of *Bifidobacterium longum* subsp. *infantis*.³⁵ The authors stated that their pilot study was not powered to look at clinical outcomes and that further studies are indicated.

In a small RCT by Murakami et al.³⁶ eight surgical infants were included (*Bifidobacterium*: 4; no *Bifidobacterium*: 4); they reported that unexpectedly there were significantly more Bifidobacteriaceae in the samples from those who did not receive probiotics ($p < 0.05$). Since the sample size was very small, the results may need to be interpreted with caution. The authors concluded that surgical stress appears to affect intestinal microbiota and that probiotic administration requires further clarification.

A meta-analysis that included 198 infants with HD (two RCTs, three observational studies) reported that the incidence of Hirschsprung-associated enterocolitis was 22.6% in the probiotic group vs. 30.5% in the controls, but the difference was not statistically significant (odds ratio 0.72; 95% confidence interval: 0.37–1.39; $p = 0.33$).³⁷ In an RCT of 30 children (<15 years) undergoing various surgeries, Okazaki et al.³⁸ reported that supplementation with *Bifidobacterium breve* BBG-001 was well tolerated without adverse effects, and postoperative infectious complications were significantly decreased. Faecal analysis showed increased levels of *Bifidobacterium* and decreased abundances of Enterobacteriaceae, *Clostridium difficile* and *Pseudomonas*.³⁸

Evidence is emerging from adult studies regarding the beneficial effects of probiotics in GI surgery.^{39,40} The meta-analysis by Lytvyn et al.,³⁹ which included 20 RCTs ($N = 1374$), concluded that probiotic/symbiotic supplementation decreases the risk of surgical site and urinary tract infections in patients undergoing abdominal surgery. Another meta-analysis by Yang et al.⁴⁰ that included 28 RCTs ($n = 2511$) involving adult patients undergoing GI surgery came to similar conclusions. The durations of hospital stay and antibiotic therapy were shorter in the probiotics/symbiotic group vs. controls.

Hence, there seems to be adequate rationale for conducting RCTs of probiotic supplementation, especially the one that contains *Bifidobacteria* in neonates with CGISCs.

Currently, two RCTs of probiotic supplementation in neonates undergoing GI surgery are underway (Howlette et al., Canada,

<https://ichgcp.net/clinical-trials-registry/NCT03266315>); Rao et al., Australia, ACTRN12617001401347).

While our study found dysbiosis in infants with CGISCs, it does not address if the dysbiosis is related to the underlying surgical condition or factors, such as surgical stress, delayed introduction of breastmilk, usage of antibiotics and being in the NICU ecosystem. Infants with CGISCs are probably at a higher risk of dysbiosis because they have more prolonged feed intolerance due to the underlying GI pathology. Additionally, gut is the place where these organisms are predominantly located and hence CGISCs are probably more prone to dysbiosis than other types of surgical conditions. Future studies need to compare the gut microbiota of neonates with CGISCs vs. other surgical conditions to address this issue.

The main strength of our study is that it is probably the first study comparing gut microbiota in neonates with CGISCs vs. healthy term infants using culture independent techniques. The only other study was the RCT by Powell et al.,³⁵ which demonstrated dysbiosis in neonates with gastroschisis; however, the gut microbiota of HIs was not investigated in that study. A limitation of our study is the short duration of follow-up (14 days) and that only two samples were collected from study infants (weeks 1 and 2). The reasons for this approach were logistic feasibility and funding.

We were concerned that there would be significant drop-out rates if a later postnatal age for the second sample collection was chosen (e.g. day 21). As experienced in the present study, even at week 2, there were significant drop-out rates (50%) from HIs. Drop outs would also have occurred in surgical infants because nearly 25% of our surgical infants were discharged home by day 17 and 50% by day 22. It is difficult for busy parents at home to collect samples on a weekly basis. Future studies should allocate adequate resources to have longer duration of follow-up and test multiple stool samples (e.g. once a week for 4–6 weeks).

The other limitation was the fact that HIs were more mature by 1 week compared to the CGISC group. Since the gut microbiota evolves rapidly in the neonatal period, the influence of this 1-week difference cannot be ruled out. Another limitation was that the week 1 samples were collected at an earlier postnatal age (median 2 day) compared to surgical infants (median 4 days). Surgical infants usually have delayed passage of meconium and infrequent passage of subsequent stools because of underlying gut anomaly, administration of narcotic analgesics, delayed commencement of enteral feeds and postoperative ileus.

In summary, during hospitalization, neonates with CGISC develop gut dysbiosis with depletion of the genera *Bacteroidetes* and *Bifidobacterium* and increased abundance of *Pseudomonas* and *Escherichia-Shigella*. They also have deficiency of biologically important SCFAs in their stools. Similar studies with larger sample size and longer duration of follow-up are essential.

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AUTHOR CONTRIBUTIONS

S.C.R.: Conception and design, acquisition of data, analysis and interpretation of data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published. M.E.: Analysis and interpretation of data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published. S.K.P.: Conception and design, interpretation of data; revising the article critically for important intellectual content;

and final approval of the version to be published. K.N.S.: Conception and design, interpretation of data; revising the draft of the article critically for important intellectual content; and final approval of the version to be published. I.G.: Conception and design, interpretation of data; revising the draft critically for important intellectual content; and final approval of the version to be published. A.K.: Conception and design, interpretation of data; revising the draft critically for important intellectual content; and final approval of the version to be published. B.W.: Analysis and interpretation of data; revising the draft critically for important intellectual content; and final approval of the version to be published. L.C.: Analysis and interpretation of data, revising the draft for important intellectual content; and final approval of the version to be published. P.L.C.: Conception and design, analysis and interpretation of data; revising the draft critically for important intellectual content; and final approval of the version to be published.

ADDITIONAL INFORMATION

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Supplemental Table S1: Relative abundance of various genera in surgical and healthy infants

Week 1			
Genus	Relative abundance (mean% and SD) at week1 in CGISC (N=36 stool samples)	Relative abundance (mean% and SD) at week1 healthy infants (N=25 stool samples)	P-value
Streptococcus	1.09 ± 1.64	4.80 ± 15.51	0.667
Staphylococcus	12.49 ± 20.76	2.60 ± 3.39	<0.001
Enterobacter	2.37 ± 9.43	1.82 ± 6.82	0.171
Enterococcus	10.00 ± 17.38	8.61 ± 16.75	0.065
E. Coli-Shigella	10.32 ± 16.88	15.44 ± 24.20	0.959
Pseudomonas	0.55 ± 2.80	0.03 ± 0.07	0.012
Klebsiella	1.34 ± 1.54	3.26 ± 9.90	0.535
Bifidobacterium	7.46 ± 7.95	9.33 ± 16.35	0.821
Bacteroides	0.39 ± 1.22	0.62 ± 1.64	0.900
Lactobacillus	1.50 ± 6.00	0.33 ± 0.35	0.407
Week 2			
Genus	Relative abundance (mean% and SD) at week2 CGISC (N=32 stool samples)	Relative abundance (mean% and SD) at week2 healthy infants (N=17 stool samples)	P-value
Streptococcus	10.11 ± 18.01	11.62 ± 16.81	0.103
Staphylococcus	15.69 ± 22.31	5.81 ± 6.35	0.171
Enterobacter	6.81 ± 14.49	8.03 ± 17.04	0.411
Enterococcus	8.35 ± 12.01	15.73 ± 25.37	0.892
E. Coli-Shigella	12.73 ± 22.62	4.72 ± 8.79	0.009
Pseudomonas	0.04 ± 0.66	0.00 ± 0.00	0.011
Klebsiella	4.14 ± 16.01	6.44 ± 12.05	0.652
Bifidobacterium	7.21 ± 13.49	28.96 ± 19.6	0.002
Bacteroides	0.12 ± 0.49	6.59 ± 8.62	0.039
Lactobacillus	0.32 ± 0.66	1.21 ± 3.02	0.288

CGISC: Congenital gastrointestinal surgical conditions

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Supplemental Table S2: SCFA levels in surgical and healthy infants

SCFA	Week1 CGISC (Median, range) ug/g wet faeces (n=35 stool samples)	Week1 Healthy Infant (Median, range) ug/g wet faeces (n=23 stool samples)	P-value
Acetic Acid	406.73, 300.65-693.89	1189.65, 1035.18-6834.05	<0.0001
Propionic Acid	0.51, 0.14-41.33	0.45, 0.03- 37.83	0.217
Isobutyric Acid	0.26, 0.20- 3.19	0.310, 0.26- 3.24	<0.0001
Butyric Acid	0.13, 0.070-1.29	0.24, 0.07-223.06	0.335
Isovaleric Acid	0.05, 0.04- 3.86	0.07, 0.04- 3.24	0.010
Valeric Acid	0.04, 0.03-0.39	0.08, 0.04- 0.82	0.0001
Hexanoic acid	0.14, 0.10-1.79	0.32, 0.13- 1.19	0.0003
Heptanoic acid	0.03,0.020-0.24	0.15, 0.03- 1.79	<0.0001
SCFA	Week2 CGISC (Median, Range) ug/g wet faeces (n=30 stool samples)	Week2 Healthy Infant (Median, Range) ug/g wet faeces (n=17 stool samples)	P value
Acetic Acid	395.39, 299.12-65.73	1546.19, 913.71-7767.68	<0.0001
Propionic Acid	0.47, 0.25-16.80	22.46,0.28-199.85	<0.0001
Isobutyric Acid	0.26, 0.21-0.47	0.94,0.28-25.23	<0.0001
Butyric Acid	0.14, 0.09-62.83	0.81, 0.15-25.08	<0.0001
Isovaleric Acid	0.06, 0.05-1.12	0.65, 0.07-15.10	<0.0001
Valeric Acid	0.04, 0.03-0.15	0.11, 0.06-0.65	<0.0001
Hexanoic acid	0.20, 0.101-0.80	0.66, 0.29-1.67	<0.0001
Heptanoic acid	0.06, 0.02-0.19	0.17,0.11-0.59	<0.0001

SCFA: Short-chain fatty acids, CGISC: Congenital gastrointestinal surgical conditions

Preface to Chapter 7

Probiotic Supplementation in Neonates with Major Gastrointestinal Surgical Conditions: A Systematic Review

The previous chapter provided evidence from our prospective study that neonates with major congenital gastrointestinal surgical conditions (CGISCs) develop gut dysbiosis and short-chain fatty acid (SCFA) deficiency during their initial hospital stay. Such gut dysbiosis can adversely affect the clinical outcomes of sick patients. One could hypothesise that probiotic supplementation may help reduce dysbiosis, improve SCFA levels, and optimise clinical outcomes of neonates with CGISCs. This chapter presents the results of our systematic review evaluating the efficacy and safety of probiotic supplementations in neonates with CGISC. It concluded that there is limited evidence in this area.

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REVIEW ARTICLE



Probiotic supplementation in neonates with major gastrointestinal surgical conditions: a systematic review

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ABSTRACT

Objectives: Neonates with major gastrointestinal surgical conditions frequently suffer from prolonged feed intolerance, infections, and need multiple courses of antibiotics. All these put them at risk of gut dysbiosis. Probiotic supplementation has the potential to minimise dysbiosis and improve clinical outcomes in such infants. Hence, we aimed to conduct a systematic review of probiotics in neonates with major surgical conditions of the gut.

Methods: Medline, Embase, the Cochrane Central Register of Controlled Trials (CENTRAL), and other databases were searched in September 2016.

Results: Two randomised controlled trials (RCTs) were included; the first was conducted in 24 neonates with gastroschisis, the second in eight neonates with various surgical conditions. In the first study, the overall microbial communities were not significantly different between groups, though analysis of the final specimens demonstrated higher Bifidobacteriaceae, lower Clostridiaceae, and trends toward lower Enterobacteriaceae, Enterococcaceae, Staphylococcaceae, and Streptococcaceae in the probiotic group. In the second study, there were significantly more Streptococcaceae in the faecal samples in the probiotic group and significantly more Bifidobacteriaceae in the no probiotic group ($p < .05$).

Conclusions: There is limited evidence regarding the role of probiotics in neonates with gastrointestinal surgical conditions. Adequately powered RCTs are needed to address this issue.

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Probiotic; dysbiosis;
 newborn infant; surgery;
 gut

Introduction

The major neonatal gastrointestinal surgical conditions include gastroschisis, exomphalos, duodenal atresia, intestinal atresia, congenital diaphragmatic hernia (CDH), tracheo-oesophageal fistula (TEF), short bowel syndrome (SBS), malrotation and volvulus, meconium ileus, hypoplastic left colon, meconium peritonitis, Hirschsprung disease (HD), and anorectal malformations [1–12]. Common morbidity in all these conditions includes feed intolerance, prolonged parenteral nutrition, increased risk of healthcare-associated blood stream infections (HABSI), and the need for multiple courses of antibiotics.

Recurrent administration of antibiotics can destroy the normal commensal gut bacteria [13]. In addition, delayed commencement of enteral feeds, living in the neonatal intensive care unit (NICU) and lack of exposure to mother's skin and breast milk microbiota can lead to intestinal dysbiosis [14–19]. Intestinal dysbiosis has been implicated as a cause or association for

many adverse outcomes in the neonatal, paediatric, and adult population [20–27]. A cohort study of 208 neonates with surgical conditions found that in majority of cases septicaemia was mainly a gut-derived phenomenon due to translocation of gut organisms, and hence suggested novel strategies for prevention of HABSI [28].

Probiotic supplementation has the potential to minimise/prevent dysbiosis, thereby improving the clinical outcomes of surgical neonates. Recent Meta analyses of randomised controlled trials (RCTs) in non-surgical preterm infants have concluded that probiotics enhance feed tolerance [29] and decrease the risk of necrotising enterocolitis [30] and late onset sepsis [31,32]. Similar beneficial effects are plausible in neonates with major surgical conditions of the gut. To our knowledge, there are no systematic reviews on this topic. Hence, we aimed to conduct a systematic review to evaluate the efficacy and safety of probiotic supplementation in newborn infants with major gastrointestinal surgical conditions.

Materials and methods

Guidelines from the Cochrane Neonatal Review Group (<http://neonatal.cochrane.org/resourcesreview-authors>) [33], Centre for Reviews and Dissemination (<http://www.york.ac.uk/crd/guidance/>), and the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statements [34] were followed for undertaking and reporting this systematic review. Ethics approval was not required.

Eligibility criteria

Types of studies: Only RCTs were included in the review. Observational studies, narrative reviews, systematic reviews, case reports, letters, editorials, and commentaries were excluded, but ready to identify potential additional studies.

Types of participants: Full-term and preterm neonates with major gastrointestinal surgical conditions.

Types of interventions

Studies comparing probiotics versus placebo or no probiotics were eligible for inclusion. Probiotics could be of any type, dose, and duration.

Types of outcome measures

Primary outcomes

Incidence of HABSI, defined as positive culture on blood sample drawn 48 h after admission to the NICU and before discharge home.

Secondary outcomes

Average number of episodes of HABSI per 1000 bed days, number of infants with at least one episode of (a) HABSI due to coagulase negative staphylococcus (CONS), (b) candida, (c) Gram-negative bacteria, (d) probiotic organism; surgical wound infections, urinary tract infection, hospital acquired pneumonia, number of courses of antibiotics, duration of antibiotic therapy (days), C-reactive protein (CRP) levels (mg/dl), mortality during initial hospitalisation, time to full enteral feeds (days), duration of hospital stay (days), z scores for weight, length, and head circumference at the time of discharge.

Search methods for identification of studies

We used standard search methods of the Cochrane Neonatal Review Group. The following databases were searched to identify relevant studies: MEDLINE (Ovid),

MEDLINE (In Process & Other Non-Indexed Citations), Embase (Ovid), CINAHL (EBSCO), and Cochrane Central Register of Controlled Trials (CENTRAL), the Cochrane Library. Databases were searched using the following terms: (Probiotics OR Probiotic OR Lactobacillus OR Bifidobacterium OR Saccharomyces) AND ((Infant, Newborn OR Newborn OR Neonate OR Neonatal OR Infan* or Neonat*) AND (Surgery)). The word Surgery was replaced by individual conditions i.e. Gastroschisis OR Exomphalos OR Duodenal atresia OR Intestinal Atresia OR Congenital Diaphragmatic Hernia (CDH) OR Tracheo-Oesophageal Fistula (TOF), OR Short Bowel Syndrome (SBS), OR Malrotation OR Volvulus, OR Meconium Ileus OR Meconium Peritonitis OR Hypoplastic left colon OR Microcolon OR Meconium peritonitis OR Hirschsprung Disease (HD) OR Anorectal Malformations. Search was repeated using the term Paediatric Surgery OR Pediatric Surgery. The clinical trial registries ClinicalTrials.gov and International Clinical Trials Registry Platform (ICTRP, <http://www.who.int/ictip/en/>) were searched using appropriate terminology. We did not apply any language restrictions.

Searching other resources

We searched the e-abstracts of the relevant perinatal meetings [including Pediatric Academy Society (PAS), Perinatal Society of Australia and New Zealand (PSANZ) and European Society of Paediatrics]. Grey literature was searched through the national technical information services (<http://www.ntis.gov/>), Open Grey (<http://www.opengrey.eu/>), and Trove (<http://trove.nla.gov.au/>). The reference lists of eligible studies and review articles were searched to identify additional studies.

Data collection and analysis

Data collection forms were compiled and completed independently by two of the reviewers (SR and SP). Reviewers SR and SP independently reviewed the results of the search and selected studies for inclusion. Disagreements were resolved by discussion among all reviewers.

Data extraction and management

Two review authors (SR and SP) separately extracted, assessed, and coded all data for each study using a form that was designed specifically for this review. For each included study, information regarding the primary surgical condition, sample size, gestational age, birth weight, time of commencement of probiotics, type/dose and duration of probiotic and other

clinically relevant information were collected. Discrepancies were resolved by discussion. Authors of the included RCTs were contacted for additional information from their study.

Assessment of risk of bias (ROB) in included studies: We assessed ROB by using the Cochrane "Risk of Bias Assessment Tool." Authors SR and SP independently assessed the ROB in all domains including random number generation, allocation concealment, blinding of intervention and outcome assessors, completeness of follow-up, selectivity of reporting, and other potential sources of bias. For each domain, the ROB was assessed as low, high, or unclear risk based on the Cochrane Collaboration guidelines. Where it was unclear, the authors of the included RCTs were contacted by email requesting clarification.

Data synthesis: Meta-analysis was planned to be conducted using Review Manager 5.3 (Cochrane Collaboration, Nordic Cochrane Centre, Copenhagen, Denmark) using fixed-effects model (FEM) (Mantel-Haenszel method). Analysis using random effects model (REM) was planned to ensure that the results and conclusions were not influenced by the type of model used for meta-analysis. Effect size was planned to be expressed as risk ratio (RR) and 95% confidence interval (CI) for dichotomous outcomes and mean difference and 95%CI for continuous outcomes. Statistical heterogeneity was planned to be assessed with the χ^2 test and I^2 statistic and by visual inspection of the forest plot (overlap of CIs). A p values $<.1$ on the χ^2 statistic was considered to indicate heterogeneity. I^2 statistic values were interpreted according to the guidelines of Cochrane Handbook as follows: 0% to 40% might not be important; 30% to 60% may represent moderate heterogeneity; 50% to 90% may represent substantial heterogeneity; 75% to 100%, considerable heterogeneity. The risk of publication bias was assessed by visual inspection of the funnel plot [35].

Subgroup analysis: Subgroup analyses were planned based on the type of surgical condition and gestation (full-term versus preterm).

Quality of evidence and summary of findings: The key information about the quality of evidence, the magnitude of effect of the intervention, and the sum of available data on the main outcome was planned to be presented in the summary of findings table according to the Grades of Recommendation, Assessment, Development and Evaluation (GRADE) guidelines [36].

Sensitivity analysis: We planned to conduct sensitivity analyses by excluding studies with high ROB on

random sequence generation and/or allocation concealment.

Results

The literature search retrieved 4889 citations, of which 4887 were excluded and two RCTs included [37,38]. The flow diagram of the study selection process is given in Figure 1. The study by Powell et al. [38] enrolled 24 infants with gastroschisis born at >34 weeks' gestation. Enrolled infants were randomly assigned to receive either *B. infantis* ATCC 15697 10^9 CFU (colony forming units) or placebo twice daily for 6 weeks or until hospital discharge (whichever came first). The supplementation with probiotic or placebo began following surgery as soon as consent was obtained. The supplement was instilled into the nasogastric (Replogle) tube which was then clamped for 1 h before returning to routine gastric decompression. The supplementation doses were given orally when the Replogle tube was removed. All caregivers and parents were blinded to the study group assignment. The primary outcome was faecal microbiota composition and the secondary outcome was length of hospital stay. Administration of the probiotic or placebo was well tolerated. The authors reported that overall microbial communities were not significantly different between groups, though analysis of the final specimens demonstrated higher Bifidobacteriaceae, lower Clostridiaceae, and trends toward lower Enterobacteriaceae, Enterococcaceae, Staphylococcaceae, and Streptococcaceae in the probiotic group. The median (IQR) duration of the supplementation was 19.5 (17.5, 29.5) versus 23 (IQR: 20.2, 36.7) days in the placebo versus probiotic group respectively. The duration (Placebo versus Probiotic) of TPN [22.5 (17.5, 29.2) versus 21 (19, 32.2); $p=.82$], antibiotic therapy [7 (5.7, 8) versus 6 (5, 7); $p=.25$], and length of hospital stay [(26.5 (21, 32.7) versus 27 (26, 42.5); $p=.44$] was similar between the two groups. There were no deaths and no cases of sepsis in both the groups [39].

The study by Mukarami et al. [37] evaluated the effect of probiotic supplementation on intestinal microbiota in neonates undergoing surgery within 3 days of birth. The probiotic strain used was *Bifidobacterium animalis* subsp. lactis LKM512 (LKM). Four infants were given and another four not given probiotics. Three healthy infants served as controls. Stool specimens (20 mg) were collected at five times (after birth, and on days 3, 7, 10, and 14 after surgery in surgical cases, and after birth, and on days 4, 8, 11, and 15 of life in controls). Among the probiotic group infants, all four had colostomies for anorectal

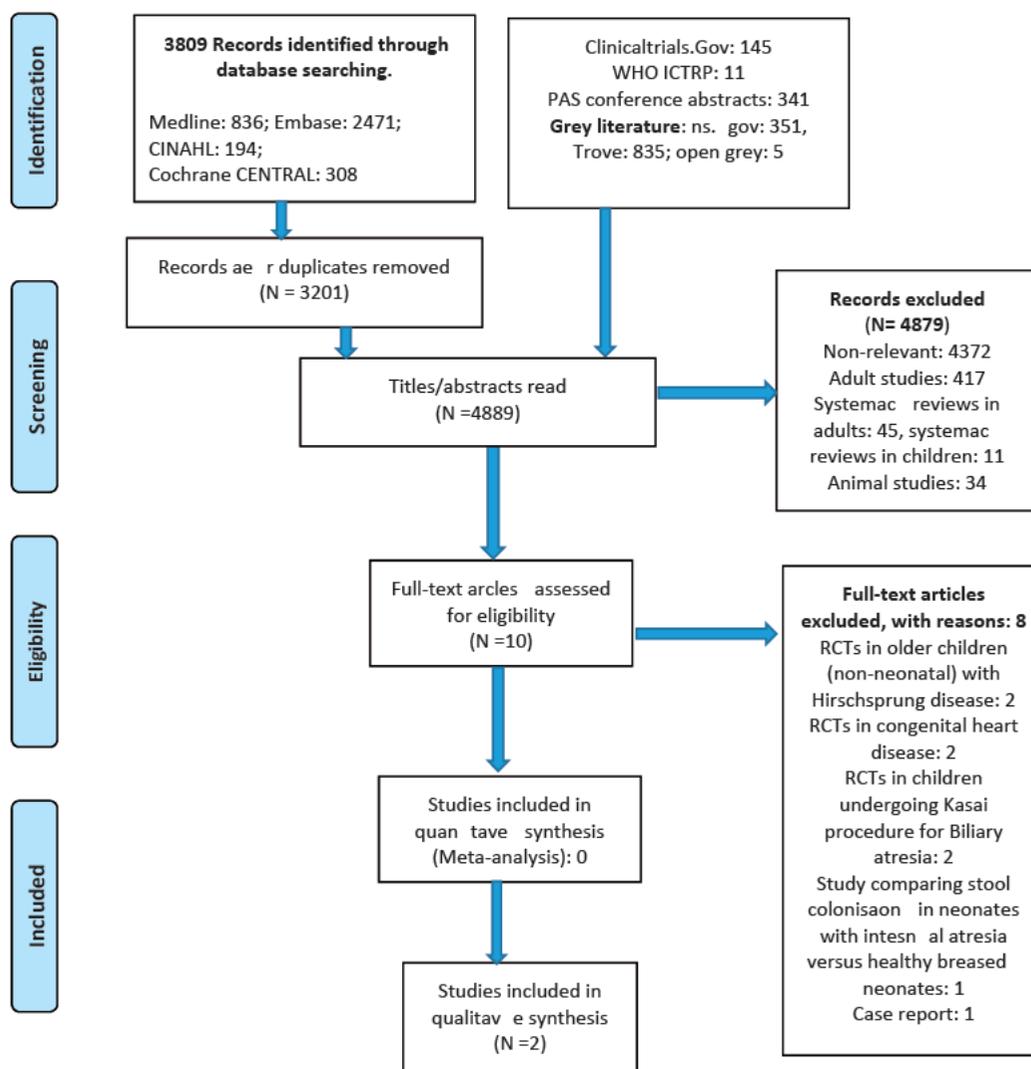


Figure 1. PRISMA 2009 flow diagram of study selection probiotic supplementation in neonates with major gastrointestinal surgical conditions: a systematic review.

malformation; among the no probiotic group infants, one had duodeno-duodenostomy for duodenal atresia and one had anorectal malformation and one had a lymphangioma requiring OK432 Injection. All surgical infants were prescribed ampicillin as a postoperative antibiotic according to the standard postoperative management protocol. Stool microbiota was analysed using 16S rRNA gene sequencing after DNA extraction. One surgical infant was a preterm infant weighing 2930g at birth and others were full-term weighing from 2426 to 3300g. Two were delivered by caesarean and the others were delivered vaginally. They observed major changes in gut microbiota in surgical infants in both the probiotic and non-probiotic group. There were significantly more Streptococcaceae in the faecal samples from those who received probiotics ($p < .05$) and unexpectedly there were significantly more Bifidobacteriaceae in the samples from those who did not receive probiotics ($p < .05$). In the

probiotic group, two cases developed egg allergy and one case developed generalised colitis at the age of ~ 12 months. In the no probiotic group, two cases developed colitis. No significant differences in weight gain were observed. They concluded that surgical stress appears to affect intestinal microbiota considerably.

ROB of included studies: Details of the ROB analysis are given in Table 1. The methods used to generate random sequence numbers and how allocation concealment was achieved was not available in both the included RCTs. Powell et al. used placebo and hence there was low risk of detection bias. Mukarami et al. did not use placebo. Follow up was complete in both the RCTs.

Results of meta-analysis: Meta-analysis could not be done due to the lack of data suitable for pooling.

GRADE evidence: The level of evidence was deemed low, mainly because of the small sample size and

Table 1. Risk of bias of the included studies.

Domains of ROB assessment	Powell 2015	Mukarami 2016
Random sequence generation (selection bias)	Unclear risk	Unclear risk
Allocation concealment (selection bias)	Unclear risk	Unclear risk
Blinding (performance bias and detection bias)	Low risk (placebo used)	Unclear (placebo not used)
Completeness of follow up (attrition bias)	Low risk	Low risk
Selective reporting (reporting bias)	Low risk	Low risk
Other bias	Low risk	Low risk

Table 2. Summary of findings (SOF) and GRADE evidence.

Outcomes	Probiotics	Placebo	Statistical analysis (e.g. relative risk, <i>p</i> value)	Number of participants	Quality of evidence (GRADE)	Comments
Mortality	0/12	0/12	Relative risk not estimable	24	Low	See footnotes
Acquired sepsis	0/12	0/12	Relative risk not estimable	24	Low	See footnotes
Duration of antibiotic therapy (days, median, and IQR)	6 (5, 7)	7 (5.7, 8)	<i>p</i> .25	24	Low	See footnotes
Duration of parenteral nutrition (days, median, and IQR)	21 (19, 32.2)	22.5 (17.5, 29.2)	<i>p</i> .82	24	Low	See footnotes
Length of hospital stay (days, median, and IQR)	27 (26, 42.5)	26.5 (21, 32.7)	<i>p</i> .44	24	Low	See footnotes

Probiotic supplementation compared with placebo in neonates with major surgical conditions of the gut.

*The evidence was deemed low in view of the very small sample size and unclear risk of bias in the domains of random sequence numbers and allocation concealment.

unclear ROB in some of the domains of assessment (Table 2).

Discussion

This first systematic review identified only two RCTs ($N=32$) that evaluated probiotic supplementation in infants undergoing gastrointestinal surgery. There is increasing number of probiotic RCTs in adult population undergoing gastrointestinal surgery. Recently three independent meta-analyses of data from adult studies have concluded that probiotic/symbiotic supplementation decreases the risk of post-operative sepsis in adults undergoing gastrointestinal surgeries [40–42]. The sample size in the included meta-analyses ranged from 1200–2500 (15–28 RCTs). There are two RCTs of probiotic supplementation in children (<18 years) with Hirschsprung disease [43,44]. While one of them showed beneficial effects of supplementation [43], the other did not [44]. In another RCT in 30 children (<15 years) with various surgical (majority gastrointestinal) conditions *Bifidobacterium Breve* (BBG-01) or placebo was administered daily from 7 days prior to surgery until discharge from hospital. Administration of the probiotic strain BBG-01 in the perioperative period was found to be safe and improved the gut flora, increased the faecal short-chain fatty acid (acetic acid) concentration, and decreased the risk of septicaemia [45].

A recent survey of 86 clinicians (70 institutions in Japan) from departments of paediatrics, newborn medicine, obstetrics and gynaecology and paediatric surgery reported that adverse events (functional ileus) occurred in two extremely preterm infants, and

B. breve bacteraemia in two surgical neonates. No serious adverse events with a poor outcome were observed [46]. The total number of patients treated with probiotics exceeded 23,000, with 169 being paediatric surgical cases [46]. This data provides additional reassurance that probiotic supplementation may be safe in infants with gastrointestinal surgical conditions.

Intestinal dysbiosis is known to be severe in critically ill patients [47,48]. Infants with gastrointestinal surgical condition are critically ill and hence are expected to have severe dysbiosis. Animal studies and *in vitro* studies have shown that probiotics improve gut barrier function [49], decrease gut bacterial translocation [50], inhibit gut colonisation with pathogenic bacteria [51], improve colonisation with healthy commensals [52] and protect from enteropathogenic infection through acetate production [53], enhance innate immunity [54], and increase maturation of the enteric nervous system [55], all of which have the potential to improve the outcomes of infants with surgical conditions of the gut. Hence, it is important to conduct adequately powered RCT to evaluate this potential therapy in this high-risk population of infants.

The strengths of our systematic review are the use of Cochrane methodology, and thorough literature search including the grey literature. The weaknesses are the lack of adequate number of RCTs and the extremely small sample size.

Conclusions

There is limited evidence regarding the role of probiotics in infants with gastrointestinal surgical conditions.

Given the biological plausibility for benefit and encouraging results from adult studies, adequately powered neonatal RCTs are needed to address this issue.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Preface to Chapter 8

Probiotic Research in Neonates with Congenital Gastrointestinal Surgical Conditions: Now is the Time

In the previous chapters, the following evidence was presented:

1. Gut dysbiosis and short-chain fatty acid (SCFA) deficiency occurs in critically ill patients, including neonates with congenital gastrointestinal surgical conditions (CGISC),
2. Probiotic supplementation has the *potential* to improve their outcomes by attenuating gut dysbiosis and improving SCFA levels, and
3. There are only two small RCTs in neonates with CGISCs with a total sample size of 32.

This chapter summarises the overall evidence so far and emphasizes the need for further research on probiotic supplementation in neonates with CGISC.

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Opinion

Probiotic research in neonates with congenital gastrointestinal surgical conditions – Now is the time

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The major congenital gastrointestinal surgical conditions (CGISC) include oesophageal atresia, gastroschisis, exomphalos, malrotation and volvulus, duodenal atresia, intestinal atresia, meconium ileus, hypoplastic colon, meconium peritonitis, intestinal stenosis, congenital short bowel syndrome, Hirschsprung disease (HD), anorectal malformations and others. In addition to surgical repair, strategies for managing such conditions include early commencement of enteral feeds, standardization of feeding advancement, strict hand hygiene and aseptic precautions for indwelling catheters (Graham, 2010; Lauriti *et al.*, 2014; Savoie *et al.*, 2016; Dama *et al.*, 2017). Despite such best practices and advances in surgical techniques, morbidities including feed intolerance, healthcare-associated infections, cholestatic jaundice, growth failure and neurodevelopmental disabilities continue to impose significant health burden on this cohort (Willis *et al.*, 2010; Bishay *et al.*, 2012; Wang *et al.*, 2014; Dwyer *et al.*, 2016; Hong *et al.*, 2018). Additional strategies are hence required to improve their outcomes.

Gut dysbiosis in infants with CGISC

Neonatal gut microbiota develops rapidly after birth and achieves an adult-like composition and stability by 2–3 years of age (Arrieta *et al.*, 2014). The evolution of gut microbiome is affected in infants with CGISC admitted in intensive care units (ICUs). These infants receive

parenteral nutrition (PN), get exposed to multiple courses of antibiotics, do not receive early enteral feeding and optimal maternal skin to skin contact. Decontamination of the skin for surgery, exposure to gastric acid suppressants, breakdown of natural barriers due to invasive procedures and indwelling tubes and catheters, colonization of the ICU room surfaces and hands of the healthcare providers also contribute to the risk of gut dysbiosis in infants with CGISC (Donnell *et al.*, 2002; van Saene *et al.*, 2003; Hussey *et al.*, 2011; Fouhy *et al.*, 2012; Ralls *et al.*, 2016; Rogers *et al.*, 2016; Kitsios *et al.*, 2017).

- (i) *PN and gut dysbiosis*: The role of PN in gut dysbiosis deserves attention as it is often the main/only source of nutrition in infants with CGISC. Lavalée *et al.* (2017) randomized neonatal piglets to receive total parenteral nutrition (TPN) or sow feeds (SF) for 14 days. Ileal segments and mucosal scrapings were used to assess the microbiota composition by 16S rRNA gene sequencing. Significant dysbiosis was noted in the TPN group, especially in those which received soy-based lipids. In another study, using a mouse model, Ralls *et al.* (2016) reported permeation of TPN-derived nutrients into the gut lumen, where they were preferentially utilized by Enterobacteriaceae, which then flourished.
- (ii) *Antibiotics and gut dysbiosis*: Fouhy *et al.* (2012) compared the gut microbiota of nine newborn infants treated with parenteral ampicillin and gentamicin, with that of nine matched healthy infants. Gut microbiota of the antibiotic-treated infants showed significantly higher proportions of Proteobacteria and lower proportions of Actinobacteria and the associated genus Bifidobacterium, as well as the genus Lactobacillus compared with the untreated controls 4 weeks after the cessation of treatment. Even by week 8, Proteobacteria levels remained significantly higher in the treated infants (Fouhy *et al.*, 2012). Increased abundance of Proteobacteria is a concern because it is considered as a potential diagnostic signature of dysbiosis and risk of disease (Shin *et al.*, 2015).
- (iii) *The ICU ecosystem and gut dysbiosis*: In a study in adult ICU patients, McDonald *et al.* (2016) showed

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evidence of extreme dysbiosis. The phylogenetic diversity at discharge was significantly lower than at admission. Faecal samples tended to have a lower relative abundance of Firmicutes and Bacteroidetes and an increased relative abundance of Proteobacteria and well-recognized pathogens such as *Enterobacter* and *Staphylococcus* (McDonald *et al.*, 2016). In a study in paediatric ICUs, Rogers *et al.* (2016) reported taxonomic alterations in the gut microbiota. These included enrichments of gut pathogens such as *Enterococcus* and *Staphylococcus* at multiple body sites and depletion of commensals such as *Faecalibacterium* and *Ruminococcus* from stool samples. Alpha and beta diversity were unstable over time (Rogers *et al.*, 2016).

Studies have shown an association between gut dysbiosis and morbidities such as hospital-acquired infections in neonates with surgical conditions (Donnell *et al.*, 2002; van Saene *et al.*, 2003) and Hirschsprung-associated enterocolitis (HAEC) (Li *et al.*, 2016).

Probiotics for CGISC

Given that gut dysbiosis occurs and is associated with morbidities in infants with CGISC, optimization of gut microbiota by probiotics is a potentially beneficial strategy to improve their outcomes.

Probiotics are defined as live microorganisms that when administered in adequate amounts confer health benefits on people with specific illnesses (Hill *et al.*, 2014). Probiotics inhibit gut colonization with pathogenic bacteria (Sassone-Corsi and Raffatellu, 2015), enhance gut barrier function (Bron *et al.*, 2017), facilitate colonization with healthy commensals (Garrido *et al.*, 2012), protect from enteropathogenic infection through production of acetate (Fukuda *et al.*, 2011), reduce antimicrobial resistance (Taft *et al.*, 2018), enhance innate immunity (Giorgetti *et al.*, 2015) and increase maturation of the enteric nervous system and promote gut peristalsis (Hyland and Cryan, 2016; De Vadder *et al.*, 2018). Through these mechanisms, probiotics have the potential to decrease the risk of sepsis, improve feed tolerance and minimize parenteral nutrition-associated cholestasis in infants with CGISC.

(i) *Evidence from studies in adult patients:* A recent meta-analysis of 20 RCTs ($N = 1374$) concluded that probiotic/symbiotic supplementation decreases the risk of surgical site and urinary tract infections in patients undergoing abdominal surgery (Lytvyn *et al.*, 2016). Another meta-analysis that included 28 RCTs ($n = 2511$) involving adult patients undergoing gastrointestinal surgery came to similar conclusions (Yang *et al.*, 2017). The durations of hospital stay and

antibiotic therapy were shorter in the probiotics/symbiotic group vs controls (Yang *et al.*, 2017). The need for caution in interpreting the results was emphasized considering the high risk of bias in included studies (Lytvyn *et al.*, 2016; Yang *et al.*, 2017).

(ii) *Evidence from studies in paediatric patients:* In a RCT, 30 children (<15 years) with various surgical (majority gastrointestinal) conditions were supplemented with probiotic *Bifidobacterium breve* BBG-01 or placebo daily from 7 days before the surgery until discharge. Probiotic supplementation was safe. It improved the gut flora, increased the concentration of faecal acetic acid and decreased the risk of septicaemia (Okazaki *et al.*, 2016). A recent meta-analysis that included 198 infants with HD (two RCTs, three observational studies) reported that the incidence of HAEC 22.6% in the probiotic group vs. 30.5% in the controls, but the difference was not statistically significant (OR 0.72; 95% CI 0.37–1.39; $P = 0.33$; Nakamura *et al.*, 2018). Majority of the infants in the included studies were outside the neonatal period.

(iii) *Evidence from studies in neonates:* A systematic review (Rao *et al.*, 2018) that focussed on CGISC exclusively in the neonatal population found only two small RCTs (Murakami *et al.*, 2016; Powell *et al.*, 2016). The Powell *et al.* (2016) RCT included 24 neonates with gastroschisis (Probiotics: 12, Placebo: 12). The probiotic supplement was administered for 6 weeks or until hospital discharge, whichever came first. Significant dysbiosis was noted in the study infants, and it was partially attenuated by administration of *Bifidobacterium longum* subsp. *infantis* (Powell *et al.*, 2016). In the RCT by Murakami *et al.* (2016), four surgical neonates (duodenal atresia, anorectal malformations) received probiotics, four received no probiotics. Bifidobacteriaceae was more abundant in neonates who had not received probiotics. It was concluded that surgical stress appeared to affect the intestinal microbiota considerably. The need for further RCTs in this area was emphasized.

Safety of probiotics

Evidence from over 35 RCTs with a total sample size of nearly 12 000 and observational studies with over 14 000 participants show that probiotics are beneficial and safe in preterm non-surgical infants (Olsen *et al.*, 2016; Rao *et al.*, 2016; Sawh *et al.*, 2016; Dermyshe *et al.*, 2017). Even a large RCT that did not show benefits of probiotic supplementation acknowledged that short-term safety of probiotics was good in preterm infants (Costeloe *et al.*, 2016). Recent meta-analyses have shown that probiotics do not increase or decrease the risk of intraventricular

haemorrhage, chronic lung disease, retinopathy of prematurity and neurodevelopmental outcomes in preterm non-surgical infants (Cavallaro *et al.*, 2017; Villamor-Martinez *et al.*, 2017; Upadhyay *et al.*, 2018). These findings provide reassurance regarding medium-term safety of probiotics in preterm infants. However, there are few case reports of sepsis due to probiotic organisms (Ohishi *et al.*, 2010; Vallabhaneni *et al.*, 2015; Brecht *et al.*, 2016). Hence, constant vigilance and quality assurance of the product while conducting RCTs of probiotic supplementation in infants with CGISC are warranted.

Ongoing RCTs of probiotics in infants with CGISC

To our knowledge, currently, there are two ongoing RCTs evaluating the role of probiotics in this area. One trial is being conducted in Calgary (Canada) and aims to recruit 88 infants born between 23 and 42 weeks of gestation who require gastrointestinal surgery (Mugarab-Samedi *et al.*, 2017). The probiotic supplement is FloraBabyTM (Renew Life Canada, Oakville, ON, Canada). Each sachet (1 g) will have 4 billion colony-forming units (CFU) of probiotics, consisting of *Bifidobacterium breve* (HA-129), *Lactobacillus rhamnosus* (HA111), *Bifidobacterium bifidum* (HA-132), *Bifidobacterium longum* subsp. *infantis* (HA-116) and *Bifidobacterium longum* subsp. *longum* (HA-135). Placebo is maltodextrin. The primary outcome of interest is length of hospital stay. Stool microbial analysis using culture independent 16S rRNA studies will be undertaken.

The other study (ours) is being conducted in Western Australia (Rao *et al.*, 2017). Sixty infants (≥ 35 weeks' gestation) with major CGISC will be recruited. The probiotic group will receive 3×10^9 CFU/day (i.e. 3 billion organisms) in 1.5 ml of the expressed breast milk or sterile water, given as a single daily dose via the orogastric/nasogastric feeding tube or orally. The probiotic sachet (Morinaga Industries, Tokyo, Japan) will contain a mixture of three strains (*B. breve* M-16V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536 (1×10^9 CFU of each strain per 1 g sachet)). Placebo is maltodextrin. Supplementation will be commenced as soon as possible after admission once the baseline stool samples are collected and will be continued until discharge. Primary outcome will be gut microbiota (using 16 s ribosomal RNA Pyrosequencing studies for phylogenetic profiling) on stool samples. Secondary outcomes will be stool short-chain fatty acids and relevant clinical outcomes.

Conclusions

In summary, probiotic supplementation has the potential to minimize gut dysbiosis and improve clinical outcomes

of neonates with CGISC. Though small, the completed and ongoing RCTs will provide important data and confidence to embark on adequately powered large RCTs in this exciting area.

Conflict of interest

None declared.

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Preface to Chapter 9

Probiotic Supplementation in Neonates with Congenital Gastrointestinal Surgical Conditions: A Pilot Randomised Controlled Trial

The previous chapters discussed the importance of conducting RCTs evaluating probiotic supplementation in neonates with congenital gastrointestinal surgical conditions (CGISCs). This chapter presents the results of our double-blind pilot RCT in which neonates with CGISCs were randomised to receive a triple-strain bifidobacterial probiotic (3 billion organisms per day) or placebo (maltodextrin) during their stay in the neonatal intensive care unit. The results confirmed our hypothesis that probiotic supplementation attenuates gut dysbiosis and improves short-chain fatty acid levels (especially acetate). It also found that probiotic supplementation may improve head growth in these infants.

*The results of this RCT were published in *Pediatric Research*. 2022 Jan 3:1–10.*

CHAPTER 9

CLINICAL RESEARCH ARTICLE



Probiotic supplementation in neonates with congenital gastrointestinal surgical conditions: a pilot randomised controlled trial

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OBJECTIVE: To evaluate whether probiotic supplementation attenuates gut-dysbiosis in neonates with congenital gastrointestinal surgical conditions (CGISC).

METHODS: Sixty-one neonates (≥ 35 weeks gestation) with CGISC were randomised to receive daily supplementation with a triple-strain bifidobacterial probiotic ($n = 30$) or placebo ($n = 31$) until discharge. Stool microbiota was analysed using 16S ribosomal RNA gene sequencing on samples collected before (T1), 1 week (T2), and 2 weeks (T3) after supplementation and before discharge (T4). The primary outcome was the sum of the relative abundance of potentially pathogenic families of Clostridiaceae, Enterobacteriaceae, Enterococcaceae, Pseudomonaceae, Staphylococcaceae, Streptococcaceae, and Yersiniaceae at T3.

RESULTS: The median gestational age [38 weeks (IQR: 37.1–38.9)] was similar in both groups. The probiotic group had lower rates of caesarean deliveries (40% versus 70%, $p = 0.02$). The relative abundance of potentially pathogenic families was lower in the probiotic group compared to placebo at T3 [(median: 50.4 (IQR: 26.6–67.6) versus 67.1 (IQR: 50.9–96.2); $p = 0.04$]. Relative abundance of Bifidobacteriaceae was higher in the probiotic group at T3 [(median: 39.8 (IQR: 24.9–52.1) versus 0.03 (IQR 0.02–2.1); $p < 0.001$]. Stratified analysis continued to show a higher abundance of Bifidobacteriaceae in the probiotic group, irrespective of the mode of delivery.

CONCLUSIONS: Probiotic supplementation attenuated gut dysbiosis in neonates with CGISC.

TRIAL REGISTRATION: <http://www.anzctr.org.au> (ACTRN12617001401347).

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IMPACT:

- Probiotic supplementation attenuates gut dysbiosis and improves stool short-chain fatty acid levels in neonates with congenital gastrointestinal surgical conditions.
- This is the second pilot RCT of probiotic supplementation in neonates with congenital gastrointestinal conditions.
- These findings will pave the way for conducting multicentre RCTs in this area.

INTRODUCTION

The common morbidities in neonates with congenital gastrointestinal surgical conditions (CGISC) are feed intolerance and healthcare-associated infections (HAI).^{1–9} Recurrent administration of antibiotics, delayed enteral feeds, use of parenteral nutrition (PN), and delayed exposure to the mother's skin and breast milk microbiota can lead to intestinal dysbiosis in these infants.^{10–15} Our previous prospective study found that neonates with CGISC develop gut dysbiosis during their stay in the neonatal intensive care unit.¹³

Experimental studies have shown that probiotic supplementation attenuates gut dysbiosis, strengthens the gut barrier, prevents enteropathogenic infections, reduces antimicrobial resistance, enhances immunity, and promotes gut peristalsis.¹⁶ Through these mechanisms, probiotics have the potential to improve the outcomes of neonates with CGISC.¹⁶ Many beneficial biological functions of probiotics are mediated via short-chain fatty acids (SCFAs).¹⁷ The major SCFAs (80–95%) in the gut are acetate, propionate, and butyrate.^{18,19}

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In infants, SCFAs are produced by the fermentation of milk oligosaccharides by gut bacteria, and the type of milk has a significant effect on the composition of the SCFA.²⁰

Our previous prospective study also found that neonates with CGISC have lower faecal SCFA levels.¹³

Systematic reviews from adult studies have concluded that probiotic/synbiotic supplementation is safe and reduces the risk of postoperative infections.²¹ A pilot randomised controlled trial (RCT) ($n = 24$) in neonates with gastroschisis reported that gut dysbiosis was partially attenuated by the probiotic *Bifidobacterium longum* subsp. *infantis*.²² Our systematic review found limited evidence regarding the role of probiotics in neonates with CGISC and recommended the conduct of RCTs.²³ Hence, we conducted this pilot RCT to evaluate the efficacy and safety of probiotics in attenuating dysbiosis and improving SCFA levels in term and near-term infants with CGISC.

METHODS

Hypothesis

Probiotic supplementation attenuates gut dysbiosis and increases stool SCFA levels in term and near term neonates with CGISC.

Design and setting

Double blind RCT in the neonatal intensive care unit of Perth Children's Hospital, Western Australia. Approval was obtained from the Institutional Human Research Ethics Committee (HREC Ref Number RGS0000002554). It was registered with the Australia and New Zealand Clinical Trials Registry (ACTRN12617001401347). The protocol is available in Supplement File 3. Infants were recruited between November 2017 and March 2020.

Eligibility criteria included

Neonates (≥ 35 weeks' gestation) with intestinal atresia, malrotation, congenital diaphragmatic hernia (CDH), tracheoesophageal fistula (TOF), gastroschisis, exomphalos, Hirschsprung disease, imperforate anus, short bowel syndrome, and other surgical conditions requiring stomas (e.g., severe meconium ileus, microcolon). Preterm infants less than 35 weeks of gestation were excluded to reduce the confounding effect of prematurity on the gut microbiota.

Intervention

A mixture of three strains (*Bifidobacterium breve* M 16V, *Bifidobacterium longum* subsp. *infantis* M 63, and *Bifidobacterium longum* subsp. *longum* BB536; 1×10^9 colony forming units (CFU) of each strain per 1 g sachet; Morinaga Milk Industry Co., Ltd., Japan]. Dose was 1 sachet per day, which provided a total of 3×10^9 (i.e., 3 billion) CFU per day. *Placebo*: Maltodextrin. The trial supplements were stored in a refrigerator at 2–8 °C.

Group assignments were allocated using a computer generated sequence in randomly ordered block sizes of 2 and 4. These sequences were generated by our institutional trial pharmacist, without the involvement of the research team. Allocation concealment was optimised through pharmacy controlled allocation; the clinical trial pharmacist generated random numbers and used them to prepare sequentially numbered individual boxes, each containing 30 sachets of trial supplements. The sachets containing probiotics or placebo were of identical design, weight, and volume; the boxes containing the sachets were also of similar design. The contents of the sachets (probiotic or placebo) were similar in texture, smell, and taste. All these steps were undertaken to ensure adequate blinding of healthcare providers and parents. Once parental consent was obtained, the chief investigator or his delegate allocated the next box of study supplement to the infant without knowledge of the contents of the box (probiotic or placebo).

In the postoperative period, once baseline stool samples were collected, infants were given trial supplements once daily until discharge. Trial supplements were dissolved in 1.5 mL of expressed breast milk (EBM) or sterile water (if EBM was not available) and administered via the feeding tube or mouth. Supplementation was continued even when the infants did not receive enteral feeds. If an infant was having continuous or intermittent gastric suctioning, once the trial supplements were given, suctioning was stopped for 3–4 h prior to recommencing.

Feeding regimen of study infants

The standard policy of our unit is to commence PN within 48–72 h of admission for surgical infants who are unable to tolerate enteral feeds. Enteral feeding with expressed breast milk were commenced as soon as possible in the postoperative period (usually on postoperative day 2 and advanced as tolerated), depending on the underlying surgical condition and consensus opinion of neonatologists and surgeons. Infant formulae or hydrolysed formulae are rarely used in our unit for surgical infants. None of our study infants received donor breast milk because it was reserved for preterm infants less than 32 weeks of gestation in our unit.

Stool sample collection, storage, and analysis

Stool samples were collected at four time points: (a) as soon as possible after admission, but before commencing trial supplements (T1); (b) 1 week (T2), and (c) 2 weeks after commencing supplements (T3); and (d) prior to discharge (T4).

The samples were collected from the nappies into sterile microvials and stored in the NICU at 20 °C for 3–4 days and subsequently at 80 °C. At the completion of full recruitment, samples were shipped on dry ice to the University of New South Wales (Sydney, Australia), where microbial analysis was undertaken. Acidified samples were frozen at 20 °C and shipped to the School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore, where SCFA analysis was performed.

The stool microbiota was assessed using the 16S ribosomal RNA gene sequencing method (Supplementary File 1). Stool short chain fatty acid assay was performed according to our previously described method¹³ with slight modifications (Supplementary File 1). The scientists who conducted microbial and SCFA assays and their respective statistical analyses were blinded to the randomisation groups. They conducted statistical analyses of data from stool samples as group 1 versus group 2. The trial pharmacist disclosed the groupings only after receiving all the results through e mail. This ensured adequate blinding of clinicians, research teams, lab scientists, and statisticians throughout all stages of the trial.

Primary outcome

The primary outcome was the sum of the relative abundance of potentially pathogenic families of Clostridiaceae, Enterobacteriaceae, Enterococaceae, Pseudomonaceae, Staphylococcaeae, Streptococcaeae, and Yersiniaceae at T3 using 16S ribosomal RNA gene sequencing methods. T3 was chosen as the main time point of interest because 2 weeks is a reasonable duration of supplementation to enable colonisation by the probiotic strains, and many study infants will still be in the hospital.

Secondary outcomes

(1) Stool microbiota at T1, T2, and T4 time points; (2) SCFA levels at all time points; (3) short term clinical outcomes during initial stay in the NICU, such as incidence of mortality, HAI, duration of antibiotics, PN, hospital stay, time to reach full feeds after surgery, and physical growth. The z scores for weight, length, and head circumference at birth were calculated using the Fenton growth charts²⁴ and at discharge using the WHO charts²⁵ through the publicly accessible PediTools website of clinical calculators.²⁶

Statistical considerations

Sample size estimation. In a recent RCT, the sum of the relative abundance of potentially pathogenic families such as Enterobacteriaceae, Staphylococcaeae, Enterococcaeae, Clostridiaceae, and Streptococcaeae was approximately 76% in the stools of infants with gastroschisis who received placebo.²² Hence, we calculated that a total sample size of 60 infants (probiotic: 30, placebo: 30) would be required to demonstrate a 50% reduction of potentially pathogenic bacterial families from 76 to 38% after 2 weeks of supplementation with probiotics with an alpha error of 0.05, and a power of 80%.

Statistical analysis of clinical data. Continuous data with normal distribution were summarised using mean and standard deviations (SD) and compared using the two sample t test. Median, interquartile range (IQR), and range were used to summarise data with skewed distribution and compared using the Wilcoxon rank sum test. Binary outcomes were compared using the Fisher's exact test. To compare the z scores of physical growth parameters at discharge versus birth, the matched pairs t test was used. For all analyses, a p value less than 0.05 was considered significant.

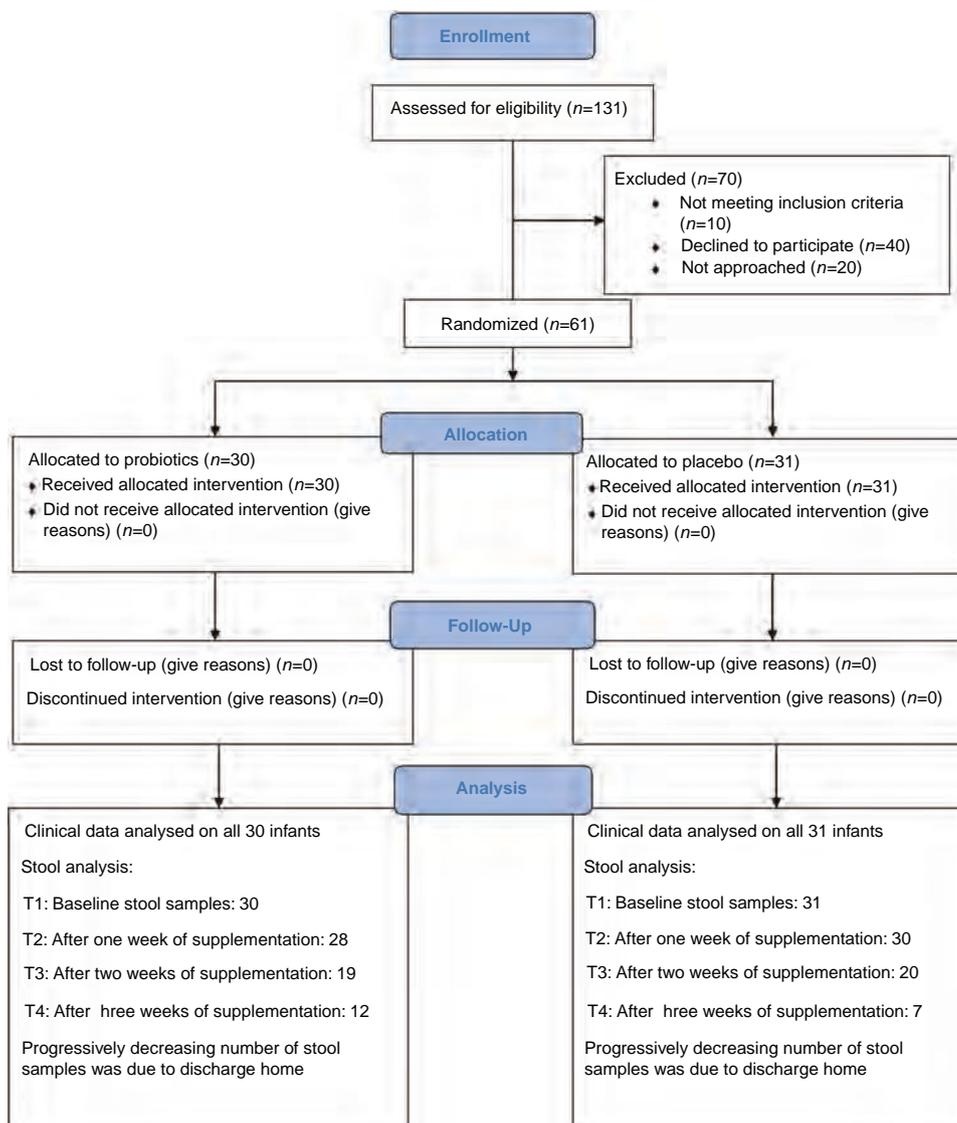


Fig. 1 CONSORT flow diagram showing participant flow through the trial.

Statistical analysis for microbiological data. Analyses were conducted with R version 3.6.0. For microbial richness, linear mixed model effects (LME) test (MASS, lme4, and lmerTest packages) was used to identify if there were significant differences between the groups over time. In our LME model, Subject ID was a random factor, while time and treatment were used as fixed factors. Post hoc pairwise comparisons were performed using Tukey's HSD method to adjust for multiple comparisons. Wilcoxon rank sum test with Benjamini Hochberg correction to adjust for multiple testing was used to identify differences between the groups at the various time points.

For beta diversity, PERMANOVA was used to check if community structures differed between the groups at the various time points followed by pairwise adonis test (<https://github.com/bwemheu/pairwise.adonis>) for pairwise comparisons between the groups. *P* values were adjusted for multiple testing using the Benjamini Hochberg correction.

Taxa significantly different between the groups were identified at the phylum, class family, and genus levels using analysis of the composition of the microbiome (ANCOM; v2.0).²⁷ ANCOM compares the log ratio abundance of each taxon among multiple groups to all the remaining taxa. It uses the Kruskal Wallis test to assess significant differences, with multiple testing corrected by the Benjamini Hochberg (BH) false discovery rate. For this study, we accepted the *W* statistic cutoff of 0.7 from the ANCOM output to define taxa as significantly different. Pairwise comparisons in the groups over time was then determined using our LME model. For pairwise comparison of the taxa at T3, Wilcoxon rank sum test with BH correction was

used. For all microbiota and SCFA analysis an adjusted *p* value of <0.05 was considered significant.

Statistical analysis of SCFA data. Wilcoxon's rank analysis with BH correction was performed to compare SCFA concentrations between the two groups (probiotics and placebo) at the different time points.

An independent data safety and monitoring committee (DSMC) reviewed the data at 50% of recruitments and advised to continue the study. The safety issues that arose during the early stages of the trial are described in Supplementary File 1. The CONSORT checklist (Supplementary File 2) was used to report the results of this RCT.²⁸

RESULTS

The study recruitment was completed in March 2020 after achieving full sample size. A total of 61 infants were randomised to receive either probiotics ($n = 30$) or placebo ($n = 31$). All infants received the trial supplements as per original randomisation without any crossovers. All infants were analysed as per the original assigned groups. Figure 1 shows a CONSORT flow diagram. Table 1 presents the clinical details of the study infants. The median gestational age, birth weight, Apgar scores, cord pH, and lactate levels were similar between the two groups. The incidence of maternal pregnancy-

Table 1. Clinical characteristics of study infants.

	Probiotic (N 30)	Placebo (N 31)	P value
Gestation (weeks)	38.1 (IQR: 37.1–39) (range: 35.1–41)	37.7 (IQR: 37.1–38.9) (range: 35.6–41.8)	0.675
Birth weight (g)	2960 (IQR: 2570–3688) (range: 1885–4130)	2985 (IQR: 2570–3270) (range: 2000–3730)	0.531
Birth length (cm)	50 (IQR: 47–51) (range: 40–57)	49 (IQR: 47–50) (range: 43–56)	0.623
Birth head circumference (cm)	33.7 (IQR: 32.5–35) (range: 31–37)	34 (IQR: 33–35) (range: 31–37.5)	0.820
Maternal PIH	0	1 (3%)	1.000
Maternal APH	7 (23.3%)	0	0.005
Maternal diabetes	1 (3.3%)	5 (16%)	0.20
Maternal chorioamnionitis	0	0	NE
Maternal intrapartum antibiotics	18 (60%)	20 (64%)	0.934
Caesarean	12 (40%)	22 (71%)	0.021
Apgar scores at 5 min	9 (IQR: 9–9) (range: 7–10)	9 (IQR: 8–9) (range: 6–10)	0.768
Cord pH	7.27 (IQR: 7.23–7.31) (range: 7.15–7.40)	7.28 (IQR: 7.23–7.33) (range: 7.1–7.36)	0.862
Cord lactates (mmol/L)	3.3 (IQR: 2.1–4.3) (range: 2–9.4)	3.8 (IQR: 2.4–5) (range: 1–8)	0.632
Major gastrointestinal conditions	CDH: 7, gastroschisis: 7; OA with TOF: 5, imperforate anus: 1; CDO: 2; exomphalos: 1; ileal atresia: 2; JIA: 2; malrotation: 1, meconium ileus: 0; colon perforation: 1; HD: 1, duplication cyst: 0	CDH: 1, gastroschisis: 5; OA with TOF: 5, imperforate anus: 6; CDO: 4, exomphalos: 1; ileal atresia: 1; JIA: 2; malrotation: 0, meconium ileus with CF: 2; colon perforation: 0; HD: 3, duplication cyst: 1	0.166
CDH	7	1	0.026
Day of life, first stool sample	6 (IQR: 5–6) (range: 2–17), N 30	6 (IQR: 3–9) (range: 1–25), N 31	0.425
Day of life, second stool sample	15.5 (IQR: 13–17.5) (range: 11–27), N 28	13 (IQR: 12–18) (range: 9–34), N 30	0.121
Day of life, third stool sample	23 (IQR: 20–25) (range: 16–30), N 19	20 (IQR: 20–26) (range: 13–42), N 19	0.463
Day of life, fourth stool sample	38.5 (IQR: 29.5–42) (range: 26–46), N 8	34 (IQR: 32–38) (range: 27–53), N 6	0.824
Day of life consent was given	5 (IQR: 4–7) (range: 2–18)	5 (IQR: 3–7) (range: 2–25)	0.535
Day of life supplements were commenced	6.5 (IQR: 5–9) (range: 4–18)	7 (IQR: 5–11) (range: 3–26)	0.820
Duration of supplementation (days)	18.5 (IQR: 11–27) (range: 3–54)	16 (IQR: 10–22) (range: 4–58)	0.337

Bold values indicate statistical significance $p < 0.05$.

SVD spontaneous vaginal delivery, AVD assisted vaginal delivery, CS caesarean section, APH antepartum haemorrhage, PIH pregnancy induced hypertension, NE not estimable, IQR interquartile range, OA oesophageal atresia, JIA jejuno ileal atresia, CF cystic fibrosis, HD Hirschsprung disease, IQR interquartile range.

induced hypertension (PIH), chorioamnionitis, and peripartum antibiotics was similar between the groups. The incidence of antepartum haemorrhage (APH) was higher, and caesarean delivery rates were lower in the probiotic group. The probiotic group had more infants with congenital diaphragmatic hernia than the placebo group. The median age at surgery was 2 days in both the groups. The median age at commencement of supplementation was approximately 7 days in both groups, and the duration of supplementation was 16–18 days.

Results of microbial analysis

Of the 61 recruited infants, stool samples were available for 61, 58, 39, and 29 infants at time points T1, T2, T3, and T4, respectively. The attrition in sample size was because 22 infants were discharged home before completing 2 weeks of supplementation (i.e., before reaching T3). We could not extend the trial beyond March 2020 to recruit additional infants because of severe restrictions that were implemented due to the COVID-19 pandemic during that period.

Alpha diversity (i.e., Richness and Shannon diversity index) was comparable between the two groups at all time points (all $p > 0.05$; Fig. 2a). Similar results were obtained on Chao1 and ACE estimations (data not shown). Observed OTUs increased in both groups over time and the probiotic group displayed significantly increased bacterial richness at T4 compared to T1 ($p = 0.015$) and

T2 ($p < 0.01$; Fig. 2a). Beta diversity analysis revealed that both groups had similar community structures at T1 ($p = 0.807$; Fig. 2b). However, at T2, T3, and T4, the community structures of the probiotic group were significantly different from those of the placebo group (all $p < 0.05$; Fig. 2b).

Comparisons at the bacterial phylum level. Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Proteobacteria were the most common phyla in the stool samples (e-Fig. 1A in Supplementary File 1). The median relative abundance of Actinobacteria was significantly higher in the probiotic group than in the placebo group at T3 (40.1% versus 0.1%; $p < 0.0001$) and at T2 and T4 (both $p < 0.01$; e-Fig. 1B in Supplementary File 1). Although the relative abundance of Proteobacteria was similar between the two groups at T3 (22.7% in probiotic versus 50.3% in placebo; $p = 0.27$) and at other time points (e-Fig. 2 in Supplementary File 1), the levels in the probiotic group decreased over time, with T2 and T4 being significantly different from T1 ($p = 0.048$ and $p = 0.046$, respectively).

Comparisons at the bacterial class level. The relative abundance of Gammaproteobacteria between the two groups was similar at all time points (e-Fig. 3 in Supplementary File 1).

Comparisons at the bacterial family level. The relative abundance of the sum of potentially pathogenic families of Clostridiaceae,

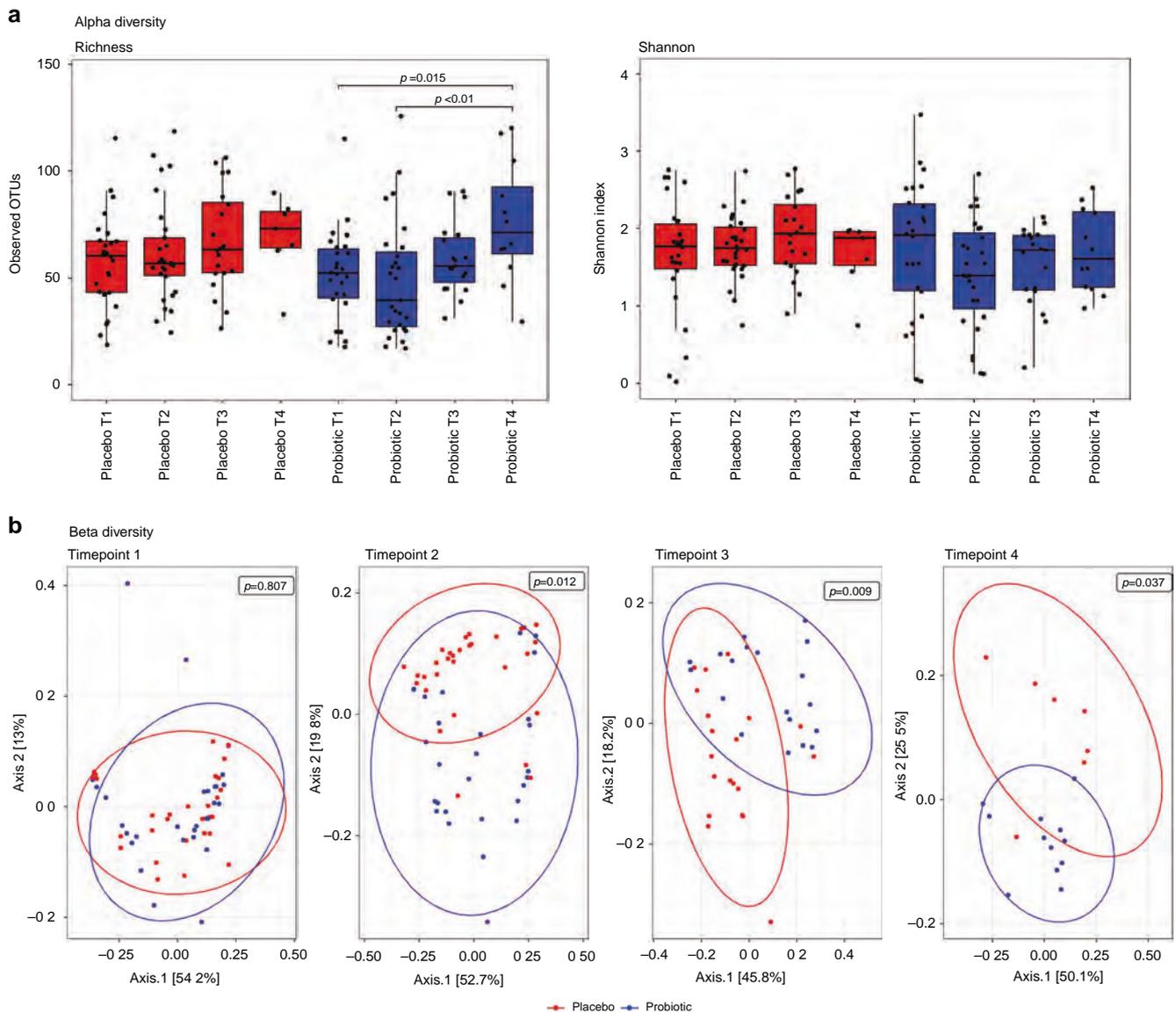


Fig. 2 Alpha and Beta diversity at various time points in the stool samples of study infants. **a** Alpha diversity. Richness and the Shannon diversity index were similar between the probiotic and placebo groups at all time points (all $p > 0.05$). **b** Beta diversity as measured by weighted unifrac by time point. At baseline, infants in the probiotic group had similar community structures to the placebo group ($p = 0.807$). However, at subsequent time points T2, T3, and T4, the community structures of the probiotic group were significantly different from the placebo group (all $p < 0.05$).

Enterobacteriaceae, Enterococcaceae, Pseudomonadaceae, Staphylococcaceae, Streptococcaceae, and Yersiniaceae were significantly lower in the probiotic group compared to placebo at T3 [(median: 50.4 (IQR: 26.6–67.6) versus 67.1 (IQR 50.9–96.2); $p = 0.044$) and at T2 and T4 ($p = 0.006$ and $p = 0.014$, respectively; Fig. 3a). Since there was a slight imbalance in the number of stool samples between the probiotic ($n = 19$) and placebo ($n = 20$) groups at T3, one sample from the placebo group was randomly removed and the data were reanalysed. The results continued to remain similar to the original analysis ($p = 0.036$).

The relative abundance of the family Bifidobacteriaceae was significantly higher in the probiotic group at T3 [(median: 39.8 (IQR: 24.9–52.1) versus 0.03 (IQR 0.02–2.1); $p < 0.001$) and at T2 and T4 (both $p < 0.001$; Fig. 3b).

Comparisons at the bacterial genus level. The relative abundance of the genus *Bifidobacterium* was significantly higher in the

probiotic group at T2, T3, and T4 (all $p < 0.001$; e-Fig. 4 in Supplementary File 1).

Stool SCFAs: The total SCFA levels were higher in the probiotic group than in the placebo group at T3 ($p = 0.008$; Fig. 4). Acetate levels were higher in the probiotic group (e-Fig. 5 in Supplementary File 1). The butyrate levels were similar between the groups at all time points except T2, when they were lower in the probiotic group (e-Fig. 6 in Supplementary File 1). The propionate levels were similar between the two groups at all time points (e-Fig. 7 in Supplementary File 1).

Post hoc subgroup analysis based on the mode of delivery

Since the probiotic group had significantly lower rates of caesarean section, we conducted a post hoc analysis separately for infants born via caesarean section and through the vaginal route. The results showed a significantly higher abundance of Bifidobacteriaceae in the

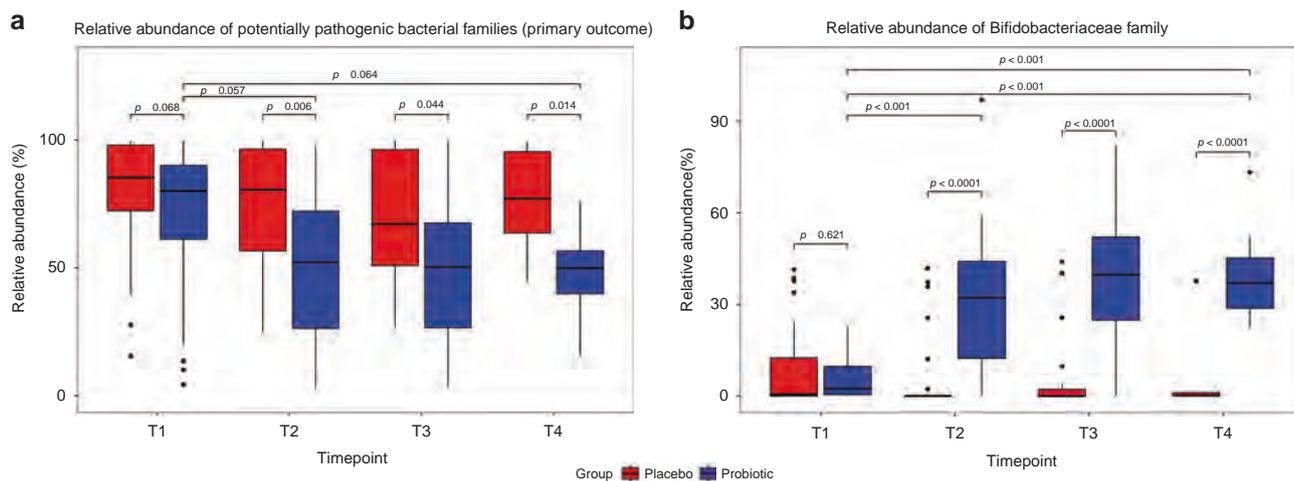


Fig. 3 Comparisons of relative abundances of bacterial families in the stool samples at various time points. **a** Comparison of relative abundance of potentially pathogenic families (sum-total of Clostridiaceae, Enterobacteriaceae, Enterococcaceae, Pseudomonadaceae, Staphylococcaceae, Streptococcaceae and Yersiniaceae) between probiotic and placebo at various time points. At the bacterial family level, the relative abundance of the sum-total of potentially pathogenic families of Clostridiaceae, Enterobacteriaceae, Enterococcaceae, Pseudomonadaceae, Staphylococcaceae, Streptococcaceae & Yersiniaceae were significantly lower in the probiotic group compared to placebo at time points T2, T3, and T4 ($p = 0.002$, 0.033 , and 0.007 respectively). **b** Comparison of relative abundance of family Bifidobacteriaceae between probiotic and placebo at various time points. The relative abundance of the family Bifidobacteriaceae was significantly higher in the probiotic group at T2, T3, and T4 (all $p < 0.001$).

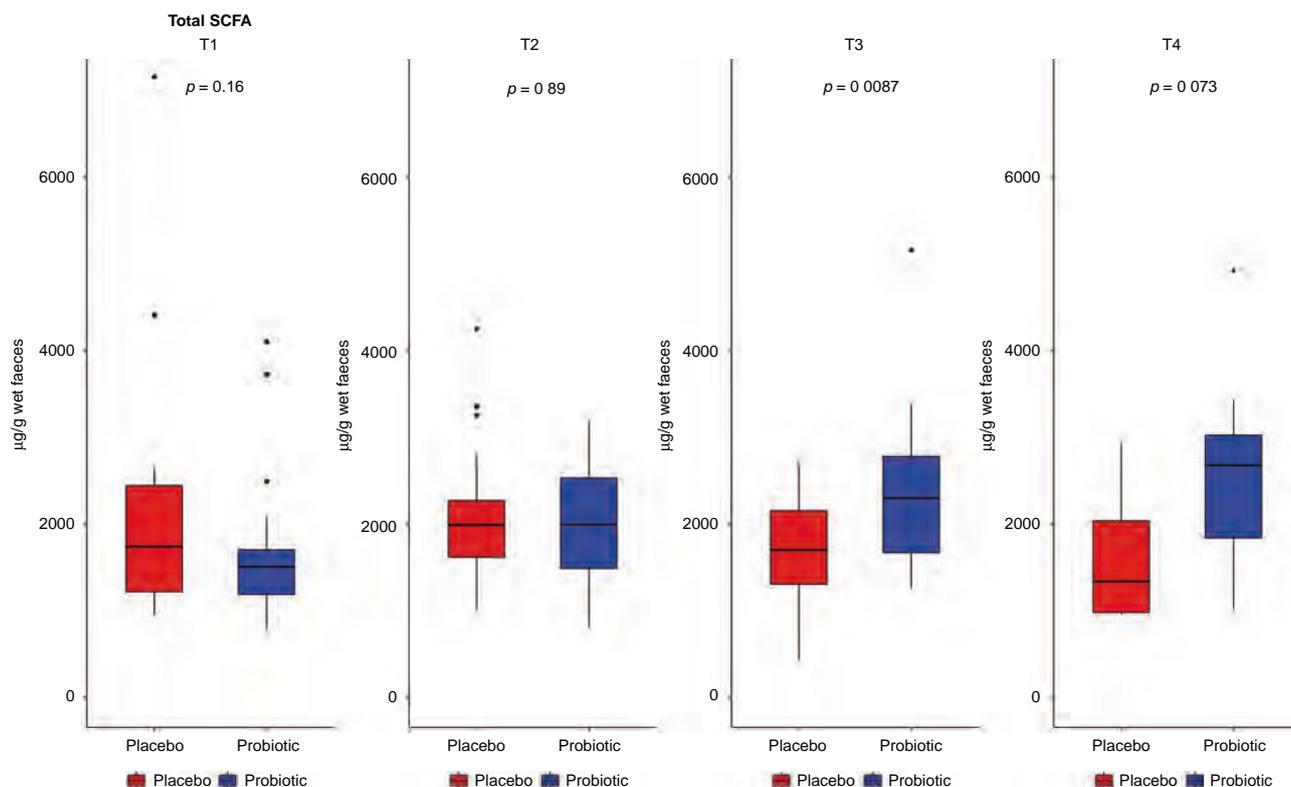


Fig. 4 Total SCFA levels in stools between probiotic and placebo groups at various time points. Total SCFA levels in stools were significantly higher in the probiotic group at time point T3 ($p = 0.008$). At other time points, the differences between the two groups were not statistically significant.

probiotic group, irrespective of the mode of delivery. However, the beneficial effects of supplementation on SCFA levels were significant only in infants born by caesarean section. The relative abundance of pathogenic families was not statistically significant in either mode of delivery, even though the trend was in favour of probiotics (Table 2).

Clinical outcomes

All participating infants survived. There were no significant differences between the groups in the incidence of HAI, duration of antibiotics, PN, hospital stay, and time to reach full feeds after surgery (Table 3). All routine clinical specimens (blood, urine, CSF,

Table 2. Effect of probiotics supplementation at T3, based on route of delivery. Effect of probiotics supplementation based on route of delivery

	Vaginal delivery			Caesarean section		
	Probiotic Median (IQR, range) N = 9	Placebo Median (IQR, range) N = 6	P value	Probiotic Median (IQR, range) N = 9	Placebo Median (IQR, range) N = 12	P value
Sum of relative abundance of potentially pathogenic families at T3	49.1 (25.9–67.4)	74.2 (49.6–89.8)	0.37	52.5 (44.2–67.7)	58.4 (51.2–95.9)	0.39
Relative abundance of Bifidobacteriaceae at T3	42.8 (23.7–55.2)	0.05 (0.03–12.9)	0.049	33.7 (28.9–45.4)	0.03 (0.01–1.51)	0.001
Total SCFA levels at T3 (µg per gram of wet faeces)	2254 (QR: 1915–2668); range: 1243–5152	1529 (QR: 1349–2208); range: 723–2647	0.18	2598 (QR: 1588–2786); range: 1399–3388	1725 (QR: 1092–2135); range: 426–2723	0.034

Bo d values indicate statistical significance $p < 0.05$. IQR interquartile range, SCFA short-chain fatty acid, T3 two weeks after commencing supplementation.

endotracheal secretions, wound swabs) from study infants were analysed using aerobic and anaerobic culture methods. There were no cases of infections due to the administered probiotics.

Physical growth outcomes

The z-scores for weight were significantly lower at discharge than at birth in the probiotic and placebo groups (both $p < 0.0001$; e-Fig. 8 in Supplementary File 1). The degree of postnatal growth restriction for weight was similar between the two groups (0.93 in the probiotic and 0.79 in the placebo; $p = 0.486$; Table 1, e-Fig. 9 in Supplementary File 1). The z-scores for head circumference were lower at discharge than at birth in both the probiotic and placebo groups (both $p < 0.0001$; e-Fig. 10 in Supplementary File 1). However, postnatal growth restriction for head circumference was less severe in the probiotic group ($p = 0.013$; Table 1 and Fig. 5). The z-scores for length at discharge were similar to those at birth in both the probiotic and placebo groups (e-Fig. 11). The degree of postnatal growth restriction for length was similar between the two groups (e-Fig. 12).

DISCUSSION

This pilot RCT found that after 2 weeks of supplementation with the three-strain bifidobacterial probiotic, neonates with CGISC had a lower relative abundance of potentially pathogenic bacterial families, higher abundance of bifidobacteria, and higher SCFA levels in their stools compared to placebo. Postnatal growth restriction for head circumference was less severe in the probiotic group than in the placebo group. Other clinical outcomes were similar between the two groups. No infections related to the administered probiotic organisms were observed. These results provide reassurance regarding the use of this probiotic in neonates with CGISC.

The only other RCT in neonates with surgical conditions was by Powell et al.,²² in which 24 infants with gastroschisis were supplemented with *Bifidobacterium longum* subsp. *infantis* ATCC 15697 or placebo. In their study, the daily dose was 2×10^9 CFU compared with 3×10^9 CFU in our study. Similar to their study, the majority of our infants received breast milk as the sole source of diet, trial supplements were commenced in the postoperative period, and given for a median duration of 3 weeks. Similar to their study, the relative abundance of Bifidobacteriaceae increased to 40% after commencing supplements in the probiotic group and remained at 3% in the placebo group.

In line with the recent literature, our study found that neonates with CGISC develop postnatal growth restriction for weight and head circumference.^{29,30} Studies in preterm infants have reported an association between postnatal growth restriction of the head circumference and adverse developmental outcomes.^{31–33} Hence, it was reassuring that postnatal growth restriction for head circumference was less severe in the probiotic group in our RCT. Similar beneficial effects of probiotics on head circumference were observed in extremely low birth weight infants in recent RCTs.^{34,35}

In our current RCT, SCFA levels were significantly higher in the probiotic group at T3 than in the placebo group, mainly due to elevated acetates. Even though the levels were also higher at T2 and T4, they were not statistically significant, probably because infants would have received only 1 week of supplementation by T2, whereas by T4, the sample size was very small. Bifidobacteria are known to ferment HMOs and produce acetate as a by-product.³⁶ Bifidobacteria are not butyrogenic, and hence butyrate levels were not elevated in the probiotic group in our study. While it has been suggested that acetate and lactate produced by bifidobacteria can be used as a substrate by other commensal bacteria (cross-feeding)³⁷ to produce butyrate, thereby increasing its levels, this was not observed in our study.

The relative abundance of Proteobacteria, considered to be the microbial signature of gut dysbiosis,³⁸ was lower in the probiotic

Table 3. Clinical course and outcomes of study infants.

	Probiotics	Placebo	P values
Mortality	0	0	NE
Healthcare associated infections (HABSI or UTI or SSI or VAP or pleural infection or peritonitis or meningitis or viral infection)	4 (13.3%)	6 (19.3%)	0.731
HABSI before commencing trial supplements	0	1 (3%)	1.000
HABSI after commencing trial supplements	0	2 (6.5%)	0.492
Duration of antibiotics before commencement of trial supplements (days)	5 (IQR: 4–7) (range: 2–10)	5 (IQR: 3–6) (range: 1–11)	0.336
Duration of antibiotics after commencement of trial supplements (days)	3 (IQR: 0–6) (range: 0–31)	2 (IQR: 0–5) (range: 0–13)	0.305
Total duration of antibiotics (days)	9 (IQR: 4–13) (range: 2–34)	6 (IQR: 5–10) (range: 1–22)	0.312
Cholestatic jaundice	1 (3.3%)	1 (3.2%)	1.000
Sepsis due to the administered probiotic organism	0	0	NE
Duration of PN (days)	13.9 (IQR: 9–24) (range: 0–46)	10.1 (IQR: 6.9–21.3) (range: 0–58.7)	0.246
EBM to commence feeds	30 (100%)	28 (93.3%)	0.492
Time to commence feeds after surgery (days)	3 (IQR: 2–5) (range: 0–10)	2 (IQR: 1–4) (range: 0–9)	0.023
Time to full enteral feeds after surgery (days)	12 (IQR: 8–16) (range: 0–46)	9 (IQR: 6–15) (range: 1–52)	0.306
Exclusive EBM at discharge	24 (80%)	22 (71%)	0.554
Use of formula milk	6 (20%)	9 (29%)	0.554
Duration of hospital stay (days)	27.5 (IQR: 16–37) (range: 10–102)	20 (IQR: 13–31) (range: 10–67)	0.094
Z scores for weight at discharge minus at birth	0.93 (SD 0.52)	0.79 (SD 1.00)	0.486
Z scores for head circumference at discharge minus at birth	0.20 (SD 0.63)	0.70 (SD 0.84)	0.011
Z scores for length at discharge minus at birth	0.40 (SD 1.02)	0.14 (SD 1.20)	0.455

Bold values indicate statistical significance $p < 0.05$.

EBM expressed breast milk, NE not estimable, HABSI healthcare associated bloodstream infections, UTI urinary tract infection, SSI surgical site infection, VAP ventilator associated pneumonia, EBM expressed breast milk, IQR interquartile range, NE not estimable, SD standard deviation.

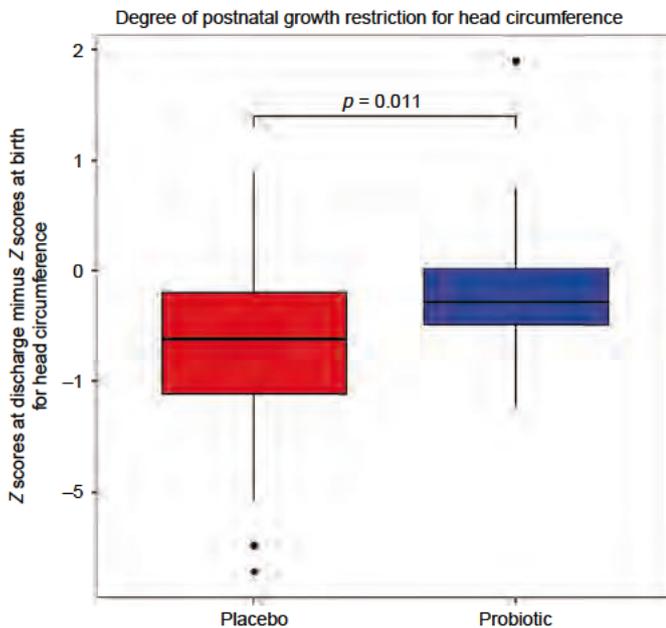


Fig. 5 Comparison of the degree of postnatal growth restriction for head circumference in study infants. The degree of postnatal growth restriction for head circumference was less severe in the probiotic group than in the placebo group.

group than in the placebo group (22.7% versus 50.3%), but the differences were not statistically significant. Studies with larger sample sizes are needed to determine if this difference is real.

Although not statistically significant, hospital stay was longer in the probiotic group (27 versus 20 days, $p = 0.095$). We speculate that this was because the probiotic group had more infants with complex conditions such as CDH and gastroschisis, and fewer infants with imperforate anus.

It was reassuring that none of the infants developed infection due to the administered bifidobacterial strains. There are case reports of bifidobacterial sepsis in surgical infants and preterm infants without surgical conditions.^{39,40} Hence, ongoing microbiological surveillance is important when conducting probiotic RCTs.

In our study infants, the use of exclusive breast milk dropped to 70–80% by discharge compared to 93–100% at birth. However, the use of formula milk was similar between the two groups (20% in the probiotic group versus 29% in the placebo group; $p = 0.554$). The common reasons for the use of formula milk were inadequate production of breast milk and mother's choice. Some studies have suggested that probiotics are more effective in infants who are breastfed rather than formula.^{41–43} Given such findings and overall benefits of breast milk, every effort should be made to encourage breastfeeding and to express breast milk during hospital stay and after discharge in these infants.

While our study did not specifically address the issue of cross-contamination (aka cross-colonisation),⁴⁴ the relative abundance of the genus *Bifidobacterium* in the placebo group was only 5% at all time points T2–T4, versus 35–45% in the probiotic group. Hence, even if there was cross-contamination, the load was not

enough to allow them to colonise adequately in the placebo group and, hence, unlikely to be clinically significant.

An important limitation of our study was the higher rates of caesarean delivery in the placebo group. Infants born by caesarean delivery are known to have a lower abundance of bifidobacteria in the first few months of life.⁴⁵ In this context, it was reassuring to know that even in subgroup analysis, the probiotic supplemented group had higher bifidobacterial counts. Our findings are similar to Frese et al.⁴⁶ who reported that supplementation resulted in higher colonisation with bifidobacteria, which persisted even after supplementation was ceased, whether the infants were born vaginally or by caesarean section.

Since many factors such as gestational diabetes,⁴⁷ mode of delivery,⁴⁵ intrapartum antibiotics^{48–50} and maternal probiotics^{51–53} can affect neonatal gut microbiota, it is difficult to achieve a balance between the two groups for all such variables in small RCTs. Future studies should consider using the technique of “allocation by minimisation”⁵⁴ to ensure adequate balance. In addition to maternal factors, important neonatal variables to be balanced include gestational age⁵⁵ and underlying surgical conditions. Since the type of milk used (breast milk versus cow’s milk based versus hydrolysed formula) can influence gut microbiota,⁵⁶ standardising their feeding regimen is desirable, but may not be feasible, given that multiple factors can influence milk production and its content. One option is to use pasteurised human donor milk, but the quantity required will be enormous because majority of these infants are born at term or near term, rather than at an extremely preterm gestation, in whom the quantity required is small. RCTs with large sample size have the potential to minimise the risk of imbalance in two groups regarding the type of milk used and other variables.

Another limitation of our study was that 16S rRNA gene sequencing allowed the allocation of reads only up to the genus level. Hence, it was not possible to confirm whether the increase in the relative abundance of *Bifidobacterium* in the probiotic group was due to the administered strains. A whole-metagenome approach that provides species- and strain-level information needs to be incorporated in future studies. Another limitation of the study was that stool samples from only 39 of the 61 study infants were available for analysis at T3, because others were discharged home by then.

While surgical conditions included in our RCT were heterogeneous, all of them had common issues such as feed intolerance, need for PN, and risk of infections. Hence, we decided to include all types of gastrointestinal surgical conditions in this pilot RCT. Another reason was because it would have taken at least 7–9 years to achieve the sample size of 60 if we had conducted it in a single surgical condition (for example, our unit admits only 10 cases of gastroschisis per year).

There is some observational evidence from adult literature that maltodextrin may lead to adverse health outcomes secondary to alterations in gut microbiota.⁵⁷ In our RCT, the probiotic supplement contained probiotic organisms and maltodextrin, whereas placebo was only maltodextrin. Hence, any effect of maltodextrin on gut microbiota would have occurred in both placebo and probiotic groups. To evaluate the effects of maltodextrin (at the small dose of 1 gram per day as used in our trial) on gut microbiota, prospective cohort studies comparing gut microbiota in healthy breastfed infants receiving maltodextrin versus healthy breastfed infants are needed.

The important strengths of our study are as follows: (a) It is the first RCT to evaluate the efficacy and safety of the three-strain bifidobacterial supplementation in CGISC in neonates and (b) all study infants had baseline stool samples prior to commencing supplements.

The current pilot RCT found that the degree of postnatal growth restriction of head circumference was less severe in the probiotic supplemented group than placebo. Hence, one could hypothesise that probiotic supplemented group will have better

neurodevelopmental outcomes. Our recent retrospective study³⁰ with a sample size of 400 found the incidence of suboptimal neurodevelopmental outcomes (SNDO) to be 16% in term and near-term neonates with CGISC. A sample size of 516 infants (258 in each arm) will be required to have 80% power at the two-sided 5% significance level to detect a 50% difference in the primary outcome (16% in controls and 8% in the probiotic group). Since nearly 30% of them are expected to be discharged home even before completing 2 weeks of supplementation, the sample size should be increased by another 154, and hence the final sample size will be around 670 infants. With such a large sample size, baseline frequencies of potential confounders such as gestational age, maternal antibiotics, maternal probiotic usage, mode of delivery, severity of illness and type of milk feeds are expected to be balanced between the two groups. Involvement of multiple centres will be crucial to achieve this sample size within a reasonable time period of 24–36 months.

In summary, this pilot RCT found that after 2 weeks of supplementation with the three-strain *Bifidobacterium*, neonates with CGISC had a lower relative abundance of potentially pathogenic bacterial families, higher abundance of *Bifidobacterium*, and higher SCFA levels in their stools. Larger studies with clinical endpoints and long-term follow-ups are necessary.

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AUTHOR CONTRIBUTIONS

Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, and final approval of the version to be published: S.C.R., M.E., L.C., A.D.K., I.J.G., K.N.S., B.W., P.L.C., and S.K.P.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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Supplementary File 1

Microbial analysis of stool samples: Stool microbiota was assessed using the 16S ribosomal RNA gene sequencing method using the following steps.

DNA extraction: DNA was extracted from thawed stool samples (0.3g) using the Qiagen Powersoil kit (cat# 1288-100; Hilden, Germany). However, instead of vortexing, samples were subjected to physical lysis in a bead-beater (TissuerLyzer11, Qiagen) for 3min at 30Hz. DNA was eluted in molecular grade water and stored at -80°C.

PCR amplification and 16S rRNA sequencing

The V3-V4 region of the 16S rRNA gene were amplified and sequenced as previously described.¹ Library preparation and pair end sequencing was performed (2x300 cycles) on the Illumina MiSeq platform at the Ramaciotti Centre for Genomics (UNSW, Sydney, Australia).

16S rRNA sequence analysis

16S rRNA sequence data were initially quality filtered and trimmed using fastp 0.20.1 truncating reads if the quality was found to be below 12. USEARCH version 11.0.667 was used to merge forward and reverse reads between 300 and 500 nucleotides. Reads with an expected error of more than 2, and more than 1 ambiguous base, were subsequently removed. All sequences of all samples were concatenated in a single file and subsequently dereplicated to form unique sequences. Unique sequences were clustered into zero-radius operational taxonomic units (zOTUs, also called ASVs) using the UNOISE3 algorithm implemented in USEARCH. Chimeras were removed *de novo*

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during clustering. Processed, concatenated sequences were mapped on the final set of zOTUs to determine their occurrence and abundance in each sample using the `otu_tab` command with an identity cut-off of 97% and termination options disabled, which means that every sequence is searched against every zOTU to find the best hit. Taxonomy was assigned to each zOTU using the SINA aligner (version 1.7.2) and the SILVA SSURef NR99 v138.1 database.

For alpha diversity measures, each sample was subsampled 100 times to a count of 20,500 counts per sample and the average was taken. OTU richness and diversity indices, Shannon, ACE and Chao1, were calculated in R (version 3.6.0) using the `vegan` package. Relative abundance analysis at the Phylum, Family and Genus levels were carried out using `phyloseq` package in R. Data were visualized using `ggplot2` and `ggpubr` packages. For beta diversity analysis, data were square root transformed. A phylogenetic tree for diversity computations was calculated with `FastTree` (version 2.1.10). Weighted unifracs distances were calculated and visualized on a principal coordinate analysis plot.

Stool Short Chain Fatty Acid assay

Quantification of SCFAs in faecal samples of study infants were carried out according to our previously described method² with slight modifications. All chemicals and standards were purchased from Sigma-Aldrich (Merck, Singapore). In brief, the faecal samples stored in 1% phosphoric acid at -20°C were first thawed and homogenized with vortex. Then, 100 µL of 10% meta-phosphoric acid solution was added to 0.5 mL of the faecal sample to adjust the pH to about 2.0. Samples were vortexed for about 10 minutes and centrifuged for 30 minutes at $20,817 \times g$, at 4°C to solidify the precipitate. Subsequently, 0.5 mL of aqueous supernatant was transferred into

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a new tube; 4-methyl valeric acid was added as internal standard (IS) to a final concentration of 500 μ M. Then, 500 μ L of ethyl acetate was added to extract SCFAs with a vortex for about 30 min and centrifuged for 30 min at $20,817 \times g$. Finally, 50 μ L of organic extracts were transferred into GC glass vials for the gas chromatography-mass spectrometry (GC-MS) analysis. One microliter of organic extracts was injected into GC-MS system (Agilent Technologies 7890B-5977B) equipped with a HP-FFAP capillary column (30m \times 0.250mm \times 0.25 μ m; Agilent). GC-MS setup was the same as described elsewhere.² The SCFA analyses were performed in duplicate. Quantifications were carried out in selected ion monitoring acquisition mode in MassHunter Acquisition software with base peak ion selected as quantifier for each compound. The calibration graphs were constructed in MassHunter Quantitative software (version B.09.00) by plotting the relative response (ratio of peak area SCFAs/peak area IS) vs. relative concentration for each individual SCFAs. The final SCFA concentrations were expressed as microgram per gram wet weight faecal sample.

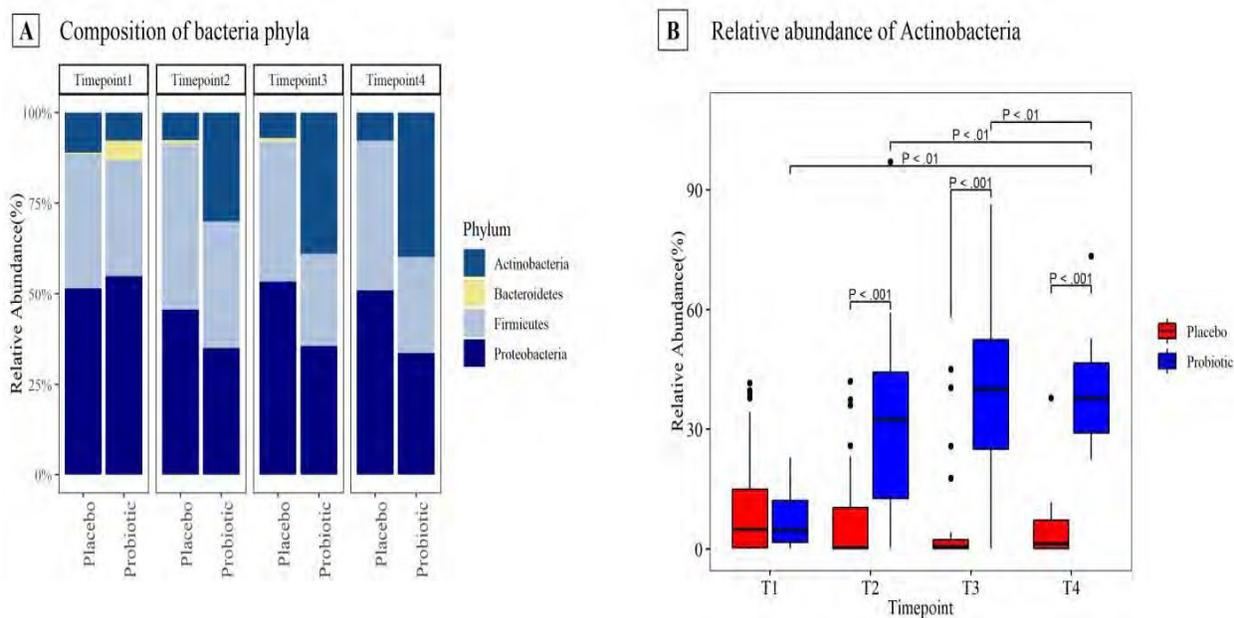
Quality assurance of the trial supplements

To ensure the trial supplements did not contain harmful pathogens, random sachets were sent by our trial pharmacist on three occasions during the study period to our microbiology laboratory at PathWest Laboratory Medicine, Western Australia. The first batch was in the second week of December 2017, within one month of commencing the trial after recruitment of three participants. The laboratory reported that one of the placebo sachets grew *Bacillus megatarium* and that the probiotic sachets did not grow any contaminating bacteria or fungi. The recruitment into the trial was withheld temporarily, and the information was conveyed to the parents of all three trial participants who had been discharged home by then; none of those three infants had developed

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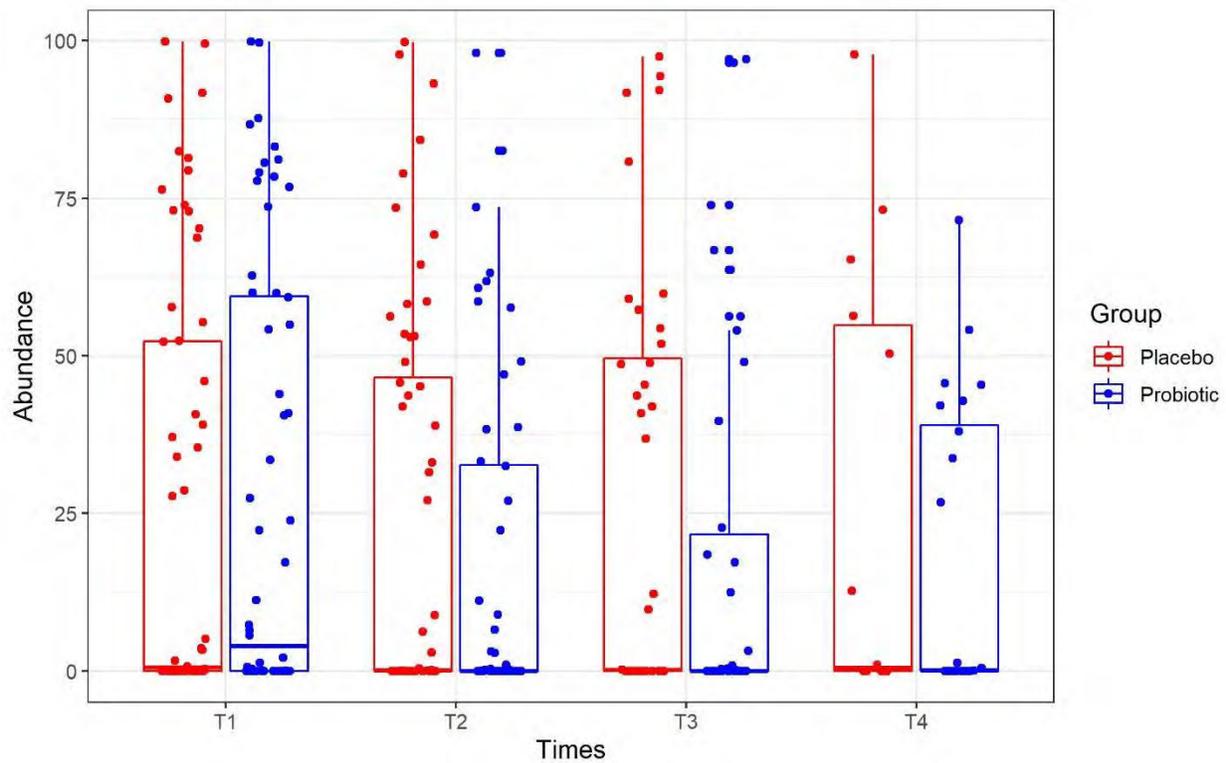
sepsis due to those bacteria or any other organism during hospital stay or after discharge. The HREC, DSMC and the manufacturers of the trial supplements (Morinaga Milk Industry; Japan) were informed. Three weeks later, five more random sachets were sent for microbiological assay of which none grew any organisms and the laboratory reported that the product complies with sterility as defined in the British Pharmacopeia, 2017. The HREC and DSMC were updated with this reassuring information and the trial was recommenced with their permission. Similar microbiological assays were undertaken on six more random samples (3 each of probiotic and 3 each of placebo) in November 2018; none of them showed growth of contaminant bacteria. The manufacturers reassured us that their probiotic/placebo products meet the specification set by referring to the CODEX standards for Powdered Formulae for Infants and Young Children. (CAC/RCP 66 - 2008) and are free of pathogenic bacteria, but just like any food supplements of probiotics, the samples are not expected to be totally sterile. In that context, our independent lab findings of the sterility of the samples were encouraging.

e-Figure 1. A. Relative abundances of common bacterial phyla. B. Comparison of relative abundance of Actinobacteria between probiotic and placebo groups at various time points.



A. Composition of the bacterial phyla with relative abundance greater than 1% in the study. B. Relative abundance of Actinobacteria, the only phyla found to be significantly different between the groups over time using ANCOM (Analysis of composition of microbiomes) with Subject as the random variable. Pairwise comparisons were conducted using Wilcoxon Rank sum test with 'BH' correction. An adjusted P value of < .05 was considered significant. Box shows interquartile range; the line, median; and the error bars, the range and the dots are outliers.

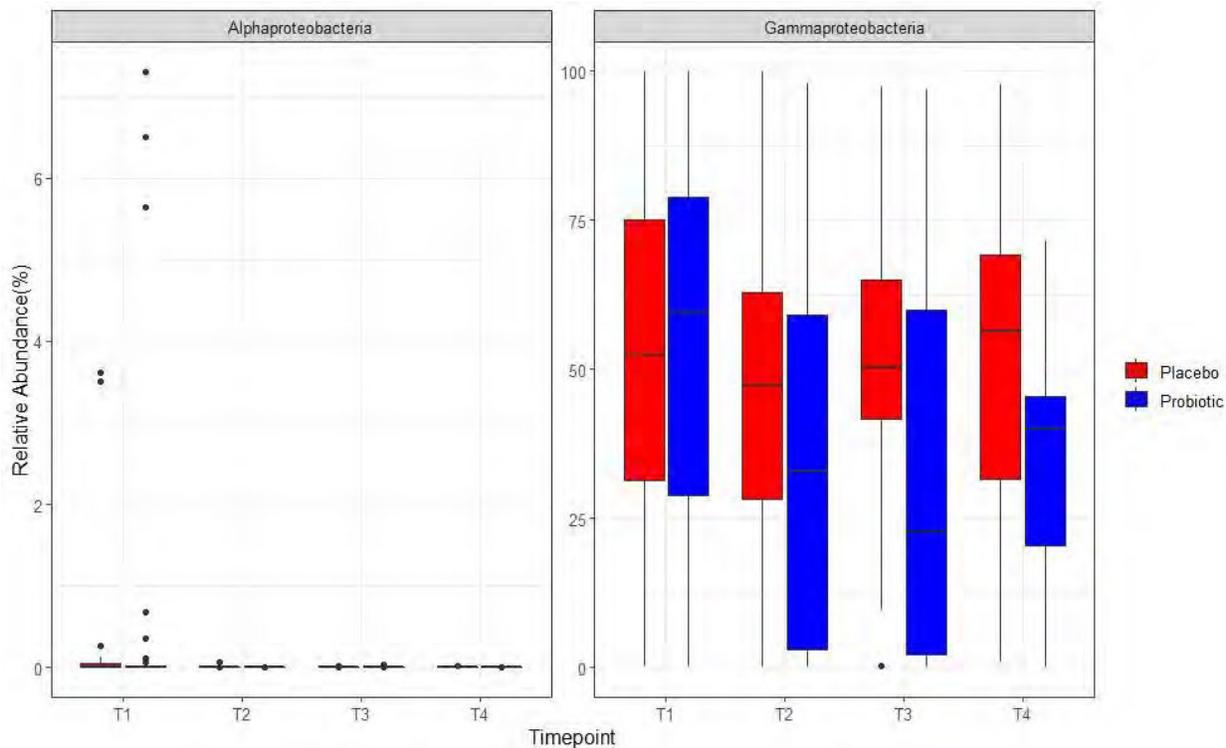
e-Figure 2. Comparison of relative abundance of Proteobacteria between probiotic and placebo at various time points.



The relative abundance of Proteobacteria decreased over time in the probiotic group, but not in the placebo. However, the relative abundance of Proteobacteria was similar between the probiotic and placebo groups at all time points. Box shows interquartile range; the line, median; and the error bars and the dots are individual values.

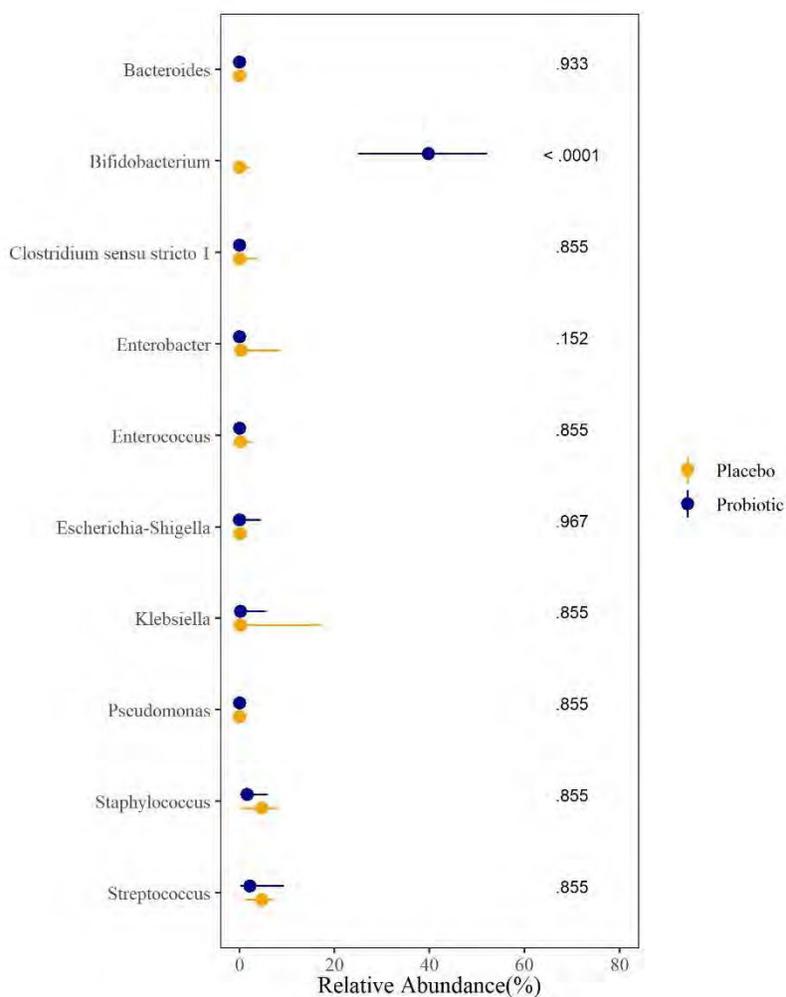
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e-Figure 3. Comparison of relative abundance of Gammaproteobacteria and Alphaproteobacteria between probiotic and placebo groups at various time points faceted by Class.



At the bacterial class level, the relative abundance of Gammaproteobacteria between the probiotic and placebo groups was similar at all time points. Similar findings were noted for Alphaproteobacteria. Box shows interquartile range; the line, median; and the error bars, the range.

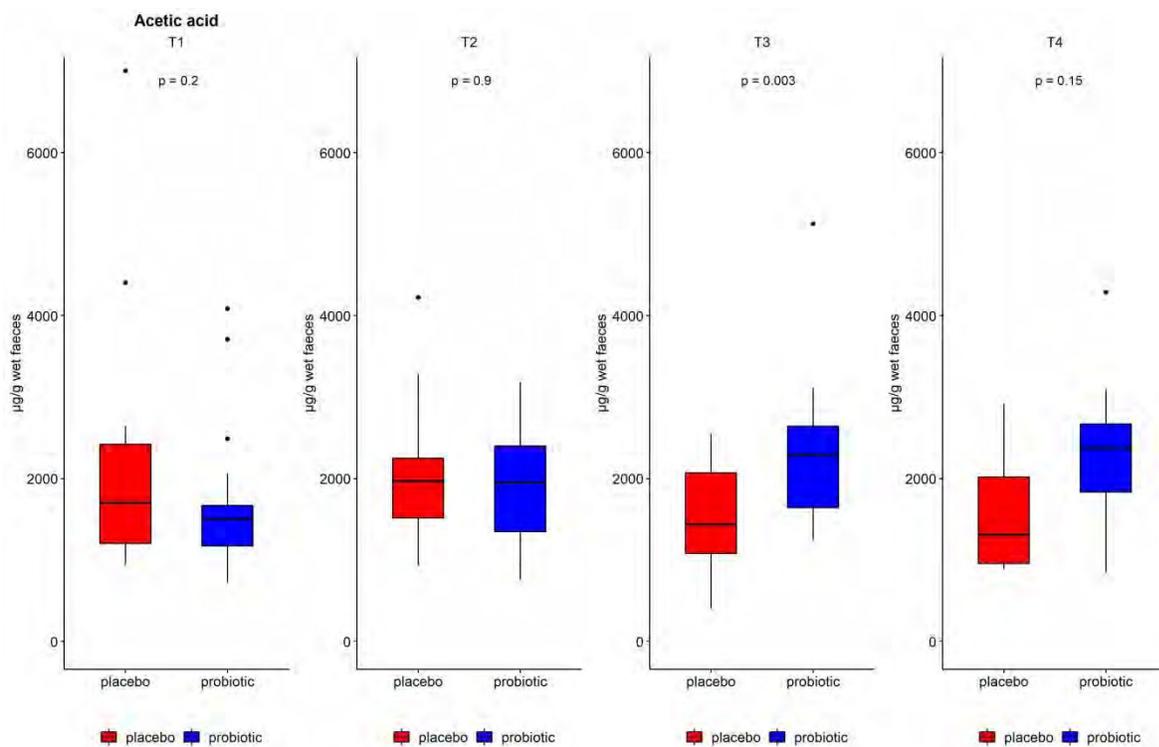
e-Figure 4. Comparison of relative abundance of bacterial genera between probiotic and placebo groups at various timepoint 3.



At the bacterial genus level, there were no significant differences in the relative abundances of common pathogenic genera between the probiotic and placebo groups at time point T3. The relative abundance of the genus Bifidobacterium was significantly higher in the probiotic group. The circles are median, and the error bars depict 1st and 3rd quartile.

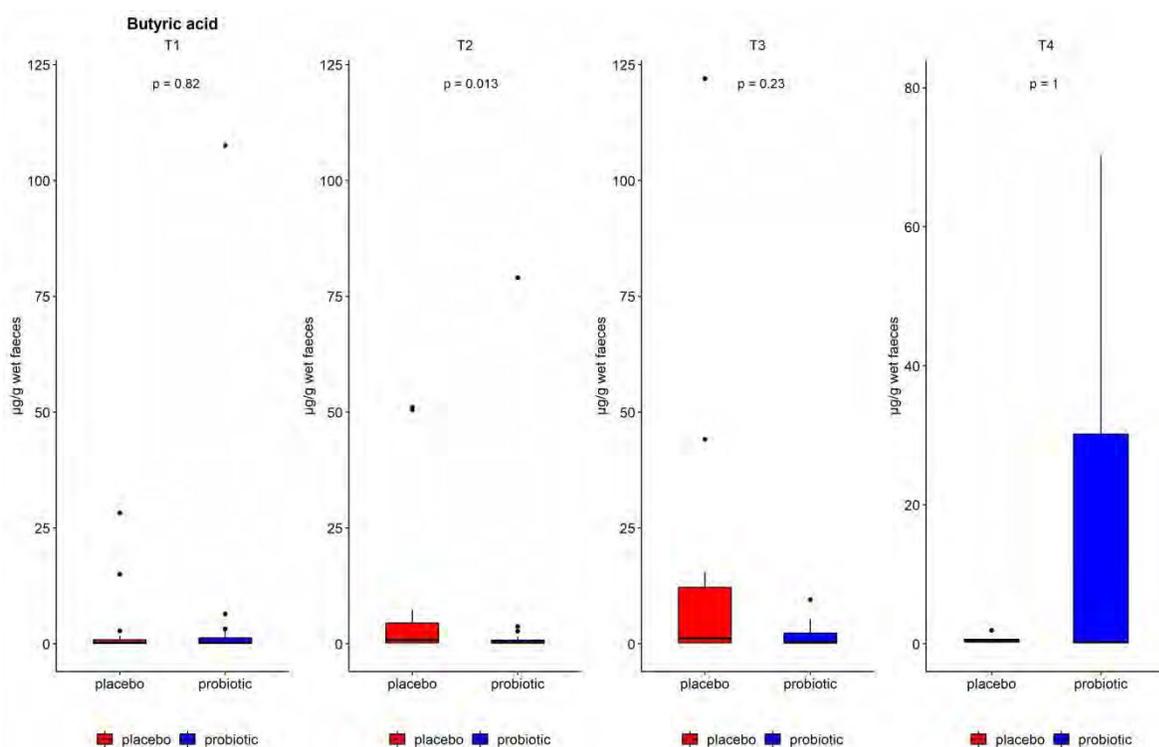
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e-Figure 5: Stool acetic acid levels between probiotic and placebo groups at various time points.



The stool acetate levels were similar between the probiotic and placebo at all time points except T3, when the levels were significantly higher in the probiotic group

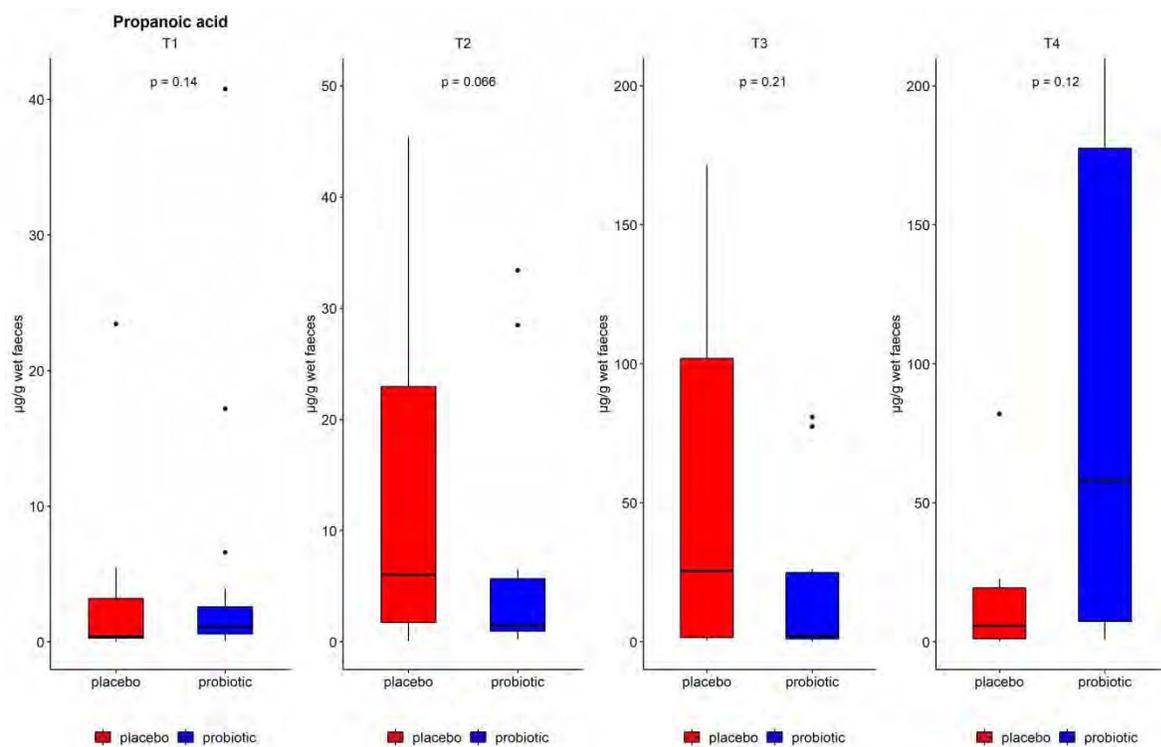
e-Figure 6: Stool Butyrate levels between probiotic and placebo groups at various time points.



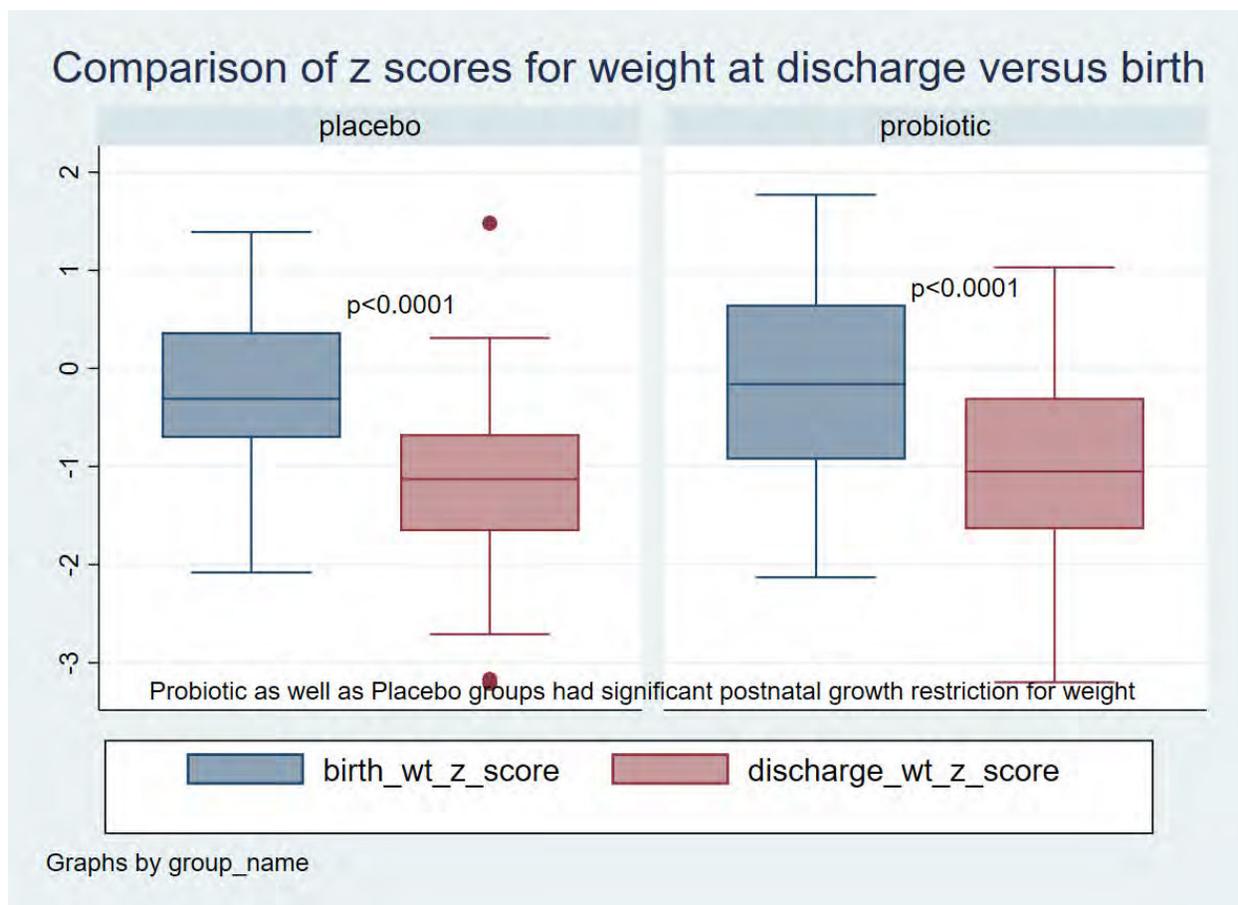
The stool butyrate levels were similar between the probiotic and placebo at all time points except T2, when the levels were significantly lower in the probiotic group.

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e-Figure 7: Stool Propionate levels between probiotic and placebo groups at various time points.



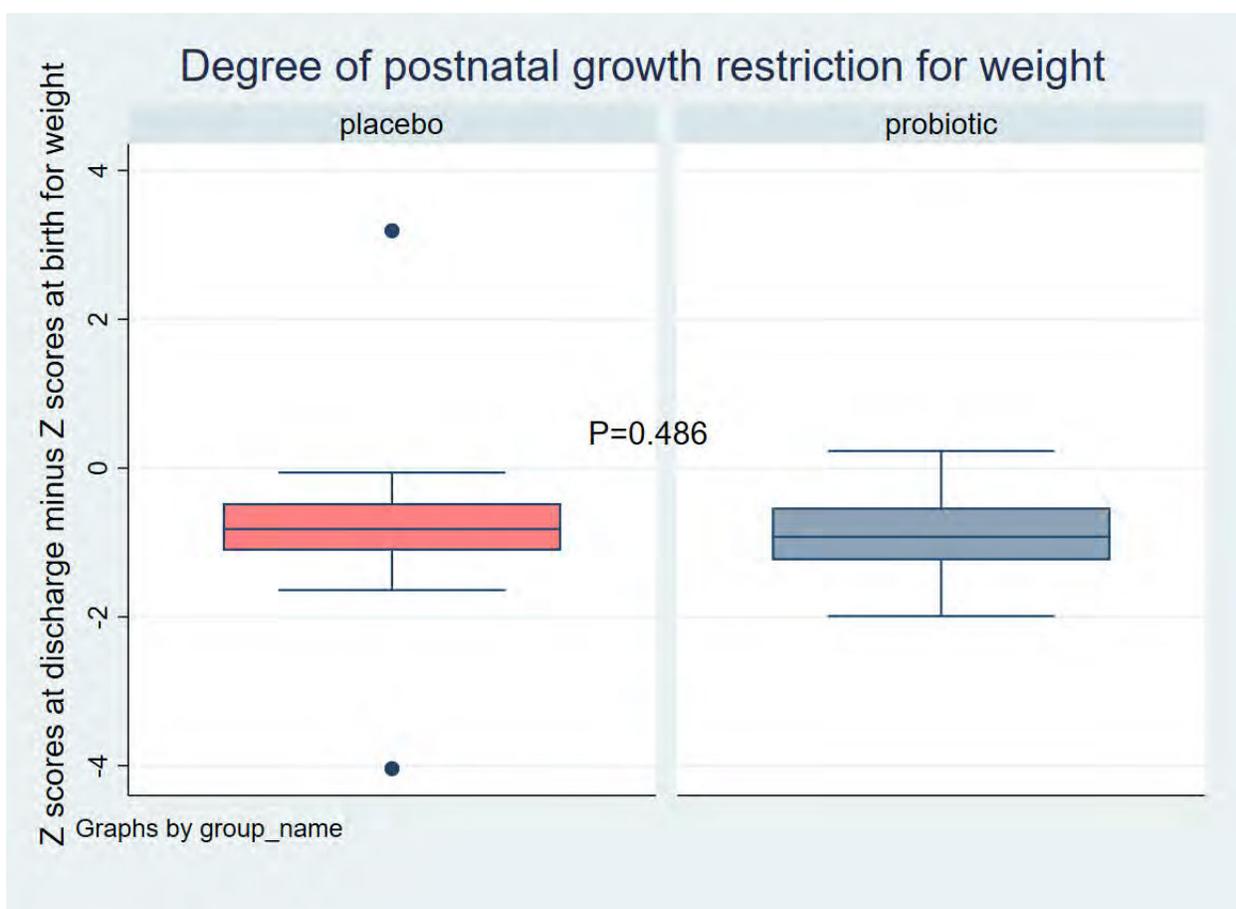
The stool propionate levels were similar between the probiotic and placebo at all time points.

e-Figure 8: Comparison of z scores for weight at discharge versus birth in study infants.

The z scores for weight at discharge were significantly lower than at birth in both probiotic and placebo groups.

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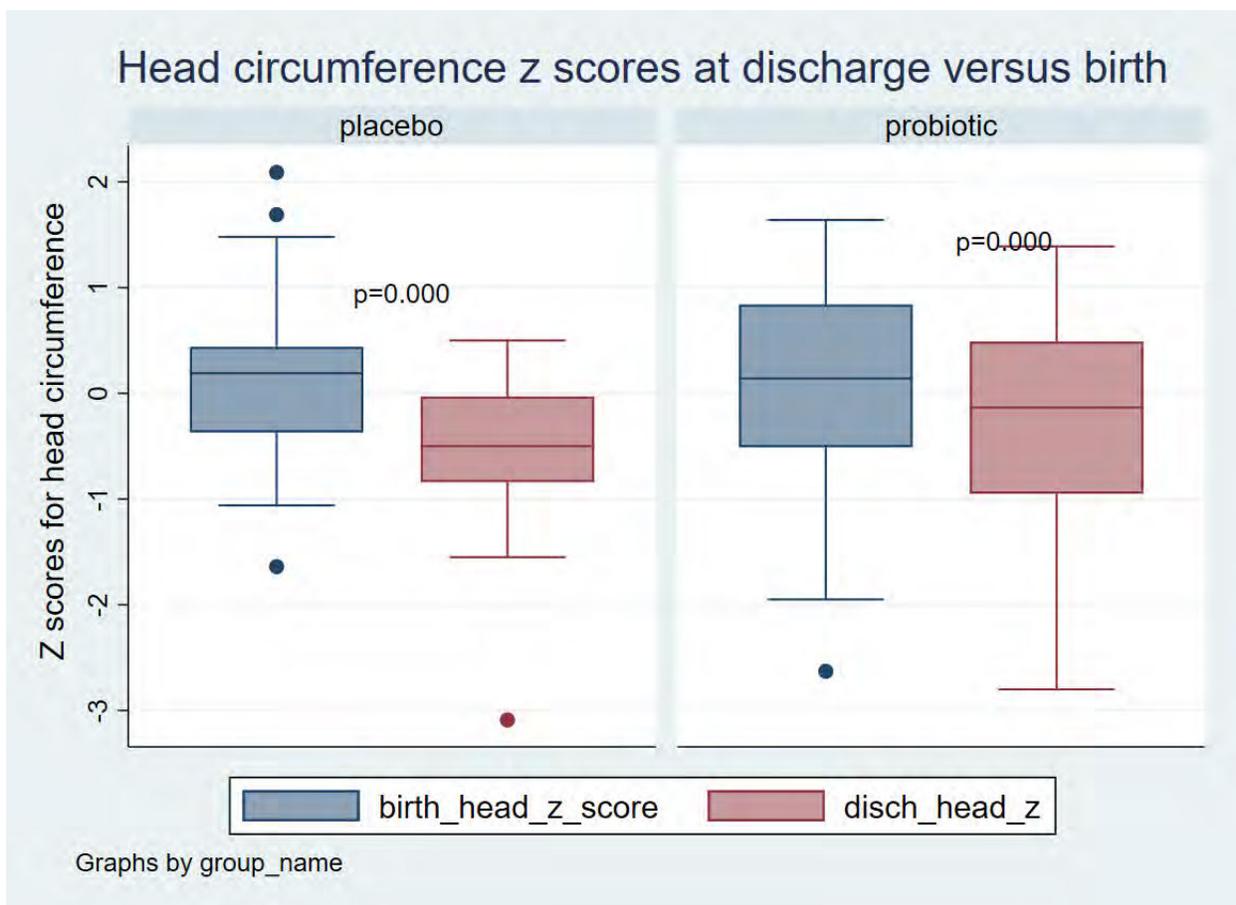
e-Figure 9: Comparison of degree of postnatal growth restriction for weight in study infants.



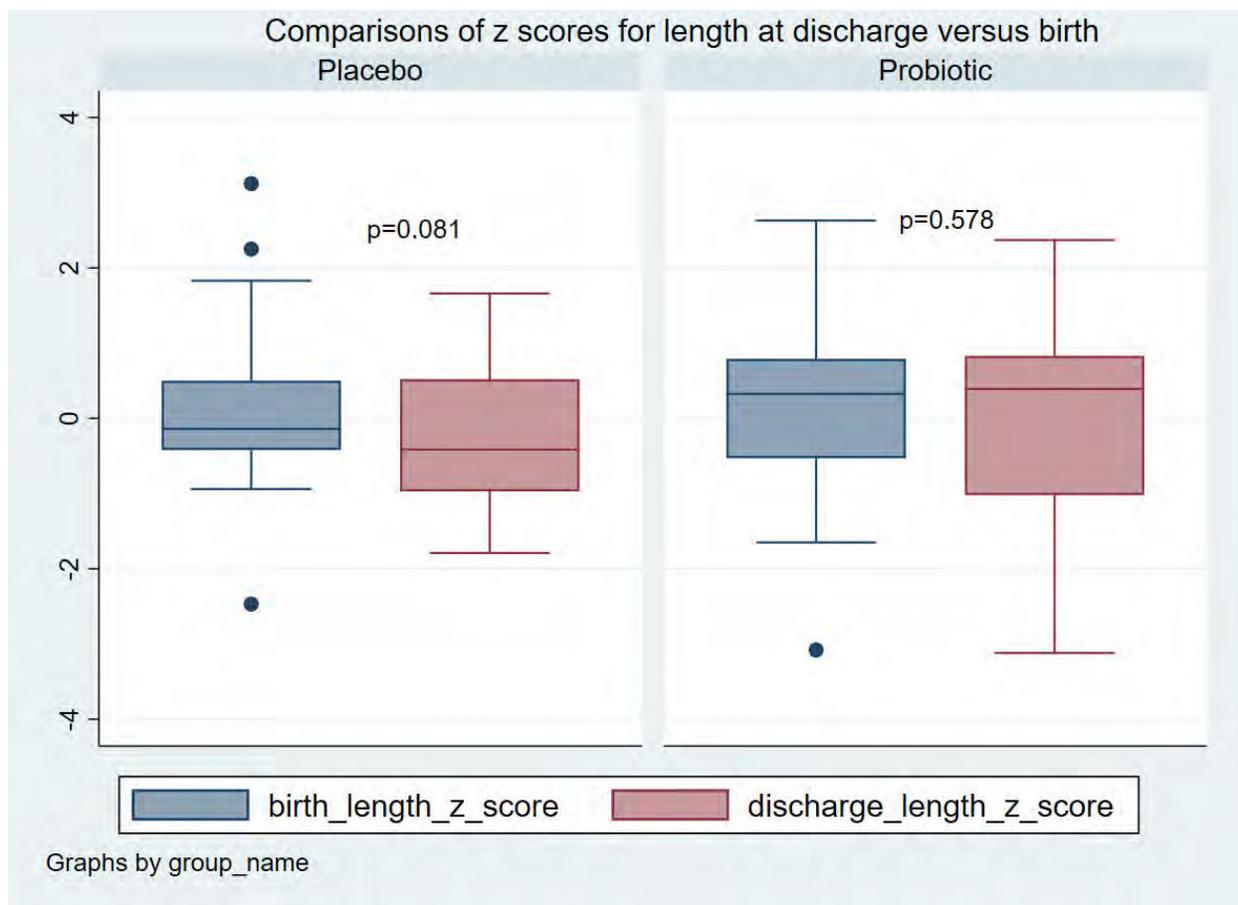
The degree of postnatal growth restriction for weight was similar in the probiotic group compared to placebo.

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e-Figure 10: Comparison of z scores for head circumference at discharge versus birth in study infants.



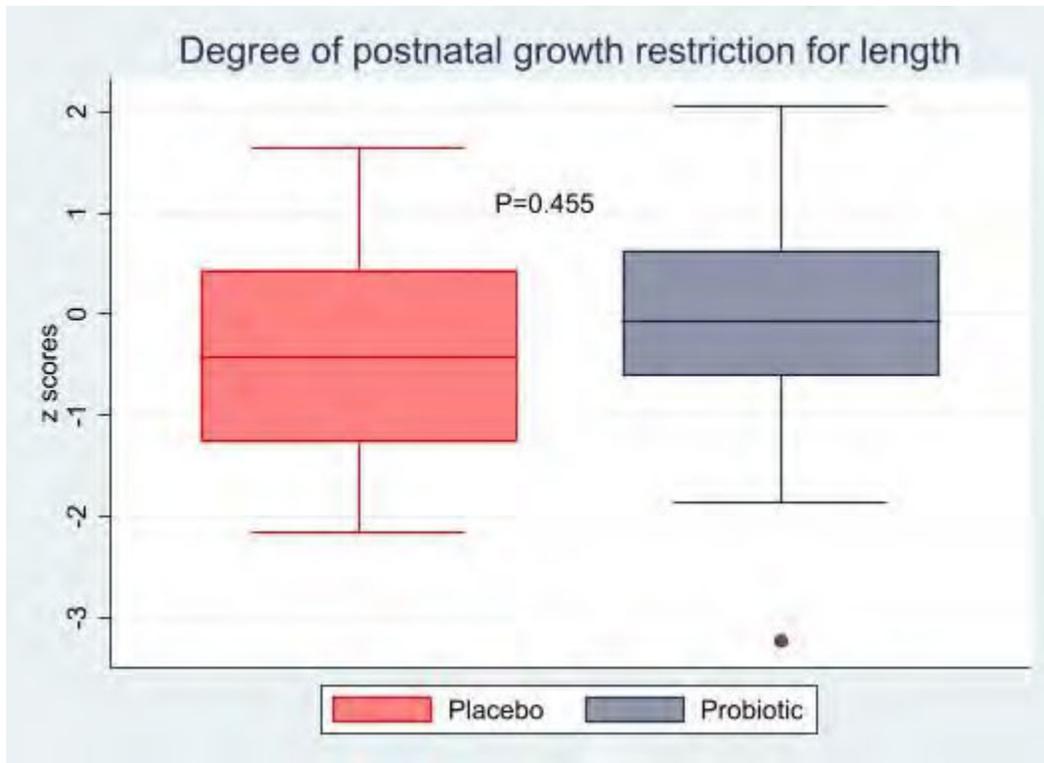
The z scores for head circumference at discharge were significantly lower than at birth in both probiotic and placebo groups.

e-Figure 11: Comparison of z scores for length at discharge versus birth in study infants

The z scores for length at discharge were similar those at birth in both the probiotic and placebo groups.

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e-Figure 12: Comparison of the degree of postnatal growth restriction for length in the study infants:



The degree of postnatal growth restriction for length was similar in the probiotic and placebo groups


A. Supplementary file 2: CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1,3
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	4
Introduction			
Background and objectives			
	2a	Scientific background and explanation of rationale	5
	2b	Specific objectives or hypotheses	5,6
Methods			
Trial design			
	3a	Description of trial design (such as parallel, factorial) including allocation ratio	6,7
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	-
Participants			
	4a	Eligibility criteria for participants	6
	4b	Settings and locations where the data were collected	6
Interventions			
	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	6,7
Outcomes			
	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	8,9
	6b	Any changes to trial outcomes after the trial commenced, with reasons	-
Sample size			
	7a	How sample size was determined	9
	7b	When applicable, explanation of any interim analyses and stopping guidelines	10
Randomisation:			
Sequence generation			
	8a	Method used to generate the random allocation sequence	6
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	6
Allocation concealment mechanism			
	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	6,7
Implementation			
	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to	6,7

		interventions	
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	7, 8
	11b	If relevant, description of the similarity of interventions	6,7
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	9, 10
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	9, 10
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	11
	13b	For each group, losses and exclusions after randomisation, together with reasons	11
Recruitment	14a	Dates defining the periods of recruitment and follow-up	6, 11
	14b	Why the trial ended or was stopped	11
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Table 1
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Table 1, Figure 1
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	11-14
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	Table 1,2,3
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	Table 2
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	13, 14
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	16, 17
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	17
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	16-18
Other information			
Registration	23	Registration number and name of trial registry	4
Protocol	24	Where the full trial protocol can be accessed, if available	supplementary file 3
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	20

Supplementary file 3: TRIAL PROTOCOL**Probiotic Supplementation in neonates with congenital gastrointestinal surgical conditions: Protocol for a Pilot Randomized Double-Blind Placebo-Controlled Trial**

Introduction: The major neonatal gastrointestinal surgical conditions are Gastroschisis, Duodenal atresia, Intestinal Atresia, Congenital Diaphragmatic Hernia (CDH), Tracheo-Oesophageal Fistula (TOF), Short Bowel Syndrome (SBS), Malrotation and Volvulus, Meconium Ileus, Hypoplastic left colon, Hirschsprung Disease (HD) and Anorectal Malformations. Common morbidity in all these conditions is feed intolerance, prolonged parenteral nutrition, increased risk of healthcare-associated bloodstream infections (HABSI), and the need for multiple courses of antibiotics.¹⁻¹¹ Recurrent administration of antibiotics can destroy the normal commensal bacteria.¹² In addition, delayed commencement of enteral feeds, living in the neonatal intensive care unit, and lack of exposure to mother's skin and breast milk microbiota can lead to intestinal dysbiosis.¹³⁻¹⁸ Intestinal dysbiosis has been implicated as a causative factor or association for many adverse outcomes in the neonatal, paediatric and adult population.¹⁹⁻²⁶ A cohort study of 208 neonates with surgical conditions found that in the majority of cases, septicemia was mainly a gut-derived phenomenon due to translocation of gut organisms, and hence suggested novel strategies for prevention of HABSI.²⁷

Probiotic supplementation has the potential to minimise/prevent dysbiosis, thereby improving the clinical outcomes of surgical neonates. A recent meta-analysis from adult studies that included 1374 patients from 20 RCTs concluded that probiotic/synbiotic supplementation decreases the risk of surgical site infections and urinary tract infections; however, the quality of available evidence was considered low.²⁸ There are two RCTs of probiotic supplementation in children (<18 years) with Hirschsprung disease.^{29,30} While one of them showed beneficial effects of supplementation²⁹, and the other did not.³⁰ In another RCT in 30 children (<15 years) with various surgical (majority gastrointestinal) conditions, BBG-01 or placebo was administered daily from 7 days before surgery until discharge from hospital. They found that administration of the probiotic strain

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Bifidobacterium Breve (BBG-01) in the perioperative period was safe and improved the gut flora and increased faecal short-chain fatty acid (acetic acid), and decreased the risk of septicemia.³¹

To our knowledge, there are only two RCTs (total sample size 24+8=32; published in October, November 2015) that have evaluated the role of probiotics in neonates with major gastrointestinal surgical conditions^{32,33} The RCT from Powell et al. included 24 neonates with gastroschisis of which 12 received probiotics and 12 received a placebo.³² The probiotic supplement was administered for six weeks or until hospital discharge (whichever came first). The supplement was given even when the infants were not fed enterally and were on a gastric decompression regimen. They found significant dysbiosis in infants with gastroschisis that was partially attenuated by administering *Bifidobacterium longum* subsp *infantis*.³² In the RCT from Murakami et al., surgical neonates (duodenal atresia, anorectal malformations) received probiotics; four received no probiotics. Interestingly, they found that Bifidobacteriaceae was significantly more abundant in the placebo group. Both authors suggested the need for further RCTs in this area. Hence, we aim to conduct a pilot RCT to evaluate the efficacy and safety of probiotics in term and late preterm infants with major gastrointestinal surgical conditions. The results of this trial will enable us to design a multicenter RCT in the near future.

Hypothesis: We hypothesize that probiotic supplementation improves the gut microbiota and clinical outcomes of term neonates with major gastrointestinal surgical conditions.

Justification for the study: Meta-analyses of RCTs in preterm infants (non-surgical) from our group and the Cochrane review have shown probiotic supplementation to decrease mortality, Necrotizing Enterocolitis (NEC) and improving feed tolerance.³⁴⁻³⁶ An updated meta-analysis³⁷ from our group has confirmed that probiotics are also beneficial in reducing the incidence of late-onset sepsis (LOS) in preterm (non-surgical) neonates. Pooled results from 37 RCTs (N = 9416) showed that probiotics significantly decreased the risk of LOS (675/4852 [13.9%] vs 744/4564 [16.3%]; relative risk, 0.86; 95% confidence interval, 0.78–0.94; P = .0007; I² = 35%. If probiotic supplementation can improve the outcomes of preterm (non-surgical) infants, they may likely improve the outcomes of neonates with major surgical conditions of the gut.

Design and setting: Double-blind Randomized placebo-controlled trial in the neonatal intensive care unit of Princess Margaret Hospital (PMH), Perth, Western Australia.

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Regulatory approvals and registration: Approval will be obtained from the human research ethics committee at Child and Adolescent Health Service, Princess Margaret Hospital for Children, Perth, Western Australia. The trial will be registered with Australia and New Zealand Clinical Trials Registry (ANZCTR). Approval from the Therapeutic Goods and Administration (TGA) and Australian Quarantine and Inspection Service (AQIS) will also be obtained for importing the trial medication from Japan.

Eligibility criteria: Inclusion criteria: (1) Neonates (≥ 35 weeks gestation) with Intestinal Atresia (duodenal, jejunal, ileal or colonic), Intestinal Malrotation, Congenital Diaphragmatic Hernia, Tracheo-Oesophageal fistula, Gastroschisis, Exomphalos, Meconium ileus or peritonitis requiring laparotomy, Hirschsprung Disease, Anal atresia, Imperforate anus, Short Bowel Syndrome, and other surgical conditions needing the creation of small or large intestinal stoma (e.g. severe meconium ileus, micro-colon), (2) Informed parental consent. Exclusion criteria: (1) non-gastrointestinal surgical conditions, (2) Infants less than 35 weeks at birth.

Intervention: Mixture of 3 strains (*B. breve* M-16V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536 (1×10^9 of each strain per 1 g sachet: 3-strains group). Placebo: Dextrin.

Justification for the chosen probiotic strains: *B. Bifidobacterium breve* is the commonest Bifidobacterium found in breastmilk.³⁸ *B. breve* M16V has been shown to achieve effective colonisation of the gut by our group.³⁹ We have also demonstrated that routine administration of this strain reduces the risk of necrotising enterocolitis (NEC) and LOS in preterm infants.⁴⁰ This particular strain has been safely used in neonates for more than a decade in Japan.⁴¹ A closely related probiotic bacteria, *B. breve* BBG-001, has been shown to be safe in a large RCT in very preterm infants.⁴² *Bifidobacterium longum* is one of the most abundant species of the Bifidobacterium genus in the gut microbiota of healthy breast-fed infants and adults.⁴³ *Bifidobacterium longum* ssp. *longum* and ssp. *infantis* are known to have a symbiotic relationship with infants.⁴³ *Bifidobacterium longum* subspecies *infantis* (*B. infantis*) is considered an essential coloniser of the neonatal gut.⁴⁴ It is unique among gut bacteria in its capacity to digest and consume any human milk oligosaccharides, enabling it to colonise and proliferate. In vitro, *B. infantis* grows better than other bacterial strains in the presence of human milk oligosaccharides,

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displays anti-inflammatory activity in premature intestinal cells, and decreases intestinal permeability. In premature infants, *B. infantis* given in combination with human milk decreases *Enterobacteriaceae* in the faeces.⁴⁴ *Bifidobacterium longum* subspecies *longum* (*B. longum*) has antioxidant properties.⁴⁵ A RCT in preterm infants found that compared with administration of one species (*B. breve M16V*), administration of three species of bifidobacteria that we will be using (*B. breve M-16V*, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536) resulted in earlier formation and maintenance of a bifidobacteria-predominant faecal microbiota.⁴⁶ Hence the multistrain probiotic product that we have selected may result in substantial clinical improvements.

Justification for the dose: An optimal dose is essential for any probiotic strain to survive and colonise the gut after overcoming the barriers such as gastric acid, bile and competing flora. In a systematic review of probiotic supplementation in preterm infants, our group found that the median dose in the RCTs was 3 billion organisms per day.^{34,47} Our unit has used this dose in nearly 1000 preterm infants (non-surgical) over the past few years. We have shown that the incidence of NEC and LOS has reduced significantly since the introduction of probiotics.⁴⁰

Randomisation, allocation concealment, and blinding: Group assignment will be allocated by a computer-generated randomisation sequence in randomly ordered block sizes of 2 and 4. Opaque, sealed, coded envelopes will be used for randomisation. Allocation concealment optimised by opening the envelope before administering the first dose of the study medication. The Clinical Trial Pharmacist (CTP) will supply the randomisation sequence and the sachets (identical design, weight, smell, and taste) containing either the probiotic or placebo (equal volume of dextrin) manufactured by Morinaga Milk Industry Co., Ltd, Japan, to the nursing staff. This will ensure the masking of all investigators, clinical and non-clinical outcome assessors, nursing staff, and parents regarding the allocation status of enrolled neonates.

Probiotic protocol: As soon as possible after admission, enrolled neonates will be supplemented with freshly reconstituted contents of the allocated sachets every day and continued until discharge. The majority of the RCTs in the adult meta-analyses and paediatric and neonatal RCTs used probiotic supplementation in the pre-operative period and continued in the postoperative period.^{28,31,32,48}

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Reconstitution of the dry powder in the sachets will be done using sterile water for injection or breast milk. Care will be taken during reconstitution to reduce the risk of cross-contamination by adhering to strict hand hygiene. The dose will be 3×10^9 CFU/day (i.e., 3 billion organisms per day) in 1.5 ml of the reconstituted solution, given as a single dose via the orogastric feeding tube. Supplementation will be continued even when the patients are not enterally fed. Supplementation will be withheld if a patient develops sepsis due to the probiotic organism. Supplementation can be withheld at the discretion of the attending clinician if the infant is deemed hemodynamically unstable.

Primary outcomes: Gut microbiota (16 s r RNA Pyrosequencing studies for phylogenic profiling) on the stools collected a) as soon as possible after admission, but before the commencement of supplementation b) 7-9 days after commencement of supplementation, c) 14-16 days after commencement of supplementation, and d) before discharge from the neonatal unit. **Secondary outcomes:** Stool Short Chain Fatty Acids (SCFA) analysis, blood culture positive sepsis, CRP, duration of antibiotic therapy, duration of hospital stay and time to achieve full enteral feeds of 120 ml/kg/day. The rationale for SCFA analysis is given in this document, two pages below.

Safety: This will be assessed by monitoring for (1) blood culture positive sepsis by the administered strains of bifidobacteria and (2) adverse effects such as abdominal distension, vomiting, and diarrhoea leading to the cessation of the supplementation. The standard Bactec culture method used in our microbiology lab can detect bifidobacterial sepsis within 48 hrs and provide an antibiotic susceptibility profile. All outcomes and safety parameters will be monitored from enrollment until death or discharge from the hospital. The occurrence of death or sepsis due to the administered probiotic organism will be considered a severe adverse event. It will be reported immediately to the HREC and the data and safety monitoring committee (DSMC). Abdominal distension, vomiting and loose stools are quite common in neonates with surgical conditions of the gut. If these symptoms persist beyond the expected duration, they will be considered adverse events and reported to the HREC and DSMC. In addition, any unexpected adverse events will also be reported to the HREC and DSMC.

Stool sample collection: Stool samples will be collected on the following days: a) As soon as possible after admission, but before commencing the trial medication b) on any one day between

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7-9 days after starting the trial medication, and c) on any one day between 14-16 days after commencing the trial medication. If the infant is an inpatient in the NICU for more than four weeks after starting the trial medication, one more stool sample will be collected before discharge. Stool samples will be temporarily stored in the refrigerator at 4-6 C for a maximum of 48 hours and subsequently transferred to small cryovials and stored at -80c.

a. Microbiota analysis (Routine cultures and 16 s r RNA Pyrosequencing studies for phylogenic profiling): Until the beginning of the last decade, culture-based techniques were the mainstay of evaluating intestinal microbes. However, it is well known that the majority of bacterial cells in faeces cannot currently be cultured in the laboratory.⁴⁹ Hence, we will be using the recently developed high throughput molecular techniques that analyse microbial DNA and RNA and enable detailed analysis of the microbiota.⁵⁰⁻⁵²

The analysis of stool microbiota of study infants will be performed by extracting the DNA from stool samples and creating an amplicon 16s rRNA library which will be analysed using high throughput Ion Torrent PGM sequencing to allow calculation of diversity indices and rarefaction curves. The resulting sequences will be subjected to comparison with published sequence libraries data for taxonomic classification.

DNA extraction: DNA will be extracted from thawed stool samples (0.3g) using the Qiagen Powersoil kit (cat# 1288-100; Hilden, Germany). However, instead of vortexing, samples will be subjected to physical lysis in a bead-beater (TissuerLyzer11, Qiagen) for 3min at 30Hz. DNA will be eluted in molecular grade water and stored at -80°C.⁵³

PCR amplification and 16s rRNA sequencing: The V3-V4 region of the 16S rRNA gene will be amplified and sequenced as previously described.⁵⁴ Library preparation and pair-end sequencing will be performed (2x300 cycles) on the Illumina MiSeq platform at the Ramaciotti Centre for Genomics (UNSW, Sydney, Australia).

Analysis of sequencing data: 16S rRNA sequence data will be initially quality filtered and trimmed using fastp 0.20.1 truncating reads if the quality was found to be below 12. USEARCH version 11.0.667 will be used to merge forward and reverse reads between 300 and 500 nucleotides. Reads with an expected error of more than two and more than one ambiguous bases will be subsequently removed. All sequences of all samples will be concatenated in a single file and subsequently

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dereplicated to form unique sequences. Unique sequences will be clustered into zero-radius operational taxonomic units (zOTUs, also called ASVs) using the UNOISE3 algorithm implemented in USEARCH. Chimaeras will be removed de novo during clustering. Processed, concatenated sequences will be mapped on the final set of zOTUs to determine their occurrence and abundance in each sample using the `otu_tab` command with an identity cut-off of 97% and termination options disabled which means that every sequence will be searched against every zOTU to find the best hit. The taxonomy will be assigned to each zOTU using the SINA aligner (version 1.7.2) and the SILVA SSURef NR99 v138.1 database.

b. Short-Chain Fatty Acid analysis: A critical way commensal microbiota communicates with the host is through the generation of metabolites, which are then absorbed into the bloodstream and sensed by host G protein-coupled receptors (GPCRs).⁵⁵ The well-studied microbial metabolites to date are short-chain fatty acids (SCFAs), the most abundant of which are acetate, propionate, and butyrate. These are a subgroup of fatty acids that contain six or fewer carbons on their aliphatic side chain. SCFAs are produced by microbial fermentation of complex polysaccharides (starches and fibre, human milk oligosaccharides) in the colon. They are absorbed via the colonic epithelium into the portal circulation of the host. The SCFA maintain intestinal acidity and inhibit colonisation by pathogenic organisms as well as activate epithelial proliferation, stimulate intestinal motility, enhance epithelial mucin secretion, and become a source of energy for epithelial cells.⁵⁶ Considering their importance, we will study the SCFA (acetate, propionate, and butyrate) in the stools using gas chromatography. SCFA data will be compared between those receiving probiotics and placebo. Differences in the bacteria population could alter the type and amount of faecal SCFAs present.^{57,58}

Statistical considerations:

Sample size estimation: A recent study found that nearly 76% of the stool bacterial flora in infants with gastroschisis was composed of pathogenic bacterial families such as *Enterobacteriaceae*, *Staphylococcaeae*, *Enterococcaceae*, *Clostridiaceae* and *Streptococcaceae*³². A total sample size of 60 infants (probiotic: 30, placebo: 30) would be required to demonstrate a 50% reduction of potentially pathogenic bacteria to 38% by two weeks of supplementation in the probiotic supplemented group with an alpha error of 0.05 and power of 80%. Our pilot study will be powered

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only to demonstrate substantial differences in the pathogenic microbiota, with the intent that if more minor differences were noted, then these data would be useful to determine the sample size and feasibility for a future larger study.

Approach to statistical analysis: Continuous variables will be compared using the t-test for normally distributed data and the Wilcoxon rank-sum test for skewed data. Categorical variables will be compared using the fisher's exact test. Logistic regression analysis will be used to analyse binary outcomes (primary outcome of interest) to derive Relative Risk and 95% confidence intervals. Linear regression analysis will be used for continuous outcomes to derive regression coefficients and respective confidence intervals. A p-value of <0.05 will be considered statistically significant.

Data monitoring and safety: An independent data and safety monitoring committee (DSMC) has been established to ensure the safety of the trial participants and to monitor the quality of the study and data collection⁵⁹. Interim analyses will be done at the end of recruitment of 20 and 40 participants. We will use an unadjusted chi-squared test to compare the mortality outcome between the probiotic and placebo groups. Since the new intervention is probiotics, if there is a statistically significant ($p < 0.0294$) increased risk of mortality in the probiotic group, the trial will be stopped. These p values are as per recommendations of Pocock et al.⁶⁰ The same p values will be used for the first as well as second interim analysis as per Pocock rule.⁶⁰ If there are excessive numbers of cases of septicemia due to the administered probiotic organism with clinical deterioration, the DSMC will advise the stoppage of the trial. In case of asymptomatic bacteremia or mild infection due to the administered strains, the study may continue but with closer monitoring as deemed by the DSMC. If the mortality is lower in the probiotic arm, we will continue the trial because the benefit could be because of chance.

The Department of Microbiology, PathWest Laboratory Medicine WA, Perth Children's Hospital Western Australia will help with microbiological safety monitoring. Safety will be assessed by monitoring for (1) blood culture positive sepsis by the administered strains of bifidobacteria and (2) adverse effects such as abdominal distension, vomiting, and diarrhoea leading to the cessation of the supplementation. Onsite product assessment and ongoing monitoring for sepsis due to the

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administered strains of bifidobacteria will be ensured by the Microbiology departments, PathWest Laboratory Medicine WA, at Princess Margaret Hospital and QEII Medical Centre. These are NATA certified laboratories. Product assessment will include confirming species identity and viability by conventional microbiological testing, MALDI-ToF species identification and 16S ribosomal RNA gene sequencing. Antimicrobial susceptibility of the three species within the product to penicillin, cefotaxime, meropenem, gentamicin and vancomycin will be determined by Etest® methodology. Detection of potential product contamination by culture for *Staphylococcus aureus*, coagulase-negative *Staphylococci*, beta-hemolytic *Streptococci*, *Enterococci*, *Bacillus* species, *Pseudomonas aeruginosa*, members of the *Enterobacteriaceae* family, and yeasts and moulds will be undertaken. The important adverse effect that can be anticipated is the rare occurrence of bacteremia or septicemia due to the administered probiotic organism. It is reassuring to know that the live probiotic organisms have been routinely administered in the NICU of KEMH and PMH, even in extremely preterm (non-surgical) infants for the past three years (>1200 babies) without any adverse events. If such an adverse event occurs, an appropriate antibiotic will be administered after discussion with the microbiology department. Parents will be informed through open disclosure approach and the details documented in medical records. The HREC will be notified within 24 hours of the event.

Data handling, storage, confidentiality: The NHMRC Australian guidelines will be followed for confidentiality and data storage⁶¹.

Reporting: A CONSORT checklist will be followed for reporting the results in a journal.⁶²

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Preface to Chapter 10

Probiotic Supplementation for Neonates with Congenital Gastrointestinal Surgical Conditions: Guidelines for Future Research

Our pilot RCT found that probiotic supplementation with the three-strain bifidobacterial product (*B. breve* M-16V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536) attenuates gut dysbiosis, increases stool short-chain fatty acid (SCFA) levels and improves the growth of head circumference in neonates with congenital gastrointestinal surgical conditions (CGISC). In this article, we have provided guidelines for designing future RCTs based on the experience gained from our pilot RCT. The recommendations include advice about sample size, potential confounders, outcomes of interest, probiotic strain selection, storage, dose, duration and microbial quality assurance, collection of stool samples, storage, and analysis and reporting. Following these guidelines will increase the internal validity of future RCTs in this area and hence confidence in their results.

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CHAPTER 10

Probiotic Supplementation for Neonates with Congenital Gastrointestinal Surgical Conditions: Guidelines for Future Research

Abstract:

Our pilot RCT found that probiotic supplementation with the three-strain bifidobacterial product (*B. breve* M-16V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536) attenuates gut dysbiosis, increases stool short-chain fatty acid (SCFA) levels and improves the growth of head circumference in neonates with congenital gastrointestinal surgical conditions (CGISC). In this article, we have provided guidelines for designing future multicentre RCTs based on the experience gained from our pilot RCT. The recommendations include advice about sample size, potential confounders, outcomes of interest, probiotic strain selection, storage, dose, duration and microbial quality assurance, collection of stool samples, storage, and analysis and reporting. Following these guidelines will increase the internal validity of future RCTs in this area and hence confidence in their results.

Background: Our pilot RCT¹ found that supplementation with the three-strain probiotic containing *B. breve* M-16V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536 attenuates gut dysbiosis and increases faecal short-chain fatty acid (SCFA) levels in neonates with congenital gastrointestinal surgical conditions (CGISCs). The head circumference growth was better in the probiotic group. Whilst the results were in favour of probiotics, there were areas of uncertainty due to the following reasons:

(a) Caesarean section^{2,3} rates were higher in the placebo group (70% vs 40%), whereas the incidence of congenital diaphragmatic hernia (CDH) was higher in the probiotic group (23% vs 3%).

(b) The relative abundance of proteobacteria, considered the signature of dysbiosis⁴, was not significantly different between the two groups after two weeks of supplementation (22.7% in probiotic versus 50.3% in placebo; $p=0.27$).

(c) Being a pilot RCT, the study was not powered to identify clinically significant differences between the groups.

Based on the experience gained from our pilot RCT¹, we make the following suggestions for designing future RCTs to overcome the above uncertainties.

1. **Sample size:** Our pilot RCT found that the head circumference growth was better in the probiotic group¹. Hence, one could hypothesise that the probiotic supplemented group will have better neurodevelopmental outcomes. Our retrospective study⁵ with a sample size of 400 found the incidence of suboptimal neurodevelopmental outcomes (SNDO) to be 16% in term and near-term infants with CGISC. A sample size of 516 infants (258 in each arm) will be required to have 80% power at the two-sided 5% significance level to detect a 50% difference in the primary outcome (16% in controls and 8% in the probiotic group). Since nearly 30% of infants are expected to be discharged home before completing two weeks of supplementation, the sample size should be increased by another 154, and hence the final size will be 670 infants. The involvement of multiple centres will be crucial to achieving this sample size within a reasonable time period.

The results of our pilot RCT¹ also provide the data for estimating the sample sizes for RCTs primarily aimed to compare the stool bacterial community structures between the probiotic and placebo groups. The SIMR package⁶ allows users to calculate sample sizes and conduct power analysis for longitudinal studies. For studies comparing beta-diversity, micropower package⁷ can be used as it calculates sample sizes using pairwise distance and PERMANOVA. It is essential to involve bioinformaticians to ensure appropriate methods for power and sample size

calculations and plan the statistical techniques for analyses based on the study hypothesis and design⁸⁻¹⁰.

2. Inclusion criteria: Our pilot RCT¹ focussed on late preterm and term infants with CGISC to avoid the confounding effect of extreme prematurity on gut microbiota. Future RCTs, if of adequate sample size, could include even very preterm (<32weeks) and extreme preterm (<28 weeks) infants with CGISC. With a large sample size, baseline characters, including gestational age, are expected to be similar between the study groups. Probiotic supplementation is safe and beneficial even in extremely preterm (non-surgical) infants and reduces the risk of necrotising enterocolitis (NEC), feed intolerance, and late-onset sepsis (LOS)¹¹⁻¹⁵.

3. Randomisation: Since our pilot RCT¹ had a small sample size of 60 infants, we used computer-generated random sequence numbers in random blocks of two and four to ensure an equal number of infants receive probiotics or a placebo. However, this method did not minimise the chance of unequal distribution of essential confounders (e.g., mode of delivery and severity of the surgical condition), which can affect the gut microbiota.

Therefore, future studies should consider using “treatment allocation by minimisation”¹⁶ or “rank minimisation”¹⁷ to achieve balance, especially if the sample size is small. The involvement of clinical epidemiologists at a very early stage of trial protocol is crucial to achieving the ideal design for the trial, including the randomisation method.

Factors such as gestational diabetes¹⁸, mode of delivery³, intrapartum antibiotics¹⁹⁻²¹ and maternal probiotics,²²⁻²⁴ gestational age²⁵ and underlying surgical conditions that can affect the neonatal gut microbiota need to be balanced between the probiotic and placebo groups. Since the type of milk used (breast milk versus cow’s milk-based versus hydrolysed formula) can influence gut microbiota²⁶, standardising their feeding regimen is desirable but unlikely to be feasible, given that multiple factors can affect milk production and mother’s choice and clinicians’ opinions.

4. Selection of probiotic product

So far, only two pilot RCTs have evaluated probiotics in neonates with CGSC. The first study was by Powell et al., who reported that daily administration of the single strain probiotic *B. longum subsp. infantis* (1×10^9 CFU per day) resulted in partial attenuation of gut dysbiosis in neonates with gastroschisis²⁷. The second RCT was ours¹, in which the multi-strain probiotic product, a mixture of *B. breve* M-16V, *B. longum subsp. infantis* M-63 and *B. longum subsp. longum* BB536 (1×10^9 of each strain per sachet; Morinaga Milk Industry Co, Japan) was safe and effective in improving gut microbiota and SCFA levels. We found the study sachets (probiotics and placebo) free of harmful bacteria by conducting regular microbial analyses on random sachets. Future RCTs in neonates with CGISC could consider using this product or the strain used by Powell et al²⁷. In the other small RCT currently being conducted in Canada (ClinicalTrials.gov identifier NCT03266315), 20 newborn infants with CGSCs will be randomised to receive the multi-strain product FloraBaby™, a mixture of bifidobacteria and lactobacilli strains. It is essential to wait for the results of that study before conducting multicentre RCTs using that product in neonates with CGISC.

While many other probiotic products are safe and effective in preterm infants without surgical conditions, it is essential to test them in pilot RCTs in neonates with CGISCs before considering them in large multicentre RCTs. This is because, unlike preterm infants without CGISCs, neonates with CGISC undergo regular bowel enemas, contrast dye studies, laparotomies and get exposed to various types of general anaesthetics, all of which could influence the effects of the administered probiotics. It is preferable to choose a strain that persists in the gut for at least some time after the supplementation is ceased. In a recent RCT, one group of healthy breastfed infants were supplemented with the probiotic *B. infantis* EVC001 from day 7 to day 28 of life ($n = 34$), and the second group did not receive any probiotics ($n = 32$). *B. infantis*-fed infants had significantly higher populations of faecal

Bifidobacteriaceae, in particular *B. infantis*, during the supplementation period, as well as 30 days after the supplementation was ceased. Infants colonized by high levels of *Bifidobacteriaceae* had 4-fold-lower faecal endotoxin levels, and lower levels of Gram-negative proteobacteria and bacteroidetes²⁸. When the study infants were followed, Fecal *B. infantis* levels were 2.5–3.5 log units higher at 6–12 months in the probiotic group compared to controls ($P < 0.01$)²⁹. Similar studies should be conducted in neonates with CGISC to identify a suitable strain and its dose. If the supplemented probiotic strain stays in the gut for at least few weeks after ceasing supplementation (i.e., after discharge from hospital), it can reduce inflammation, infection and toxemia that can occur during supplementation period while in the hospital and in the immediate post-discharge period. Such short-term benefits could translate into better neurodevelopment even if the supplemented strains do not result in long term colonisation.

Another factor to consider is the safety of supplemented probiotics, especially the potential for translocation and causing sepsis. Unlike preterm infants without surgical problems, neonates with CGISC undergo laparotomy. Hence, there is a risk that the administered live probiotic bacteria could spill over into the peritoneal cavity and systemic circulation resulting in sepsis and seeding in various organs. While infants in our pilot RCT¹ or Powell et al²⁷ did not experience such a complication, a large sample size study would be needed to ensure safety.

In that context, it is also reassuring that infection due to administered probiotic organisms is infrequent even among extremely preterm infants and can be treated with antibiotics. Sakurai et al³⁰ reported that bifidobacterial bacteraemia occurred in 6 out of 298 neonates (i.e. 2%), but none had severe illnesses due to bacteraemia. They speculated that the reason behind the high incidence of *B. breve* bacteraemia in their cohort was rigorous laboratory methods.

Alternatives to probiotics such as prebiotics^{31,32} and para-probiotics (dead probiotic bacteria)³³ that are unlikely to carry the risk of probiotic sepsis should also be tested in future RCTs.

5. Dose of probiotic supplements:

To our knowledge, there are no dose-finding studies in neonates with surgical conditions. There are two studies evaluating the dose-response and one study on the dosing interval of probiotics on colonisation rates in preterm infants.

Dutta et al³⁴. randomly allocated 149 preterm infants to groups A–D (received 12-hourly probiotic supplements of 10^{10} cells for 21 days, 10^{10} cells for 14 days, 10^9 cells for 21 days and placebo, respectively). They reported that colonisation with *Lactobacillus* and *Bifidobacterium* by day 28 was significantly higher in groups A, B, and C versus placebo, respectively. They also reported that there were trends toward more CFU of *Lactobacillus* and *Bifidobacterium* per ml of stool in group A versus B and group B versus C.

Underwood et al³⁵. randomly allocated 12 preterm infants to receive either *B. infantis* or *B. lactis* in increasing doses over a 5-week period. The dose was 5×10^7 , 1.5×10^8 , 4.5×10^8 , 1.4×10^9 , 4.2×10^9 , at weeks 1, 2, 3, 4 and 5, respectively. There was a greater increase in faecal bifidobacteria among infants receiving *B. infantis* than those receiving *B. lactis*. This difference was most marked at a dose of 1.4×10^9 CFU twice daily. The relative abundance of bifidobacteria declined with increasing dosage over time in the *B. lactis* group and showed a statistically nonsignificant trend toward an increase in the *B. infantis* group. It demonstrates that the colonisation response is not only dependent on the dose but also on the strain.

Watkins et al.³⁶ investigated the appropriate dosing interval of a dual strain probiotic given daily (n = 8), biweekly (n = 8) and weekly (n = 10) in preterm infants <32 weeks' gestation. The control group consisted of 12 preterm infants who did not receive the probiotic. *Bifidobacterium bifidum* (1×10^9 CFU) and *Lactobacillus acidophilus* (1×10^9 CFU), was administered (2×10^9 CFU of bacteria in total), until 34 weeks postmenstrual age (PMA). Stool samples were collected at 31-, 34-, 41- and 44-weeks PMA. At all ages assessed, colonisation levels of administered probiotic organisms were higher in the once daily group. They concluded that a daily dose is a suitable dosage for preterm infants.

Our study¹ used a total of 3 billion probiotic organisms per day (3×10^9 CFU), whereas Powell et al²⁷ had used 2 billion probiotic organisms per day (2×10^9 CFU). Powell et al²⁷. reported partial attenuation of gut dysbiosis after probiotic supplementation, whereas our study showed a higher attenuation level. Hence, a dose of at least 3×10^9 CFU per day should be used in future RCTs. Doses higher than 3×10^9 CFU per/day may offer further benefits but need to be tested in pilot RCTs initially. Smaller quantities less than 1 billion (i.e., $<1 \times 10^9$) CFU may not be enough to colonise the gut adequately, limiting their effectiveness^{37,38}.

Overall, given that there is insufficient evidence regarding the dose of probiotics, dose-finding studies with the chosen strain/s need to be conducted prior to embarking on large RCTs in neonates with CGISCs.

While conducting such dose-finding RCTs on probiotics, the relative abundance of *bifidobacterium* shouldn't be used to determine the amount of dysbiosis because it is not independent of the treatment (since the probiotic contains this DNA). One should also be familiar with the uncertainties around the definition of dysbiosis³⁹. Broadly defined, dysbiosis is any change to the composition of resident commensal communities relative to the community found in healthy individuals⁴⁰. However, researchers haven't yet agreed on what constitutes a healthy microbiome.⁴¹ The inability to define balance, in turn, makes it difficult to specify what constitutes an imbalance in the microbiota or dysbiosis⁴². There is also a concern that increasing the depth of microbiome measurements has failed to provide genuinely needed definitions for homeostasis and dysbiosis⁴² Experts lament that increasing the amount of available multiomic microbiome profiling data from terabytes to petabytes holds little promise for clarifying how to define dysbiosis, and raise the question of where microbiome science should go from here^{41,42}. Given these uncertainties and controversies, we chose a prespecified and clinically relevant, though simplistic definition of dysbiosis: an expansion of pathobionts or potentially harmful microbes⁴⁰. Hence, for our pilot RCT, we chose the sum of relative abundance of

potentially pathogenic families of *Clostridiaceae*, *Enterobacteriaceae*, *Enterococcaceae*, *Pseudomonaceae*, *Staphylococcaeae*, *Streptococcaceae*, and *Yersiniaceae* as the primary outcome.

The pathogenic families that we chose contain all the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp.*) for the following reasons: *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Enterobacter spp* belong to the family *Enterobacteriaceae*; *Staphylococcus aureus* belongs to the family *Staphylococcaceae*; *Enterococcus fecium* belongs to the family *Enterococcaceae*; *Pseudomonas aeruginosa* belongs to the family *Pseudomonaceae*.

The ESKAPE pathogens' are capable of 'escaping' the biocidal action of antibiotics and mutually representing new paradigms in pathogenesis, transmission, and resistance⁴³⁻⁴⁵. Hence they are considered as the most critical pathogens.⁴⁴ The future dose-finding studies of probiotics could use the relative/absolute abundance of ESCAPE pathogens as the criteria to define dysbiosis.

6. Storage of probiotics/placebo sachets: We stored the main stock of probiotic/placebo sachets in the trial-pharmacy department in the refrigerator at 2 to 8-degree C. To enable ease of access, at least five such sequentially numbered boxes were kept in the automated dispensing machine (ADM) within the neonatal intensive care unit (NICU) at refrigerator temperatures of 2 to 8 degrees C. Once parental consent was obtained, the box next in order was labelled with the Unique Medical Record Number (UMRN) sticker of that infant, thereby declaring that particular package belongs to that infant for the entire hospital stay. Each day, one sachet from that box was taken out from the ADM by the nurses and administered to that particular infant.

A similar approach could be undertaken in future RCTs. Keeping the trial supplements at room temperatures may affect their viability and longevity.

7. Timing of starting the trial supplements: In our study¹, trial supplements were commenced predominantly in the immediate postoperative period. The main reason for the delay was difficulty collecting baseline stool samples for reasons discussed earlier. In our RCT and the RCT by Powell et al., probiotics were commenced in the immediate post-operative period and found it to attenuate dysbiosis²⁷. The majority of the studies involving adults who underwent gut surgeries found benefits of probiotics when administered in the preoperative period⁴⁶. To obtain maximum benefit, the best time to commence probiotics is probably in the pre-operative period, but collection of stool samples before starting probiotics may not be feasible in all cases.

8. Administration of probiotics while infants are fed nil-enterally: We administered the study supplements even when the infants were fed nil-enterally and found no side effects¹. Waiting until enteral feeds are commenced may decrease the efficacy of probiotics by delaying their gut colonisation. Considering the small dose volume and low osmolarity (320–350 mOsm/L) when reconstituted in expressed breast milk⁴⁷, it is reasonable to start the supplement even if the infant is fed nil-enterally.

9. When to withhold supplementation: In our study¹, we continued supplementation even when infants were critically ill as long as there were no significant abdominal symptoms. We suggest that supplements only be withheld if there is a gut perforation or suspicion of abdominal compartment syndrome with a distended, firm, and tender abdomen to minimise the risk of gut translocation by the supplemented probiotic organisms.

10. Care immediately after administering the trial supplements: We administered the trial medication as a single daily dose for convenience. Many infants with CGISC will have nasogastric tubes (NGTs) on free drainage or suction for gastric decompression. We clamped

the NGT for at least one hour and preferably 3-4 hours to prevent the retrograde flow of the administered probiotics/placebo into the free-drain container²⁷.

11. Hand hygiene precautions: Rigorous hand hygiene needs to be followed to prevent the risk of cross-contamination. Whilst our pilot RCT¹ did not specifically address the issue of cross-contamination (aka cross colonisation)⁴⁸, we were reassured that the relative abundance of the genus *Bifidobacterium* in the placebo group was only about 5% at all time points T2-T4. In contrast, it was around 35-45 per cent in the probiotic group. Hence, even if there was cross-contamination, the load was not enough to allow them to colonise adequately and, therefore, unlikely to be clinically significant.

The relative abundance of 5% for the genus *Bifidobacterium* in the placebo arm after two weeks of supplementation and also prior to discharge in our study¹ was lower than the Australian PROPREAMS trial in preterm infants, in which it was 17.5% (SD 27.4) in the placebo group and 36.4% (32.5) in the probiotic group⁴⁹. The UK PIPS trial⁵⁰ in preterm infants (non-surgical) reported high cross-colonisation rates because 49% of infants in the placebo group were colonised (culture positive) with the administered strain. However, they did not report on the relative abundances, and hence it is difficult to know if such cross colonisation impacted the clinical outcomes of the trial⁵⁰. Future studies should report cross-colonisation rates and relative abundances from study infants.

Some researchers have recommended that future multicentre studies may have to adapt a cluster-RCT design to overcome the issue of cross-contamination⁵⁰. Irrespective of whether the trial design is conventional or a cluster RCT, strict hand hygiene is essential while caring for neonates.

12. Medication reconciliation: Our method was to record doses administered, omitted, and wasted sachets. It was matched against the number of unused sachets in the package after it was collected by the trial pharmacists when the infant had completed the intervention.

13. Safety of probiotics: Researchers need to inform the parents and research ethics committees that probiotics are live bacterial organisms, and there are reports of sepsis due to the administered probiotic organism⁵¹⁻⁵⁷. However, researchers should also reassure parents that most cases of probiotic sepsis were successfully treated by antibiotics^{30,51}. In addition, it is essential to inform parents that 63 RCTs, 30 observational studies and many meta-analyses including the Cochrane review have found probiotics to be safe in preterm (non-surgical) infants^{11-15,37,58-64}. Our pilot RCT¹ and the RCT by Powell et al²⁷ found probiotics to be safe in neonates with CGISC. The only published case report of mortality after probiotic supplementation in a preterm (non-surgical) infant was because of contamination of the product⁶⁵. Independent assessment of the *product quality* is of paramount importance⁶⁶, and probiotics should be free of contaminants and from companies with a high safety track record.

14. Ongoing microbiological quality assurance: Having a well-resourced microbiology lab is essential for any centre planning to conduct RCT of probiotics⁶⁶. It is crucial to conduct microbial analysis of random sachets of study supplements to rule out the presence of harmful pathogens and to check viability and colony counts of the probiotic strain. All routine clinical specimens (blood, urine, CSF, endotracheal secretions, wound swabs) from study infants should be analysed using culture methods to enable diagnosis of infections due to supplemented probiotic organisms. As per our standard practice for routine clinical care, we used the Becton Dickinson BACTECTTM PEDS PLUSTM/F Medium aerobic blood culture bottles with incubation monitored in the Bactec 9120 system⁶⁷. No special culture bottles were used for the study. Our laboratory's automated blood culture system detects bifidobacteria (if present) within the standard 5-day (120 hours) incubation period. Whilst some studies have shown that if incubation in aerobic culture bottles is ceased after 120 hours, a few cases of bifidobacterial bacteremia could be missed³⁰, we decided to restrict to 120 hours because going beyond that period would require larger capacity size incubators. The other issue if incubation goes beyond

120 hours is the likely recovery of slow-growing contaminant organisms, which can affect the clinical interpretation of the results. On the other hand, incubation of fewer than 120 hours will miss many cases and hence cannot be recommended.

15. Collection of stool samples: In our study¹, we collected stool samples into 0.5 ml sterile micro-tubes (sarstedt.com). If the sample is collected in a different container and subsequently transferred to the micro-tubes, there is a risk of microbial contamination. We used sterile wooden spoons to scoop fresh samples from the nappies of study infants. There were many challenges during the collection of stool samples. (a) Since the nappies are checked only once in 3-4 hours, in many cases, stools had dried up by then. (b) Watery stools got absorbed into the nappies, so the collection was impossible. (c) Delayed passage of meconium and infrequent stooling in the pre-operative and immediate post-operative periods due to intestinal obstruction, postoperative ileus, and the use of morphine/fentanyl. (d) Use of radio-contrast enema or upper GI contrast for diagnostic purposes. If stool samples are collected after the contrast study, it will not represent the actual gut microbiota of the infant. (e) Missed opportunity to collect samples because most infants pass only one stool in the pre-operative period and none until 3-5 days in the post-operative period. Hence, extra vigilance and cooperation by the bedside nurses are essential to ensure the timely collection of stool samples.

A recent study found rectal swabs correlated well with the simultaneously collected faecal samples in neonates⁶⁸. In contrast, a similar study in critically ill adults reported systematic differences in gut microbial profiles between simultaneously collected rectal swabs and stool samples⁶⁹. Further studies are needed to confirm the reliability of rectal swabs for microbial analysis. Such studies should also evaluate the effect of collection mode on stool SCFA levels.

16. Labelling of stool samples: Accurate labelling of the micro-tubes is essential to maintain the integrity of the data. They could be labelled the samples as follows if the total sample size is 100-999:

The first three digits to represent the study number of the infant, the second two digits refer to the sample number, and subsequent alphabets represent the purpose of the sample.

Example: 003-01-DNA means study infant number 3, first stool sample (i.e., before commencing supplements), and the sample is for DNA sequencing.

003-01-SCFA means study infant number 3, stool sample before commencing supplements, and the sample is for SCFA analysis.

17. Storage of stool samples: In our study¹, we stored the samples in a -20 degree C freezer immediately after collection and subsequently transferred them in a cold esky for final storage at -80 degrees C within next 96 hours. While rapid freezing to -80 degrees C is considered the best practice, it is not feasible even in the most resourceful settings. On the other hand, storing the samples at room temperatures is not recommended as it is known to lower Shannon diversity and evenness⁷⁰. It is suggested that the samples should be preserved at -20°C within 15 min after collection and then transferred on dry ice within 24 h of collection and stored at -80°C thereafter¹⁰.

Recent studies have shown that specific commercially available reagents allow for stool samples collection, preservation, and storage at ambient temperatures for longer periods⁷¹. Many laboratories provide their vials to collect stool samples that have DNA preservation agents in the vials. Hence, it is important to discuss with the laboratory at the protocol stage of the RCT. It is also essential to ensure that the sample preservation and storage methods are consistent across all samples to minimise variations in results^{10,70,72}.

18. Shipping of stool samples: Given that stool samples are biological specimens, only accredited couriers should be used for shipping such samples. Maintaining a cold chain at -80 degrees C using dry ice is essential, especially while sending the samples that do not contain preservation media. Transportation logistics, including temperatures, need to be discussed with the receiving laboratory well in advance.

19. Method of analysing stool samples for gut microbial data

There are excellent guidelines on the best approaches for analysing microbial data⁷³. Briefly, the methods used in microbiome research include amplicon, metagenomic and metatranscriptomic sequencing. The amplicon sequencing involves the 16S rRNA gene sequencing for bacteria. It is relatively inexpensive, but the analysis is limited to genus level taxonomic resolution. On the other hand, the metagenomic sequencing method sequences all microbial genomes (DNA) within a sample. It extends the taxonomic resolution to species or strain level. If there is adequate funding, metagenomic sequencing is preferred. Metatranscriptomics uses RNA sequencing to profile transcription in microbiomes, providing information on gene expression and the active functional output of the microbiome. It gives better insight into the functional activity of a microbial community. The pros and cons of each method are well described by Knight et al.⁷³ It is important to decide whether to limit to 16s ribosomal RNA gene sequencing or metagenomics during the early stages of protocol development in collaboration with the lab scientists. Even if the aim is to restrict to the former, it is helpful to collect additional stool samples and store them at -80 degrees C so that metagenomic sequencing can be undertaken in future when funding becomes available.

20. Method of analysing stool samples for SCFA

SCFAs are produced mainly by intestinal microbiota and play an important role in many biological processes in humans. Gas chromatography-mass spectrometry⁷⁴⁻⁷⁶ is the commonly used method for SCFA assay. Alternative methods are high-performance liquid chromatography, nuclear magnetic resonance and capillary electrophoresis⁷⁷.

Since SCFAs are volatile, keeping the stool samples in appropriate conditions after collection is important. Samples are usually kept at -80 degrees C, although many researchers have successfully used -20 degree C⁷⁷. It is important to screw the lid tight and not open it until it

reaches the laboratory for analysis. The stool samples for SCFA analysis should be collected in vials separate from those used for microbial analysis.

21. Ensuring blinding of the data: It is important to ensure blinding of the group allocation till the full results are available. Only the trial pharmacist or a similar professional with no vested interest in the project should know which sachets are probiotic and placebo. When the stool samples are sent to laboratories for microbial and SCFA analyses, they should be labelled as groups 1 and 2 to enable comparison without disclosing the groups. Clinical data also should be collected and compared as group-1 and group-2. Only in the end, unblinding should be done by the trial pharmacist.

Once the analysis comparing the microbiota of group 1 versus group 2 is completed, the bioinformatician may be able to guess group allocations (even when blinded) if the relative abundance of the supplemented bacteria is higher in one group. If researchers and statisticians assessing clinical outcomes become aware of those results, bias might be introduced. Hence statistical analysis of clinical data must be done by people who are blinded to the results of the microbial analysis and vice versa. This is especially important when the primary outcome of interest is clinical (sepsis, mortality, duration of hospital stay, time to full feeds, neurodevelopment).

22. Data safety and monitoring board (DSMB): Establishing a DSMB is essential before recruitment into the RCT⁷⁸. The charter should have pre-defined stopping rules both for efficacy and safety. While p values around stopping rules are essential, they should not be the sole criteria while deciding whether the trial should continue or stop.

23. Reporting: Reporting metagenomic analysis of stool samples should follow the recently published STROBE-Metagenomics guidelines⁷⁹. Given that the study design will be an RCT, CONSORT guidelines help report clinical outcomes.

Conclusions: In summary, following these guidelines will increase the internal validity of future RCTs in this area hence confidence in their results.

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Thesis Overview

Neonatologists worldwide have focused their research on gut microbiota/dysbiosis and probiotic supplementation in preterm infants. As a result of such studies, it is well established that preterm infants suffer from significant dysbiosis¹⁻³. Many RCTs and systematic reviews have shown the efficacy and safety of probiotics in preterm infants without surgical conditions⁴⁻⁶. Hence, probiotic supplementation of preterm infants has become standard practice in many parts of the world, including our neonatal intensive care units.

Like preterm infants, neonates with congenital gastrointestinal surgical conditions (CGISCs) are exposed to risk factors such as feed intolerance, delayed exposure to breast milk, inadequate skin to skin contact with their mothers, frequent infections, parenteral nutrition, gastric acid suppressants and multiple courses of antibiotics. Hence one could expect that they are also susceptible to developing gut dysbiosis like preterm infants. In addition to the above risk factors, neonates with CGISCs undergo multiple gut surgeries and invasive procedures and receive enemas and radiocontrast dye studies. Since their fragile gut will be handled at the time of surgery by surgeons, it is plausible that gut dysbiosis is more severe in them than in infants without underlying surgical conditions.

Hence, we were interested in assessing the gut microbiota of neonates with CGISCs. Our prospective study confirmed our hypothesis that these infants indeed suffer from gut dysbiosis and have very low levels of bifidobacteria⁷.

While doing the initial literature search on the subject, we realised that there was hardly any information on stool fatty acid levels in neonates with CGISCs. Given the importance of short-chain fatty acids (SCFA) in human health and the interplay between gut microbiota and SCFA, we decided to investigate stool SCFA levels in these infants. Our prospective study confirmed our hypothesis that neonates with CGISCs have low SCFA levels compared to healthy infants of similar age⁷.

Once we established that neonates with CGISCs have very low faecal bifidobacteria and SCFA levels compared to healthy breastfed infants, we undertook the next step by conducting a pilot RCT evaluating the efficacy and safety of supplementation with the triple strain bifidobacteria in them⁸. Our trial protocol has described the justification behind choosing this probiotic product. Due to our collaboration with the Morinaga Milk Industry (Japan) over the past 15 years due to our probiotic related research in preterm infants, we were quite confident with the quality of their probiotic supplements. We debated whether to choose their single strain product containing *B. Breve* M16-V, which we had used in our preterm infants for nearly a decade, or whether to use their triple strain product containing *Bifidobacterium breve* M-16V, *Bifidobacterium longum* subsp. *infantis* M-63, and *Bifidobacterium longum* subsp. *longum* BB536. Around that time, evidence was emerging that multistrain probiotics may have an advantage over single strain products⁹, and hence we decided to use the multistrain product. Another reasoning was that the three-strain product contains *Bifidobacterium longum* subsp. *infantis*, which has a strong ability to consume complex carbohydrates such as human milk oligosaccharides found in human milk^{10,11}. The results of our pilot RCT confirmed that supplementation with the triple strain bifidobacteria attenuated gut dysbiosis and improved stool SCFA levels in neonates with CGISCs⁸. It also found that head growth was better in the probiotic supplemented group.

In the recent decade, evidence is emerging that neonates with CGISCs are at risk adverse outcomes such as cognitive delay, blindness, deafness, cerebral palsy, attention deficit hyperactivity disorder and autism in the long run¹². Our neonatal units have been conducting developmental assessments in neonates with CGISCs using the Griffiths Mental Developmental Scales since the early 2000s. Hence, we decided to review their outcomes using a retrospective study design and found that nearly 16% of them had suboptimal

neurodevelopment at one year of age¹³. We also found that neonates with CGISCs have postnatal growth restriction during initial hospitalisation.

Based on our studies, it was clear that neonates with CGISCs have gut dysbiosis⁷ and also suffer from suboptimal neurodevelopment¹³. Given the role of healthy bacteria in gut-brain axis¹⁴⁻¹⁷, one could speculate that dysbiosis could contribute to their suboptimal neurodevelopment¹⁶ and probiotics could attenuate dysbiosis and improve their neurodevelopmental outcomes. Hence, we plan to compare the developmental outcomes of the 61 neonates with CGISCs who participated in our pilot RCT⁸ of probiotic supplementation.

The definitive answer to the main question, i.e., “does probiotic supplementation improve developmental outcomes of neonates with CGISCs?” requires well-designed RCTs with an adequate sample size of at least 700 infants. We have provided guidelines for conducting such RCTs drawing upon the experience gained from our pilot RCT.

It is well known that initial RCTs report a larger effect estimate for the primary endpoint than subsequent ones¹⁸. In many situations, subsequent trials report an effect size in the opposite direction compared to the original trial¹⁸. Hence, we stress the importance of repeating RCTs in different settings to ensure that the results are reproducible and more reliable.

We would like to highlight the crucial limitations of this PhD related research projects. The neurodevelopmental outcomes of infants in our retrospective study were available only until one year of age. It is well known that developmental assessments in early infancy are a weak predictor of outcomes at later years¹⁹⁻²¹. Hence future RCTs should follow study infants for developmental assessments until five years of age. Since our pilot RCT had a small sample size of 61, we could not achieve a perfect balance in baseline characters between the probiotic and placebo groups. With a large sample size (~700), one could expect a better balance between the groups. A third limitation was that we used 16s R-RNA gene sequencing methods which

allow the allocation of reads only up to the genus level. Future studies should consider using a whole-metagenome approach that provides species- and strain-level information.

In summary, the research projects in this thesis have confirmed that neonates with CGISCs are at risk of developing gut dysbiosis, infections, postnatal growth restriction and suboptimal neurodevelopment. They have also demonstrated that gut dysbiosis in such infants can be attenuated by probiotic supplementation. These findings are expected to pave the way for the conduct of large multicentre RCTs of probiotic supplementation and hopefully routine use of probiotics in them.

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Appendix 1

Approvals from Human Research Ethics Committees and hospital quality activity committees



THE UNIVERSITY OF
WESTERN AUSTRALIA

**Human Ethics
Office of Research Enterprise**

The University of Western Australia
M459, 35 Stirling Highway
Crawley WA 6009 Australia

T +61 8 6488 4703 / 3703
F +61 8 6488 8775
E humanehics@uwa.edu.au

CRICOS Provider Code: 00126G

Our Ref: RA/4/1/7743

11 August 2015

Professor Karen Simmer
School of Paediatrics and Child Health
MBDP: M551

Dear Professor Simmer

HUMAN RESEARCH ETHICS OFFICE – NOTIFICATION OF ETHICS APPROVAL FROM ANOTHER ETHICS COMMITTEE

Project: Gut Microbiota in Neonates with Gastrointestinal Surgical Conditions: A Prospective Cohort Study

Thank you for your correspondence notifying this office of your project's review and approval by a non-UWA Research Ethics Committee.

It is noted that you have ethics approval from Princess Margaret Hospital, approval number 2015-042.

The UWA students and researchers identified as working on this project are:

UWA Researchers:

<i>Name</i>	<i>Faculty / School</i>	<i>Role</i>
Professor Karen Simmer	School of Paediatrics and Child Health	Chief Investigator
Dr Sanjay Patole	School of Paediatrics and Child Health	Co-Investigator

Student(s): Shripada Rao - PhD - 21660354

Although The University of Western Australia reserves the right to subject any research involving its staff and students to its own ethics review process, in this case, the UWA Human Ethics Office recognizes the existing approval of the non-UWA ethics committee.

1. *Approving HREC to receive annual reports, amendments and notification of adverse events*

You are reminded that the approving ethics committee remains the monitoring committee for this project. You must correspond with them for matters regarding amendments, adverse events, annual and final reporting.

If you have any queries, please contact the HEO at humanethics@uwa.edu.au.

Please ensure that you quote the file reference – RA/4/1/7743 – and the associated project title in all future correspondence.

Yours sincerely

Dr Caixia Li
Manager, Human Ethics



THE UNIVERSITY OF
**WESTERN
AUSTRALIA**

Human Ethics

Office of Research Enterprise

The University of Western Australia
M459, 35 Stirling Highway
Crawley WA 6009 Australia

T +61 8 6488 3703 / 4703

F +61 8 6488 8775

E humanethics@uwa.edu.au

CRICOS Provider Code: 00126G

Our Ref: RA/4/20/4051

11 October 2017

Dr Shripada Rao
Medical School
MBDP: M561

Dear Doctor Rao

HUMAN RESEARCH ETHICS OFFICE – NOTIFICATION OF ETHICS APPROVAL FROM ANOTHER ETHICS COMMITTEE

Project: Probiotic supplementation in neonates with major gastrointestinal surgical conditions: A Pilot Randomized Double Blind Placebo Controlled Trial - Recognition Princess Margaret Hospital HREC Approval 2016086EP

Thank you for your correspondence notifying this office of your project's review and approval by a non-UWA Research Ethics Committee.

It is noted that you have ethics approval from Princess Margaret Hospital, approval number 2016086EP.

The students and researchers identified as working on this project are:

Name	Institution Details	Role
Dr Shripada Rao	Medical School	Chief Investigator
Clinical Professor Sanjay Patole	WA Department of Health	Co-Investigator
Professor Karen Simmer	Medical School	Co-Investigator
Dr Ian Gollow	Princess Margaret Hospital for Children	Co-Investigator
Dr Anthony Keil	PathWest Laboratory Medicine WA	Co-Investigator
Professor Patricia Conway	University of New South Wales	Co-Investigator

Student(s): Shripada Rao - PhD - 21660354

Although The University of Western Australia reserves the right to subject any research involving its staff and students to its own ethics review process, in this case, the UWA Human Ethics Office recognizes the existing approval of the non-UWA ethics committee.

1. *Approving HREC to receive annual reports, amendments and notification of adverse events*

You are reminded that the approving ethics committee remains the monitoring committee for this project. You must correspond with them for matters regarding amendments, adverse events, annual and final reporting.

If you have any queries, please contact the HEO at humanethics@uwa.edu.au.

Please ensure that you quote the file reference – RA/4/20/4051 – and the associated project title in all future correspondence.

Yours sincerely

Mark Davies
Manager, Human Ethics



Government of **Western Australia**
 Department of **Health**
North Metropolitan Health Service
 Women and Newborn Health Service

Research Governance Office

A/Professor Shripada Rao
 Neonatal Intensive Care Unit
 Princess Margaret Hospital for Children
 Roberts Rd
 SUBIACO WA 6008

Dear Professor Rao

Project Title: Gut Microbiota in neonates with gastrointestinal surgical conditions: A prospective cohort study
HREC Reference: 2015042EP

On behalf of the Women and Newborn Health Service, I give authorisation for your research project to be conducted at the following site(s):

King Edward Memorial Hospital for Women

This authorisation is based on the approval from the PMH HREC and the review from the Research Governance Office. This authorisation is valid subject to the ongoing approval from the HREC.

This authorisation is based on the ethical approval from the HREC, and on the basis of compliance with the 'Conditions of Authorisation to Conduct a Research Project at Site' (attached) and with the compliance of all reports as required by the Research Governance Office and approving HREC. Non compliance with these requirements could result in the authorisation being withdrawn.

The responsibility for the conduct of this project remains with you as the Principal Investigator at the site.

Yours sincerely

Dr Sayanta Jana
 Executive Director
 Medical Services

11/08/2015



Government of **Western Australia**
 Department of **Health**
 Child and Adolescent Health Service

Our Ref: 2015042EP

A/Professor Shripada Rao
 Neonatal Intensive Care Unit
 Princess Margaret Hospital for Children
 Roberts Road
 Subiaco WA 6008

Dear A/Professor Rao

HUMAN RESEARCH ETHICS COMMITTEE (HREC)

HREC REF 2015042EP

STUDY TITLE Gut Microbiota in neonates with gastrointestinal surgical conditions: A prospective cohort study

The ethics application for the project referenced above was reviewed by the PMH Human Research Ethics Committee (HREC) at its meeting on 11/06/2015. It has been approved and the following documents have been approved for use in this project.

Research Protocol Version 4 dated 15 July 2015
 Form of Consent Version 4 dated 15 July 2015
 Parent Information Sheet Version 5 dated 5 August 2015.

Approval of this project from PMH HREC is valid to 29/06/2018 and on the basis of compliance with the 'Conditions of HREC Approval for a Research Project' (attached).

Note: If additional sites are recruited prior to the commencement of, or during the research project, the Coordinating Principal Investigator is required to notify the HREC. Notification of withdrawn sites should also be provided to the HREC in a timely fashion.

A copy of this ethical approval letter must be submitted by all site Principal Investigators to the Research Governance Office or equivalent body or individual at each participating institution in a timely manner to enable the institution to authorise the commencement of the project at its site/s.

This letter constitutes ethical approval only.

This project cannot proceed at any site until separate site authorisation has been obtained from the CE, or delegate, of the site under whose auspices the research will be conducted at that site.

The PMH HREC is registered with the Australian Health Ethics Committee and operates according to the NHMRC National Statement on Ethical Conduct in Human Research and International Conference on Harmonisation – Good Clinical Practice.

The HREC's Terms of Reference, Standard Operating Procedures, membership and standard forms are available from <http://www.pmh.health.wa.gov.au/development/resources/ethics.htm> or from the Ethics Office. Should you have any queries about the HREC's consideration of your project, please contact Ethics Office.

Please quote the above trial number 2015042EP on all correspondence associated with this trial.

Yours sincerely



Dr Mark Salmon
Executive Director
Medical Services

11/08/2015

* The Ethics Committee is constituted, and operates in accordance with the National Health and Medical Research Council's National Statement on Ethical Conduct in Research Involving Humans



Government of **Western Australia**
Department of **Health**
Child and Adolescent Health Service
Research Governance Office

Our Ref: 2015042EP

A/Professor Shripada Rao
Neonatal Intensive Care Unit
Princess Margaret Hospital for Children
Roberts Road
Subiaco WA 6008

Dear A/Professor Rao

HREC REF 2015042EP
STUDY TITLE Gut Microbiota in neonates with gastrointestinal surgical conditions: A prospective cohort study

On behalf of the Child and Adolescent Health Service, I give authorisation for your research project to be conducted at the following site(s):

Princess Margaret Hospital for Children - CAHS

This authorisation is based on the approval from PMH HREC and the review from the Research Governance Office. This authorisation is valid subject to the ongoing approval from the HREC.

This authorisation is based on the ethical approval from the HREC, and on the basis of compliance with the 'Conditions of Authorisation to Conduct a Research Project at Site' (attached) and with the compliance of all reports as required by the Research Governance Office and approving HREC. Non compliance with these requirements could result in the authorisation being withdrawn.

The responsibility for the conduct of this project remains with you as the Principal Investigator at the site.

Yours sincerely

A handwritten signature in black ink, appearing to be 'M Salmon', written in a cursive style.

Dr Mark Salmon
Executive Director
Medical Services

11/08/2015



Government of Western Australia
Child and Adolescent Health Service

Research Governance Office
Our Ref: 2016086EP

23 May 2017

A/Professor Shripado Rao
Neonatal Unit
Princess Margaret Hospital
Roberts Rd
SUBIACO WA 6008

Dear Professor Rao

HREC Reference: 2016086EP
**Project Title: Probiotic Supplementation in neonates with major
gastrointestinal surgical conditions: A Pilot Randomized Double
Blind Placebo Controlled Trial**

On behalf of the Child and Adolescent Health Service, I give authorisation for your research project to be conducted at the following site(s):

Princess Margaret Hospital for Children
Perth Children's Hospital

This authorisation is based on the approval from the Child and Adolescent Health Service Ethics Committee (PMH HREC) and the review from the Research Governance Office. This authorisation is valid subject to the ongoing approval from the HREC.

This authorisation is based on the ethical approval from the HREC, and on the basis of compliance with the 'Conditions of Authorisation to Conduct a Research Project at Site' (attached) and with the compliance of all reports as required by the Research Governance Office and approving HREC. Non compliance with these requirements could result in the authorisation being withdrawn.

The responsibility for the conduct of this project remains with you as the Principal Investigator at the site.

Yours sincerely

A handwritten signature in black ink, appearing to read 'Dhanvee'.

Dr Dhanvee Kandadai
A/Director Clinical Services



Government of Western Australia
Child and Adolescent Health Service

Our Ref: 2016086EP

A/Professor Shripada Rao
Neonatal Intensive Care Unit
Princess Margaret Hospital
Roberts Road
Subiaco WA 6008

Dear A/Professor Rao

HUMAN RESEARCH ETHICS COMMITTEE (HREC)

HREC REF 2016086EP

STUDY TITLE Probiotic Supplementation in neonates with major gastrointestinal surgical conditions: A Pilot Randomized Double Blind Placebo Controlled Trial

The ethics application for the project referenced above was reviewed by the PMH Human Research Ethics Committee (HREC) at its meeting on 18/08/2016. It has been approved and the following documents have been approved for use in this project.

Protocol, Version 3, Dated 21 September 2016
Participant Information Sheet/Consent Form –
Parent/Guardian, Version 3, Dated 21 September 2016
Checklist for Inclusion and Exclusion Criteria Version 1 dated 18 July 2016
Checklist for Onsite Management of Adverse Events that May Occur in a
Clinical Trial Version 1 18 July 2016
Consent Form – Parent/Guardian, Version 3, Dated 21 September 2016

Approval of this project from PMH HREC is valid to 07/10/2019 and on the basis of compliance with the 'Conditions of HREC Approval for a Research Project' (attached).

The nominated participating site(s) in this project is/are:

Princess Margaret Hospital

Note: If additional sites are recruited prior to the commencement of, or during the research project, the Coordinating Principal Investigator is required to notify the HREC. Notification of withdrawn sites should also be provided to the HREC in a timely fashion.

A copy of this ethical approval letter must be submitted by all site Principal Investigators to the Research Governance Office or equivalent body or individual at each participating institution in a timely manner to enable the institution to authorise the commencement of the project at its site/s.

This letter constitutes ethical approval only.

This project cannot proceed at any site until separate site authorisation has been obtained from the CE, or delegate, of the site under whose auspices the research will be conducted at that site.

The PMH HREC is registered with the Australian Health Ethics Committee and operates according to the NHMRC National Statement on Ethical Conduct in Human Research and International Conference on Harmonisation – Good Clinical Practice.

The HREC's Terms of Reference, Standard Operating Procedures, membership and standard forms are available from <http://www.pmh.health.wa.gov.au/development/resources/ethics.htm> or from the Ethics Office. Should you have any queries about the HREC's consideration of your project, please contact the Ethics Office.

Please quote the above trial number 2016086EP on all correspondence associated with this trial.

Yours sincerely



Dr ~~Mark Salmon~~ *DHANVEE KANDADAI*
on behalf of PMH Human Research Ethics Committee

23/05/2017

* The Ethics Committee is constituted, and operates in accordance with the National Health and Medical Research Council's National Statement on Ethical Conduct in Research Involving Humans



Summary of changes document (4 December 2017)

The Chairperson
 Human Research Ethics Committee
 Child and Adolescent Health Service
 Princess Margaret Hospital for Children

Dear Madam/Sir

Re: Request for minor amendment to the trial protocol: Probiotic Supplementation in neonates with major gastrointestinal surgical conditions: A Pilot Randomized Double Blind Placebo Controlled Trial (HREC Ref No: 2016086)

We have begun the above mentioned pilot RCT, and recruited three infants so far. We would like to request the following minor amendments to the trial protocol. These amendments will clarify and ensure that the first stool sample is collected prior to the commencement of supplementation and there will be one week gap between the commencement of supplementation with the trial medication and the collection of second stool sample; and two week gap prior to the collection of third sample. The amendments will also enable us to collect a stool sample prior to discharge in those who are inpatients for more than four weeks.

Accepted version by the HREC: Primary outcomes: Gut microbiota (conventional cultures and 16 s r RNA Pyrosequencing studies for phylogenic profiling) on the stools a. as soon as possible after admission, b) Between D12-14 of life, and c) Between D19-21, during hospital stay.

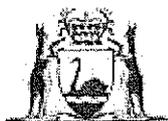
Amendment requested: Primary outcomes: Gut microbiota (conventional cultures and 16 s r RNA Pyrosequencing studies for phylogenic profiling) on the stools collected a. as soon as possible after admission, but prior to the commencement of supplementation b) 7-9 days after commencement of supplementation, c) 14-16 days after commencement of supplementation, and d) prior to discharge from the neonatal unit (in those who stay more than 4 weeks after commencing supplementation).

Accepted version by the HREC: Stool sample collection: Stool samples will be collected a). As soon as possible after admission, b) Between D12-14 of life, and c) Between D19-21, during hospital stay.

Amendment requested: Stool sample collection: Stool samples will be collected on the following days: a). As soon as possible after admission, but prior to commencing the trial medication b) on any one day between 7-9 days after commencing the trial medication, and c) on any one day between 14-16 days after commencing the trial medication. If the infant is an inpatient in the NICU for more than four weeks after commencing the trial medication, one more stool sample will be collected prior to discharge.

All the relevant documents (with track changes and clean copies) have been enclosed with this letter. These amendments have been approved by the coinvestigators of the trial.

Yours Sincerely,
 A/Prof Shripada Rao (CPI) 
 Neonatologist; Princess Margaret Hospital for Children
 Subiaco, WA, 6008



Government of Western Australia
Child and Adolescent Health Service

Our Ref: 5469/2016086EP

A/Professor Shripada Rao
Neonatal Intensive Care Unit
Princess Margaret Hospital
Roberts Road
Subiaco WA 6008

Dear A/Professor Rao

AMENDMENT OF PROJECT APPROVAL

HREC Reference No:	2016086EP
Approval Expiry Date for this Project:	7/10/2019
Project Title:	Probiotic Supplementation in neonates with major gastrointestinal surgical conditions: A Pilot Randomized Double Blind Placebo Controlled Trial

Approval Date: 19/12/2017

Thank you for submitting your amendment. The Child and Adolescent Health Service Human Research Ethics Committee (CAHS HREC) received the amendment on 5/12/2017 and their review is now complete. The following modifications were included in this amendment and have been approved for use in this project:

New and/or Revised Project Documentation:
Protocol Version 4 dated 28 November 2017
Participant Information Sheet/Consent Form Version 4 dated 28 November 2017

It should be noted that all other aspects of the project approval remain unchanged, particularly in relation to the submission of progress reports and any further amendments to the protocol (National Statement S3.3.22 and S5.5).

The responsibility for the overall conduct of this project remains with you as the Co-ordinating Principal Investigator (CPI) and with the Principal Investigator (PI) at the site/s.

Please quote the HREC Reference for this project 2016086EP on all correspondence associated with this project.

I have been delegated the authority to approve this amendment on behalf of the CAHS HREC in accordance with Standard Operating Procedures.

Yours sincerely

Clinical Prof Catherine Choong
Chair, Scientific Advisory Sub-Committee
25/01/2018



Government of Western Australia
Child and Adolescent Health Service

I have reviewed this amendment and there are no governance implications for CAHS.

I have reviewed this amendment and the governance implications associated with this amendment have been addressed to the satisfaction of the CAHS Research Governance Office.

Signature: *Lesley Beaufield* Date: *25 JAN 2018*

The Child and Adolescent Health Service Human Research Ethics Committee is constituted, and operates in accordance with, the National Health and Medical Research Council's Statement on Ethical Conduct in Human Research, 2007 (Updated May 2015).

Rao, Shripada

From: GEKO Application <GEKO.Application@health.wa.gov.au>
Sent: Wednesday, 7 December 2016 7:16 AM
To: Batta, Vamsi; Rao, Shripada; Hughes, Nina; Whelan, Sue; Jervis, Julie; Vines, Pippa; Robertson, Tracy; Keating, Karen
Subject: Quality Activity 10446 Proposal Approved - Health Care Associated Blood Stream Infections (HABSI) in neonates with major abdominal surgical conditions: A retrospective cohort study

Quality Activity 10446 Proposal Approved

Activity Area	Quality improvement activity (NSQHSS/EQuIP)
Organisation	WNHS
Title of Activity	Health Care Associated Blood Stream Infections (HABSI) in neonates with major abdominal surgical conditions: A retrospective cohort study
Committee	QI Neonatology WNHS

Activity has been approved by **QI Neonatology WNHS** on **07-Dec-2016**. and has been flagged with the intent to publish in the future.

You may now commence your Quality Activity.

[NOTE: ACCESS TO PIMS](#)

This email provides evidence of approval for your access to medical records for this audit. Please print this email and take it with you to PIMS in order to obtain the relevant records.

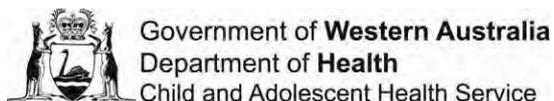
Further details are available online : [Quality Activity 10446](#).

For further assistance, please contact the [GEKO Administrator](#) for your site

Please do not respond to this email, it is automatically generated by the system and the address is not monitored.

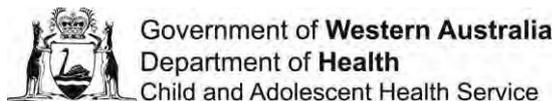
Appendix 2

Parent information sheet and consent form for the study titled *Gut Microbiota in Neonates with Congenital Gastrointestinal Surgical Conditions: A Prospective Study*

**NOTE:**

Information sheets are required for all participants in research studies. Information sheets are clear descriptions of the study and what will be required of the participant.

The information sheets should be written in appropriate language for the age of the participant therefore most studies will require a parent/guardian information sheet and a child information sheet.



PARENT INFORMATION SHEET

Project title: Gut Microbiota in neonates with major gastrointestinal surgical conditions: A prospective cohort study

Principal Investigator: A/Prof Shripada Rao, Neonatal consultant, Princess Margaret Hospital for Children and King Edward Memorial Hospital for Women, Subiaco, Western Australia

Student researcher: A/Prof Shripada Rao, Neonatal consultant, Princess Margaret Hospital for Children and King Edward Memorial Hospital for Women, Subiaco, Western Australia

Why are we doing the study?

The intestines of healthy breastfed newborn babies are colonised by the 'beneficial bacteria'. This colonisation is highly essential for the health and wellbeing of babies. The establishment of such healthy colonisation is facilitated by breastfeeding and the baby being in skin to skin contact with the mother.

Newborn babies who have major surgical conditions of the gut are at a disadvantage compared to healthy breastfed babies because of the following reasons: 1). they require staying in the intensive care unit and hence there will not be enough opportunity for skin to skin contact with the mother. 2). because they are sick, these babies usually do not receive breast milk in the first few days of life. 3). they receive antibiotics, which can kill these 'beneficial bacteria'.

All these factors can lead to the colonisation of the surgical baby's intestine by decreased number of beneficial bacteria and increased number of harmful bacteria. While such an outcome is likely, no researchers have examined intestinal bacteria in newborn babies with surgical conditions.

Hence, we are doing this study to assess the bacteria in the stools (poo) of newborn babies with surgical conditions of the gut and compare to that of stools from healthy breastfed babies. The bacteria in the stool sample are a good representative of bacteria in the intestines. We will also assess a special type of fatty acid called short chain fatty acids (SCFA) in the stools of your baby.

If our study confirms that the stools of these babies have increased number of harmful bacteria and decreased number of healthy bacteria, and abnormal SCFA concentrations, there will be scope for supplementing these babies with the beneficial bacteria, also known as probiotics.

Supplementation with such probiotic bacteria has been shown to be beneficial in premature babies and hence there is a possibility that they could be of benefit in babies with surgical problems of the gut also.

Who is carrying out the study? A/Prof Shripada Rao, Winthrop Professor Karen Simmer and Prof Sanjay Patole, who are newborn baby specialists at the Princess Margaret Hospital for Children and King Edward Memorial Hospital for Women, are carrying out this study. They are also researchers with the University of Western Australia. The results of this research project will be used by A/Prof Shripada Rao to obtain a Doctor of Philosophy at the University of Western Australia.

What will the study tell us? The study will tell us if the stools of newborn babies with surgical condition have predominantly beneficial bacteria or harmful bacteria.

Does my child have to take part?

No, it is not compulsory that your child has to take part in this study

What will you be asked to do if you decide to take part in this study?

Once you are satisfied with the information provided and decide to take part in the study, we would request you to sign the consent form. In addition, we would also request your permission to allow us to store the samples for five years. This will enable us to conduct more extensive bacterial and metabolic analyses on the stool samples using more sophisticated methods, when we get more research funding.

If your baby has a surgical condition: we will collect one stool sample of your baby within 24 hours of birth or as soon as possible after admission.. We will also collect another stool sample between day 10 and 14. If your baby is discharged home by day 10, we will request you to collect one stool sample (any day between 10 and 14) into a container provided by us and store it in the freezer compartment of your refrigerator. Our team will come and collect the sample on the same day.

If your baby is healthy without any surgical condition and breastfed: we will collect one stool sample of your baby within 24 hours of birth or as soon as possible after admission. We will also request you to collect one more stool sample (any day between 10 and 14) into a container provided by us, and store it in the freezer compartment of your refrigerator. Our team will come and collect the sample on the same day.

What does my child need to do to be in the study?

Your baby does not need to do anything to be in the study. The samples will be used for analysing the bacterial flora as well as SCFA at the laboratories at the University of Western Australia. The stool samples will also be stored for five years at -80 degree Celsius in a deep freezer located at the King Edward Memorial Hospital for Women, Perth, Western Australia. This will enable us to conduct more extensive bacterial and metabolic analyses on the stool samples using more sophisticated methods, when we get more research funding. At the end of the period, the stool samples will be disposed of as per the standard hospital protocol at that time.

Is there likely to be a benefit to my child?

Your child will not benefit by participating in this study.

Is there likely to be a benefit to other people in the future?

If this study confirms that babies with surgical conditions of the gut have less number of beneficial bacteria and more number of harmful bacteria in their stools, the next step for us will be to conduct a study to find out if supplementation with probiotics (beneficial bacteria) helps improve their outcomes.. If that study confirms the expected benefits, we will make probiotics available for routine use in such babies.

What are the possible risks and/or side effects?

There are no risks or side effects to your baby.

What are the possible discomforts and/or inconveniences?

There are no discomforts or inconveniences to your baby. There will be slight inconvenience to you, if you are required to collect the sample of stool on the day 10-14 of life at home and keep it in the freezer section for few hours, prior to our team picking it up.

Where is your information kept?

The information belonging to your baby will be kept in a secure database on the computer of Princess Margaret Hospital for Children.

What about my privacy?

No information related to you or your baby will be revealed to any external body.

Who has approved the study?

The research ethics committee of the Child and Adolescent Health Service has approved this study. They have also informed the research ethics committee of the Women and Newborn Health Services at King Edward Memorial Hospital for Women.

Who to contact for more information about this study:

If you would like any more information about this study, please do not hesitate to contact one contact one of the research team. They are very happy to answer your questions.

Name Dr Shripada Rao (PMH) Title A/Prof Contact Number: 0893408672
Dr Sanjay Patole (KEMH) Title Prof Contact Number: 0893401260

Who to contact if you have any concerns about the organisation or running of the study?

If you have any concerns or complaints regarding this study, you can contact the Director of Medical Services at PMH (Telephone No: (08) 9340 8222). Your concerns will be drawn to the attention of the Ethics Committee who is monitoring the study.

What to do next if you would like your child to take part in this research:

If you would like to take part in this research study, please read and sign the consent form provided.

THANK YOU FOR YOUR TIME

Appendix 3

Parent information sheet and consent form for the study titled *Probiotic supplementation in neonates with congenital gastrointestinal surgical conditions: A pilot randomised controlled trial*

Perth Children's Hospital, Western Australia

Participant Information Sheet/Consent Form – Parent/Guardian

Interventional Study - Parent/Guardian consenting on behalf of participant

Perth Children's Hospital

Project Title: Probiotic Supplementation in neonates with major gastrointestinal surgical conditions: A Pilot Randomized Double Blind Placebo Controlled Trial

Principal Investigator: A/Prof Shripada RAO, Neonatal Consultant

Student Researcher: A/Prof Shripada RAO

Associate Investigator(s): Prof Sanjay Patole, Prof Karen Simmer, Dr Ian Gollow, Dr Anthony Keil, Prof Patricia Conway

Location: Neonatal Intensive Care Unit, Perth Children's Hospital, Nedlands, Western Australia, 6008

1. What is the research about?

Newborn babies with major surgical conditions of the gut need admission to neonatal intensive care units. They undergo surgery, and receive intravenous drips and strong antibiotics. All these interventions interfere with the normal development of healthy bacteria in their intestines and enhance the proliferation of harmful bacteria. Such an imbalance between healthy and unhealthy bacteria in the gut can be harmful to the well-being of these babies. Administration of healthy bacterial supplements known as probiotics may prevent such an imbalance, thereby improving the outcomes. Probiotics are used routinely in premature babies (non-surgical) in our unit and many units in Australia, New Zealand and abroad, and have been shown to improve their outcomes. However, the role of probiotics in babies that have had surgery is not yet well studied.

In this study, we plan to give probiotics orally for 30 newborn babies with surgical conditions of the gut and placebo for 30 babies with similar surgical conditions. These supplements will be given once a day until discharge from our unit. A placebo is a medication with no active ingredients and without any medical benefit. We think that babies who receive probiotics will have better intestinal bacteria and overall health compared to those who receive placebo. The only definitive way one could prove if probiotics are beneficial (or not) is by conducting this type of research, which is known as randomized double blind placebo controlled trial. The results of this trial have the potential to improve the outcomes of newborn babies with surgical conditions.

As part of this study, we will collect 2-4 stool (poo) samples from your baby: The first one will be as soon as possible after admission but prior to commencing the trial medication. The second sample will be on any one day between day 7-9 days after commencing the trial medication. The third one will be on any one day between 14-16 days after commencing the trial medication. If your baby is in our unit for more than four weeks after commencing the trial medication, we will

collect one more stool sample prior to discharge. All samples will be collected only while your baby is admitted in our unit. In case of early discharge from our unit prior to the due date of collection, there will be no need for you to collect the samples at home. Initially, the samples will be kept in a deep freezer at King Edward Memorial Hospital, Subiaco, Western Australia. Subsequently they will be sent to an expert laboratory in Sydney where the stool samples will be analysed for bacterial flora and also special substances called short chain fatty acids using advanced lab technology.

The laboratory that performs these sophisticated analyses of stool samples is located at the University of New South Wales and headed by Prof Patricia Conway. Your baby's stools will not be sold by the University of New South Wales; however, lab will charge us a fee to recover the costs of analysing the stool samples. Any remaining stool samples will be stored in deep freezer at -80c in a deep freezer located at the King Edward Memorial Hospital for Women, Subiaco, Western Australia for five years. This will enable us to conduct more extensive analysis on the stool samples, when we get more research funding. At the end of the period, stool samples will be disposed of as per the standard protocol of the hospital.

The results of the current study will help us design a much larger study in the future. If that larger study confirms the benefits, probiotics will then be used routinely for all newborn babies with surgical conditions of the gut.

This study has received approval from the human research ethics committee of Princess Margaret Hospital.

The probiotic supplement (A combination of three bacteria called *B. breve M-16V*, *B. longum subsp. infantis M-63* and *B. longum subsp. longum BB536*) is an experimental treatment. It is not an approved treatment for newborn babies with major surgical conditions of the gut in Australia. This means that it must be tested to see if it is an effective treatment for these conditions.

2. Who is doing the research?

This research is a collaborative research between the neonatal and surgical teams of Perth Children's Hospital, Western Australia.

This research has been initiated by the study doctor, Dr/A/Professor Shripada Rao together with colleagues from the neonatal and surgical teams of Perth Children's Hospital, Perth, Western Australia.

The results of this research will be used by the study doctor A/Prof Shripada Rao to obtain a PhD (Doctor of Philosophy) degree from the University of Western Australia.

This research has been submitted for funding by the Raine Foundation.

3. Why is my baby invited to take part and what will they have to do?

Your baby is being invited to take part in the study because she/he has a major surgical condition of the gut, which is the primary focus of this research. Your baby does not need to do anything to be in the study. If you give consent, we will give the study supplement (probiotic or placebo) orally, once a day to your baby for the entire duration of his/her stay in our neonatal unit. This will be done by chance, like tossing a coin. Neither you nor the researcher can choose which group your baby goes in. For the length of the study neither the doctors nor you will know what group the baby is in. However, in certain exceptional circumstances, the study doctor can find out which treatment the participant is receiving.

We will collect his/her stool samples as described above and send to the laboratory in Sydney for analysis. We will also collect routine clinical information on your baby until she/he stays in our unit.

There will be no cost to you for taking part in this research and you will not be paid for taking part.

4. Does my baby have to take part in this research project?

Taking part in a research project is voluntary. It is your choice for your baby to take part or not. You do not have to agree if you do not want to. The standard of care of your baby will not be affected in anyway if you decide not to participate in the study or withdraw from the study.

5. What if I withdraw the baby from this research project?

If you decide to let your baby take part and then change your mind, that is okay, you can withdraw them from the project. You do not have to give us a reason; just tell us that you want your baby to stop. If you chose not to let your baby take part or start and then stop the study, it will not affect your baby's access to treatment or your relationship with the doctors and staff at PMH in any way. Your baby will continue to receive all the standard treatment required for their care. If you do withdraw your baby during the research project, the study doctor and relevant study staff will not use the baby's data in the analysis.

6. Are there any benefits to my baby from being in the research project?

There will be no clear benefit to the baby from their participation in this research.

7. Are there any risks, side-effects, discomforts or inconveniences from being in the research project?

Probiotic supplementation has been extensively studied in premature (non-surgical) babies. No major adverse effects attributable to the probiotic supplementation were noted in those studies. There are occasional case reports of premature babies developing bloodstream infection due to the probiotic organism itself, but they are very rare. Since probiotic use in newborn babies with

surgical conditions has not been well studied, we will monitor your baby very closely for such infections and treat them appropriately, if they occur.

Some babies develop abdominal distension and regurgitation with probiotics. Usually they are mild and babies get used to the supplementation quickly. If the problem becomes significant, we may have to stop giving the trial medication.

It is possible that your baby may develop a side effect that we do not expect or do not know about yet, and that this side effect may be serious.

8. Who will have access to my baby's information?

The following people will have access to the information we collect in this research: the research team and a representative of the PMH Ethics Office.

The information collected in this research will be re-identifiable (coded). This means that we will remove identifying information on any data or sample and replace it with a code. Only the research team have access to the code to match the data or sample to your baby's name, if it is necessary to do so. No personal information about your baby will leave the hospital. Any information we collect we will treat as confidential and only use in this project unless you have agreed to another use. We can let others know this information only if you say so or if the law says we must.

9. Will you tell us the results of the research?

We will write to you at the end of the research (in about 24 months) and let you know the results of the research. Results will not be individual, but based on all the information we collect and review as part of the research

10. Could this research project be stopped unexpectedly?

This research project may be stopped unexpectedly for a variety of reasons. These may include reasons such as:

1. Unacceptable side effects
2. While our research is underway, if a research done in other parts of the world with a very large sample size suggests that probiotic supplementation is harmful.

11. What happens if my baby needs emergency medical treatment while enrolled in the study?

If your baby suffers an accident or illness while at the hospital and requires emergency medical care, your baby will be offered all full and necessary treatment from the hospital. If the medical emergency occurs during your baby's participation in the study, the research team will ensure that the necessary medical care is given.

12. What happens next and who can I contact about the research?

If you decide to let your baby take part in this research, we will ask you to sign the consent form. By signing you are telling us that you understand what you have read and what has been discussed. Signing the consent indicates that you agree for your baby to be in the research project and have their health information used as described. Please take your time and ask any questions you have before you decide what to do. You will be given a copy of this information sheet and the consent form to keep.

The study doctor, A/Prof Shripada Rao can be contacted on 0893408672/0415508658 to answer any questions you have about your baby's participation in this research. All Research in Australia involving humans is reviewed by an independent group of people called a Human Research Ethics Committee (HREC). The ethical aspects of this research project have been approved by the HREC of Perth Children's Hospital, Western Australia.

This project will be carried out according to the *National Statement on Ethical Conduct in Human Research (2007)*. This statement has been developed to protect the interests of people who agree to participate in human research studies.

If you have any concerns and/or complaints about the project, the way it is being conducted or your baby's rights as a research participant, and would like to speak to someone independent of the project, please contact: The Executive Director of Medical Services at PCH on 6456 8222. Your concerns will be drawn to the attention of the Ethics Committee who is monitoring the study.

Consent Form – Parent/Guardian

Title: Probiotic supplementation in neonates with major gastrointestinal surgical conditions: A Pilot Randomized Double Blind Placebo Controlled Trial

Principal Investigator: A/Prof Shripada RAO

Associate Investigator(s): Prof Sanjay Patole, Prof Karen Simmer, Dr Ian Gollow, Dr Anthony Keil, Prof Patricia Conway

Student researcher: A/Prof Shripada RAO

- I have read, or had read to me, the information statement version listed above and I understand its contents.
- I believe I understand the purpose, extent and possible risks of my baby's involvement in this project.
- **I voluntarily consent to my baby taking part in this research project.**
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I understand that this project has been approved by Perth Children's Hospital Human Research Ethics Committee and will be carried out in line with the National Statement on Ethical Conduct in Human Research (2007) – updated March 2014.
- I understand I will receive a copy of this Information Statement and Consent Form.

OPTIONAL CONSENT: I do/ I do not consent to the storage and use of my baby's information in future ethically-approved research projects that are related to this project.

Baby's Name: _____

Parent's Name

Parent's Signature

Date

Declaration by researcher: I have supplied an Information Sheet and Consent Form to the participant who has signed above, and believe that they understand the purpose, extent and possible risks of their involvement in this project.

Research Team Member's Name

Research Team Member's Signature

Date

Note: All parties signing the Consent Form must date their own signature.

Interventional Study Participant Information Statement and Consent Form

Appendix 4

Thesis research papers presented at national and international conferences

Rao, Shripada

From: Abstract Submission 2019 Perinatal Society of Australia New Zealand
<noreply@xcdsystem.com>
Sent: Monday, 3 December 2018 1:47 PM
To: Rao, Shripada
Subject: PSANZ 2019 Abstracts



Dear Shripada,

We are delighted to advise that your abstract as detailed below has been accepted for an oral presentation in one of the 2019 PSANZ Congress Concurrent Abstracts Session.

Presentation Type: ORAL

Title: Gut Microbiota in Neonates with Congenital Gastrointestinal Surgical Conditions

ID#: 2019PSANZ128

Presenting Shripada Rao

Please note: This is a 10 minute oral presentation with an additional 5 minute question time (total 15 minutes).

Details of date and time for your oral presentation will be supplied shortly.

Registration:

- All presenting authors MUST be registered by December 14th 2018
- All presenting authors can access the early bird discount to the full conference registration if registered by December 14th 2018.
- If you have already registered and received the early bird discount no further discount will be applied.

You were provided with the opportunity to self-nominate for several PSANZ awards at the time of your submission. Thank you to those that did, we have your details and will ensure that judges are present at your presentation. However, we have noticed that a

Rao, Shripada

From: Abstract Submission 2019 Perinatal Society of Australia New Zealand
<noreply@xcdsystem.com>
Sent: Monday, 3 December 2018 1:38 PM
To: Rao, Shripada
Subject: PSANZ 2019 Abstracts



Dear Shripada,

We are delighted to advise that your abstract has been accepted for a Poster at the 2019 PSANZ Congress.

Poster displayed in poster showcase area in exhibition area on either the Monday/Tuesday OR Tuesday/Wednesday sessions.

Please note:

- Authors must be available to be at their poster during poster showcase sessions for judging & discussions.
- Posters for showcase area must be paper size 'AO Portrait'.

Please find below details of your accepted abstract:

Presentation Type: Poster

Title: Physical Growth Of Infants With Gastrointestinal Surgical Conditions During Stay In The Neonatal Intensive Care Unit

ID#: 2019PSANZ15

Presenting Shripada Rao

Rao, Shripada

From: Abstract Submission <noreply@xcdsystem.com>
Sent: Friday, 17 February 2017 7:36 AM
To: Rao, Shripada
Subject: PSANZ 2017 Abstracts



Dear Shripada,

Below please find details confirming your AO Poster Display which is to be hung in the poster showcase area of the exhibition arena at this year's PSANZ program.

There will be two poster hanging session times.

1. **Sunday 2nd 18:00 - Monday 3rd 19:30**
2. **Tuesday 4th 08:30 - Wednesday 5th 13:30**

Please note: Authors must be beside their poster during your 'Poster Viewing Sessions' for discussion. Your poster viewing time is outlined below.

Confirming your Poster Presentation details below:

Presentation Type: **Poster Only (AO size)**

Title: *Title: Probiotic supplementation in neonates with major gastrointestinal surgical conditions: A systematic review*

ID#: **PSANZ2017-86**

Date: **Monday April 03, 2017**

SessionTime: **5:45 PM - 7:15 PM**

Room: **Exhibition Hall**

Posters must be AO size, in Portrait orientation. Your hanging position is noted in the program, which you will be provided at registration.

Posters must meet these guidelines:

Rao, Shripada

From: contact-gutmicrob <contact-gutmicrob@inrae.fr>
Sent: Tuesday, 3 August 2021 9:13 PM
To: Rao, Shripada
Subject: Decision on abstract by the INRAE scientific committee of the 12th INTERNATIONAL SYMPOSIUM ON GUT MICROBIOLOGY

Importance: High

CAUTION External Communication: This email originated from outside of the organisation. Do not click links or open attachments unless you recognise the sender and know the content is safe.

Dear Shripada,

The INRAE scientific committee of the 12th INTERNATIONAL SYMPOSIUM ON GUT MICROBIOLOGY is pleased to inform you that your abstract has been accepted for a 15-minutes oral presentation.

The symposium will be held entirely online, using Converia Virtual Venue. To access the virtual venue, we remind you that you must register for the symposium via our website <https://gutmicrobiology-2021.symposium.inrae.fr/forms/registration>.

We are expecting to open the virtual venue on September 1st, 2021. Early in September, you will receive an email from Converia with a link to create your profile. Oral presentations will be held as a live stream of a Zoom meeting and will be strictly limited to 15 minutes, followed by 5 minutes for questions. We will send you an invitation to the Zoom meeting in a timely manner and will propose you a tryout. All platform presentations will be recorded and made available to registrants. If you do not want your talk to be recorded, please inform the organizing committee.

We are working on providing a program filled with innovative sessions as well as interactive opportunities. In addition to oral communications chosen by our scientific committee, we give the opportunity to all participants to vote and select posters for a short 3-minutes oral presentation. So, please connect to the virtual venue from September 18th to October 1st and vote for your preferred poster presentation!

Please notify us as soon as possible if you decide to withdraw your abstract for any reason or in the event that none of the authors can attend the meeting. If none of the authors registers for the meeting, the organizers may consider the abstract withdrawn.



Yours sincerely,

The INRAE Local Organizing Committee,

Email : contact-gutmicrob@inrae.fr

De : noreply@inrae.fr <noreply@inrae.fr>

Envoyé : samedi 5 juin 2021 11:30

À : shripada.rao@health.wa.gov.au

Objet : 12th INTERNATIONAL SYMPOSIUM ON GUT MICROBIOLOGY - Confirmation of submission of your abstract

Abstract : Probiotic Supplementation in neonates with gastrointestinal surgical conditions: A Pilot Randomized Double Blind Placebo Controlled Trial

Medical School Research Day Program

5th May 2021, Wednesday, 8:30 AM - 3:30 PM

McCusker Auditorium, Harry Perkins North, QEII Medical Centre

Session 1: HDR1 830-1000	Presenter	Title	Chair: Prof Anna Nowak
830-840	Welcome and arrangements for the day by Carl Schultz, <i>Chairperson</i> , Research Committee		
840-850	Brown, Rikki	<i>Axing Axl to Treat Drug Resistant Head and Neck Cancers</i>	
850-900	Dart, Sarah	<i>Donor lymphocytes are retained following tissue mismatched but not MHC mismatched solid organ transplantation</i>	
900-910	Allahham, Amira	<i>The effect of burn injuries on behaviour and the brain</i>	
910-920	Ong, Huan Ting	<i>Hypoxic Regulation of Wound Healing Activity from Adipose-derived Mesenchymal Stem Cells: Identifying Wound Healing Targets Using Ligand-Receptor Interactome</i>	
920-930	Alkhatib, Farah	<i>Abdominal Aortic Aneurysm Biomechanical Longitudinal Expansion (ABLE)</i>	
930-940	McGrath, Francesca	<i>Small nucleolar RNA networks are upregulated during human anaphylaxis</i>	
940-950	Rothzerg, Emel	<i>12 Survival-related differentially expressed genes based on the TARGET-osteosarcoma database</i>	
950-1000	Rao, Shripada	<i>Probiotic Supplementation in neonates with gastrointestinal surgical conditions: A Pilot Randomized Double Blind Placebo Controlled Trial</i>	
1000-1020	Morning Tea (Harry Perkins Foyer)		
Session 2: HDR2 1020-1200	Presenter	Title	Chair: Prof Markus Schlaich
1020-1030	Athikarisamy, Sam	<i>Clinical and Economical burden of Retinopathy of Prematurity in India</i>	
1030-1040	Carlin, Emma	<i>Risk and resilience: a mixed methods investigation of Aboriginal Australian women's perinatal mental health screening assessments</i>	
1040-1050	Jamieson, Emma	<i>Prediabetes and pregnancy: Early pregnancy glycosylated haemoglobin identifies Australian Aboriginal women with high-risk of gestational diabetes mellitus and adverse perinatal outcomes</i>	
1050-1100	Lee, Sing Ching	<i>Reproductive factors and breast arterial calcification - a systematic review and meta-analysis</i>	
1100-1110	Nolde, Janis Marc	<i>Capillary vascular density in the retina of hypertensive subjects is associated with a non-dipping pattern independent of mean ambulatory blood pressure</i>	
1110-1120	Ying, Qidi	<i>Mechanisms for the reduction in lipoprotein(a) with PCSK9 inhibition</i>	
1120-1130	Almutairi, Khalid Bander	<i>The Temporal Association Between Hospital Admissions and DMARD Usage in Rheumatoid Arthritis Patients</i>	
1130-1140	Bertot, Luis Calzadilla	<i>ABIDE: an accurate predictive model of liver decompensation in patients with non-alcoholic fatty liver-related cirrhosis</i>	
1140-1150	Vo, Uyen Giao	<i>Controlling the controls: What is negative pressure wound therapy compared to in clinical trials?</i>	
1150-1155 (mini)	Chakraborty, Anindita	<i>Effectiveness of proprotein convertase subtilisin/kexin-9 monoclonal antibody treatment on plasma lipoprotein(a) in patients with elevated lipoprotein(a) attending a clinic</i>	
1155-1200 (mini)	Reid, Allison	<i>Is there a case for targeted congenital cytomegalovirus screening in Western Australia?</i>	
1200-1230	Lunch (Harry Perkins Foyer)		
Session 3: Senior Researchers 1230-1335	Presenter	Title	Chair: Prof Trevor Mori
1230-1250	Parizel, Paul/ Francis, Roslyn	<i>Expanding the capacity for imaging research in WA</i>	
1250-1300	Clynick, Britt	<i>Biomarker Signatures for Progressive Idiopathic Pulmonary Fibrosis</i>	

Rao, Shripada

From: Ben Thompson <ben@corporatecommunique.com.au>
Sent: Friday, 13 May 2016 5:08 PM
To: Rao, Shripada
Subject: PSANZ 2016 Oral Presentation



Perinatal Society of Australia and New Zealand

Abstracts Gallery Partner



Dear Shripada Rao

Confirming your presentation details below:

Abstract ID#: 227761886

Title: *Probiotic Supplementation and Late-Onset Sepsis in Preterm Infants: A Meta-Analysis*

Presentation type: Oral presentation 10min (plus 5 min question time)

Presentation Date: Tuesday 24th May 1330-1500 session

1 Feeding & Nutrition #1

Oral Presentations guidelines and 'tips':

Appendix 5

Papers published during PhD enrolment that constituted thesis chapters



CLINICAL RESEARCH ARTICLE

Gut microbiota in neonates with congenital gastrointestinal surgical conditions: a prospective study

Shripada C. Rao^{1,2}, Meera Esvaran³, Sanjay K. Patole^{1,2}, Karen N. Simmer^{1,2}, Ian Gollow⁴, Anthony Keil⁵, Bernd Wemheuer³, Liwei Chen⁶ and Patricia L. Conway^{3,6}

BACKGROUND: There is limited information on gut microbiota of neonates with congenital gastrointestinal surgical conditions (CGISCs) available.

METHODS: This study compared stool microbiota and short-chain fatty acids (SCFAs) of 37 term infants with CGISCs with 36 term healthy infants (HIs). Two stool samples were collected from each infant: as soon as possible after birth (week 1) and 10–14 days of life (week 2).

RESULTS: Bacterial richness and alpha diversity were comparable between CGISCs and HIs at week 1 and week 2 (all $p > 0.05$). Beta diversity analysis revealed that at week 1, CGISCs had similar community structures to HIs ($p = 0.415$). However, by week 2, community structures of CGISCs were significantly different from HIs ($p = 0.003$). At week 1, there were no significant differences in the relative abundances of genera *Bifidobacterium* and *Bacteroides* between CGISCs and HIs. At week 2, the relative abundance of *Bifidobacterium* was significantly lower in CGISCs (mean percentage 7.21 ± 13.49 vs. 28.96 ± 19.6 ; $p = 0.002$). *Bacteroides* were also less abundant in the CGISC group (mean percentage 0.12 ± 0.49 vs. 6.59 ± 8.62 ; $p = 0.039$). Relative abundance of genera *Pseudomonas* and *Escherichia-Shigella* were higher in CGISCs. At week 2, stool concentrations of all SCFAs were lower in CGISCs (all $p < 0.001$).

CONCLUSIONS: During hospitalization, neonates with CGISCs develop gut dysbiosis and deficiency of SCFAs.

Pediatric Research (2020) 88:878–886; <https://doi.org/10.1038/s41390-020-0824-7>

IMPACT:

- During hospitalisation, neonates with congenital gastrointestinal surgical conditions develop gut dysbiosis with deficiency of Bifidobacteria and Bacteroides and increased abundance of Escherichia-Shigella and Pseudomonas. They also have low levels of short chain fatty acids in their stools compared to healthy infants.
- This is the first study evaluating the gut microbiota using 16S ribosomal RNA sequencing methods and stool short chain fatty acids in neonates with congenital gastrointestinal surgical conditions and comparing them to healthy infants.
- The findings of this study will pave the way for randomised trials of bifidobacterial supplementation in neonates with congenital gastrointestinal surgical conditions.

INTRODUCTION

The major congenital gastrointestinal surgical conditions (CGISCs) are gastroschisis, exomphalos, duodenal atresia, small and large intestinal atresia, oesophageal atresia, congenital short bowel syndrome (SBS), malrotation and volvulus, meconium ileus, hypoplastic left colon, Hirschsprung disease (HD), anorectal malformations and others.

Common morbidities in these conditions are feed intolerance and increased risk of infections. Infants with CGISCs are cared for in intensive care units and do not receive breastmilk in the first few days of life. They undergo invasive procedures and do not receive adequate skin-to-skin contact with their mothers. They get exposed to gastric acid suppressants, parenteral nutrition and

multiple courses of antibiotics. All these factors have the potential to increase the risk of gut dysbiosis.^{1–3}

While the gut microbiota of extremely preterm non-surgical infants has been well studied using culture-independent genomic approaches,⁴ there is very limited information on gut microbiota of term infants with CGISCs. The studies that evaluated gut flora in neonates with surgical conditions in the past were based on the conventional culture-dependent techniques.⁵ However, a growing body of evidence in the recent decade has shown the importance of culture-independent, genomic approaches in understanding the role of the human microbiota in health and disease.⁶ Hence, we conducted this prospective study to investigate the gut microbiota in term neonates with CGISCs using culture-independent techniques.

¹Neonatal Intensive Care Unit, Perth Children's Hospital and King Edward Memorial Hospital for Women, Perth, WA, Australia; ²Centre for Neonatal Research and Education, University of Western Australia, Perth, WA, Australia; ³Centre for Marine Science and Innovation at the University of New South Wales (UNSW), Sydney, NSW, Australia; ⁴Department of Paediatric Surgery, Perth Children's Hospital, Perth, WA, Australia; ⁵PathWest Laboratory Medicine, Perth, WA, Australia and ⁶School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore, Singapore

Correspondence: Shripada C. Rao (shripada.rao@health.wa.gov.au)

Received: 17 May 2019 Revised: 31 December 2019 Accepted: 1 January 2020

Published online: 16 March 2020

Many of the biological functions of healthy gut microbiota are mediated via short-chain fatty acids (SCFAs), such as acetic acid, butyric acid and propionic acid. SCFAs have important biological functions in humans, such as immune modulation, anti-inflammatory, anti-tumorigenic and antimicrobial effects and enhancement of gut integrity.⁷ They are thought to play a key role in microbiota–gut–brain crosstalk.⁸ In infants, SCFAs are the by-products of fermentation of human milk oligosaccharides (HMOs) by the anaerobic bacteria in the colon. HMOs are not digested by the small intestine of infants and hence they reach the colon intact, where they are utilized as nutrition by bacteria such as *Bifidobacteria* and *Bacteroides*. Since not all bacteria have the necessary enzymes to utilize HMOs, these milk glycans facilitate the establishment of a highly specialized microbial ecosystem dominated by *Bifidobacteria* and *Bacteroides* among others, while indirectly limiting growth of other bacteria.⁹ It is possible that neonates with CGISCs have insufficient amounts of anaerobes such as *Bifidobacteria* and *Bacteroides*, which in turn could result in decreased utilization of HMOs, and hence lower amounts of SCFAs in the colon. Given the importance of SCFAs in human health and the interplay between gut microbiota and SCFAs,⁷ we investigated stool SCFA levels in these infants.

METHODS

This was a prospective cohort study in which neonates with CGISCs were recruited from the neonatal intensive care unit of Perth Children's Hospital (PCH) and healthy infants (HIs) were recruited from the postnatal ward of King Edward Memorial Hospital (KEMH), Perth, Western Australia.

The study was approved by the institutional human research ethics committees of both hospitals. Informed parental consent was obtained for all studied infants.

Eligibility criteria

Neonates (≥ 36 weeks) with gastroschisis, exomphalos, Hirschsprung disease, duodenal atresia, other intestinal atresia, congenital diaphragmatic hernia, oesophageal atresia, congenital SBS and conditions needing enterostomy. Controls were healthy term newborn infants (≥ 36 weeks). We chose to include infants born at 36 weeks also instead of the conventional definition of 37 weeks because many infants with surgical conditions are born at 36 weeks and would not have been eligible for inclusion, thereby resulting in difficulty in achieving the sample size. Preterm infants < 36 weeks were excluded to avoid the confounding effect of prematurity, which itself is a significant risk factor for gut dysbiosis. Even though congenital diaphragmatic hernia is not truly a gastrointestinal (GI) condition, we decided to include it because the intestines are not in the abdominal cavity throughout pregnancy in this condition.

Outcomes

Stool microbiota using 16S ribosomal RNA gene sequencing, and SCFAs using modified gas chromatography-mass spectrometry (GC-MS) were measured on samples collected as soon as possible after birth/admission (week 1) and 10–14 days of life (week 2).

Stool sample collection

Two stool samples were collected in sterile containers from each consented infant. The first sample was collected as soon as possible after birth/admission (week 1) and the second sample was taken between 10 and 14 days of life (week 2). All neonates with CGISCs were inpatients at the time of week 1 as well as week 2 sample collection. The week 1 samples of healthy term infants were collected while in hospital, whereas the week 2 samples were collected in sterile containers using sterile spatula at home by parents and kept in the refrigerator at home. Parents were advised to do thorough hand washing before collecting the

samples and to screw the lid tightly immediately after collection to prevent contamination. Accredited couriers retrieved the samples from infants' homes in cooler bags with ice packs within 48 h of collection by parents. Subsequently, all stool samples were initially stored at 20 °C for 3–5 days and subsequently at 80 °C. At the completion of recruitment of all study participants, samples were shipped on dry ice (carbon dioxide) to the University of New South Wales (Sydney, Australia), where microbial analysis was undertaken. Acidified samples were shipped on dry ice to the School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore, where SCFA analysis was carried out.

DNA extraction

DNA was extracted from stool samples using the method of Matsuki et al.¹⁰ Briefly, stool samples were thawed on ice, diluted 10-fold with sterile water and bacterial cells harvested by centrifugation. DNA was subsequently extracted from the bacterial cells using chemical and physical lysis methods. DNA was stored at 80 °C.

PCR amplification and 16S rRNA sequencing

Polymerase chain reaction (PCR) amplification of the V3–V4 region of the 16S ribosomal RNA (rRNA) gene was conducted using the 341 F' (5'-CCTACGGGNGGCWGCAG-3') and 785 R' (5'-GACTAC HVGGGTATCTAATCC-3') primers with the Nextera indexes. Purified PCR products were submitted to the Ramaciotti Centre for Genomics (UNSW, Sydney, Australia) for library preparation and sequencing on the Illumina MiSeq platform using the MiSeq Kit v3 (2 × 300 cycles).

rRNA sequence analysis

16S rRNA sequence data were initially quality filtered and trimmed using TRIMMOMATIC VERSION 0.36 truncating reads if the quality was found to be below 12 in a sliding window of 4 bp. Reads shorter than 100 bp were discarded after quality trimming. USEARCH version 11.0.667 was used to merge forward and reverse reads between 350 and 550 nucleotides. Primer sequences were truncated with cutadapt version 2.5.¹¹ Reads with no detectable primers were removed from the further analysis. Afterwards, reads were quality filtered using USEARCH. All reads with an expected error of more than 2 and more than 1 ambiguous base were removed. All sequences of all samples were concatenated in a single file and subsequently dereplicated to form unique sequences. Unique sequences were clustered into zero-radius operational taxonomic units (zOTUs, also called ASVs) using the UNOISE3 algorithm implemented in USEARCH. Chimeras were removed de novo during clustering and in reference mode using the UCHIME2 algorithm together with the SILVA SSURef NR99 v132. Processed, concatenated sequences were mapped on the final set of zOTUs to determine their occurrence and abundance in each sample using the `otu` tab command with an identity cut-off of 97% and termination options disabled, which means that every sequence is searched against every zOTU to find the best hit. Taxonomy was assigned to each zOTU using the SINA aligner version 1.6¹² and the SILVA SSURef NR99 v132 database.

For alpha diversity measures, each sample was subsampled 100 times to a count of 25,400 counts per sample and the average was taken. OTU richness and diversity indices, Shannon, ACE and Chao1, were calculated in R (version 3.5.1) using the *vegan* package. Relative abundance analysis at the Phylum, Family and Genus levels were carried out using *phyloseq* package in R. Data were visualized using *ggplot2* and *ggpubr* packages.

For beta diversity analysis, data were square root transformed. To generate a phylogenetic tree for diversity computations, zOTUs were aligned with *muscle* (version 3.8.31)¹³ and the tree was calculated with *RaxML* (version 8.2.10)¹⁴ using the GTRGAMMA model. Weighted unifracs distances were calculated and visualized on a principal coordinate analysis plot.

Statistical considerations

At the time of commencing this study, to our knowledge, there were no studies that had evaluated gut microbiota in neonates with CGISCs using culture-independent techniques. In the absence of baseline data, we aimed to study 35 neonates with CGISCs and 35 healthy term infants. Since the gut microbiota changes rapidly in the first few months of life, we attempted to collect the stool samples from cases and respective controls within ± 2 days of each other.

Statistical analysis of clinical data

Summary data for continuous variables with normal distribution were reported using mean \pm SD. Median and range were used to report data with skewed distribution. Continuous variables were compared using the *t* test for normally distributed data and Wilcoxon' rank-sum test for skewed data. Binary outcomes were compared using the Fisher's exact test. A *p* value of <0.05 was considered statistically significant.

Statistical analysis of microbiota data

All data analyses were conducted with R version 3.5.1. For microbial richness, linear mixed model effects (LME) test (*MASS*, *lme4* and *lmerTest* packages) was used to identify if there were statistical differences between the groups over time as well as between the groups at the two timepoints. In our model, Patient ID was a random factor, while time and treatment were used as fixed factors. Post hoc pairwise comparisons between the groups were performed using Tukey's HSD (honestly significant difference) method to adjust for multiple comparisons.

Differential abundance of phyla and genera were examined using the Wilcoxon's rank-sum test.

For beta diversity, PERMANOVA (permutational multivariate analysis of variance) was used to check if community structures differed between the groups at the two timepoints followed by pairwise.adonis test (<https://github.com/bwemheu/pairwise.adonis>) for pairwise comparisons between the groups. *P* values were adjusted for multiple testing using the Benjamini–Hochberg correction.

For all analyses, a *p* value of <0.05 was considered statistically significant.

Quantification of SCFAs in faecal samples of study infants

Faecal SCFA analyses were performed using a modified GC-MS method.¹⁵ Acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid and 4-methyl valeric acid were purchased from Sigma-Aldrich (Merck, Singapore). First, 1 g of the faecal sample was suspended in 5 mL of 1% phosphoric acid and frozen at -20°C immediately after collection. Once thawed, the faecal suspensions were homogenized with vortex. Then, 100 μL of 10% meta-phosphoric acid solution was added to 0.5 mL baby faecal sample to adjust the pH to about 2.0. Samples were vortexed for about 10 min and centrifuged for 30 min at $20,817 \times g$, 4°C to solidify the precipitate. After that, 0.5 mL of aqueous supernatant was transferred into a new tube; 4-methyl valeric acid was added as internal standard (IS) to a final concentration of 500 μM . Then, 250 μL of ethyl acetate was added to extract SCFAs with a vortex for about 30 min and centrifuged for 20 min at $20,817 \times g$; lastly, 50 μL volume of organic extracts were transferred into GC glass vial for GC-MS analysis. One microliter of organic extracts was injected into GC-MS system (Agilent Technologies 7890B-5977B) equipped with a HP-FFAP capillary column (30 m \times 0.250 mm \times 0.25 μm ; Agilent). Helium was used as the carrier gas at 1 mL/min. Split ratio is 30:1. The oven temperature was initially held for 1 min at 80°C , then increased to 120°C at $20^{\circ}\text{C}/\text{min}$, and finally to 210°C at $6.13^{\circ}\text{C}/\text{min}$ and held for 2 min. The temperature of the injector, ion source, quadrupole, and interface were 250, 230, 150, and 280°C respectively. The SCFA data analyses were performed in duplicate. Identification of the SCFAs was based on the retention time of standard compounds and with the assistance

of the NIST 17 library. Quantifications were carried out in selected ion monitoring acquisition mode in MassHunter Acquisition software with base peak ion selected as quantifier for each compound. The calibration graphs were constructed in MassHunter Quantitative software (version B.09.00) by plotting the relative response (ratio of peak area SCFAs/peak area IS) vs. relative concentration for each individual SCFAs. The final SCFA concentrations were expressed as microgram per gram wet weight faecal sample. Since the data were not of normal distribution, Wilcoxon's rank analysis was performed to compare SCFA concentrations between the two groups (CGISCs and HIs).

Linearity and sensitivity

A stock solution containing the mixture of standards (20 mM final concentration each) in ethyl acetate was diluted to obtain a calibration curve ranging from 2 to 15,000 μM . IS was added to each diluted standards mixture (500 μM final concentration).

The calibration graphs were constructed by plotting the ratio peak area SCFAs/peak area IS vs. concentration for each individual SCFAs. By normalizing the peak area to that of the IS, the variability in the instrument response was corrected (in particular, the injection volume variability and the MS response). Each point of the calibration graph corresponds to the mean value from independent replicate injections.

The limits of detection (LOD) and limits of quantification (LOQ) of the individual analytes were obtained by injecting successively more diluted standard solutions and were calculated according to the International Union of Pure and Applied Chemistry¹⁶ method based on a signal-to-noise ratio of 3 for the LOD and of 10 for the LOQ.

Reporting

STROBE checklist was followed for reporting the results of this observational study.¹⁷

RESULTS

In total, 37 CGISCs and 36 HIs were recruited into the study. The surgical conditions in the CGISC group were oesophageal atresia: 4; gastroschisis: 9; malrotation: 4; duodenal atresia: 4; small intestinal atresia: 2; colonic atresia: 1; imperforate anus: 2; HD: 3; meconium ileus needing enterostomy: 2; and congenital diaphragmatic hernia: 6. The relevant clinical details are given in Table 1.

For microbial analysis at week 1, 36 stool samples from CGISCs and 25 samples from HIs were available; at week 2, 32 stool samples from CGISCs and 17 samples from HIs were available.

For SCFA analysis at week 1, 35 stool samples from CGISCs and 23 samples from HIs were available; at week 2, 30 stool samples from CGISCs and 17 samples from HIs were available.

Microbial analysis

For CGISCs, the total number of reads were 2,904,691 (median 76,125; range: 34,601–116,020) at week 1 and 2,313,892 (median 73,228; range: 25,460–126,106) at week 2. For HIs, total reads were 2,424,727 (median 80,631, range: 49,494–131,223) at week 1 and 1,247,024 (median 74,409, range: 54,056–104,347) at week 2.

Richness

Week 1 samples (CGISC vs. HI): There were no statistically significant differences in the number of OTUs between neonates with CGISCs and HIs at week 1 (mean OTU: 121 vs. 100; $p = 0.07$) (Fig. 1a).

Week 2 samples (CGISCs vs. HIs): There were no statistically significant differences in the number of OTUs between neonates with CGISCs and HIs (mean OTU: 83 vs. 73; $p = 0.82$) (Fig. 1a).

Bacterial richness decreased significantly in both CGISC and HI groups from week 1 to week 2 ($p < 0.001$ and $p = 0.048$, respectively) (Fig. 1a).

Table 1. Baseline clinical data of surgical and healthy infants.

	Neonates with CGISCs (N 37)	Healthy term infants (N 36)	P value
Gestational age (weeks)	37.2 ± 1.2	38.9 ± 1.3	<0.0001
Birth weight (weeks)	2946 ± 489.9	3344.9 ± 399.8	0.0002
Female, N (%)	13 (35%)	15 (42%)	0.634
Maternal pregnancy induced hypertension	3 (8%)	0 (0%)	0.240
Chorioamnionitis	2 (5.4%)	0 (0%)	0.493
Antepartum haemorrhage	1 (2.7%)	0 (0%)	1.000
Caesarean section, N (%)	16 (43.2%)	12 (33%)	0.472
Apgar at 5 min	9 (IQR: 9 9; range: 5 10)	9 (IQR: 9 9; range: 8 10)	0.026
Age at admission (days)	1 (IQR: 1 2; range: 1 9)	1 (IQR: 1 1; range: 1 1)	<0.0001
Age at initial surgery (days)	4 (IQR 2 7; range: 1 15)	NA	NA
Day of life enteral feeds commenced	6 (IQR: 3 9; range: 1 18)	1 (IQR: 1 1; range: 1 1)	<0.0001
Time to full enteral feeds (days)	15 (IQR:9 25; Range: 4 65)	1 (IQR: 1 1; range: 1 1)	<0.0001
Duration of parenteral nutrition (days)	13 (IQR: 7 24; range: 1 62)	0	<0.0001
Duration of antibiotic therapy (days)	10 (IQR: 6 21; range: 2 64)	0	<0.0001
Duration of ventilator support (h)	55 (IQR:38 137; range: 0 616)	0	<0.0001
Duration of hospital stay (days)	22 (IQR: 16 38; range: 6 167)	3 (IQR: 2 4; range: 1 7)	<0.0001
Use of proton pump inhibitors	15 (40.5%)	0	<0.0001
Use of H2 receptor blockers	0	0	NA
Number of surgeries during NICU stay	1 (IQR: 1 2; range: 1 5)	NA	NA
Mortality	0	0	NA
Early onset sepsis	0	0	NA
Hospital acquired blood stream infection (HABSI) ^a	8 (21.6%)	0	0.005
Organisms causing HABSI	CONS: 4, <i>E. cloacae</i> : 1, <i>E. coli</i> and <i>E. fecalis</i> : 1; CONS and <i>E. fecalis</i> : 1, <i>E. coli</i> : 1	NA	NA
Use of breastmilk	32 (89%)	26 (72%)	0.135
Day of life at collection of first stool sample	4 (IQR: 2 6; range: 1 10)	2 (IQR: 2 3; range: 1 6)	<0.0001
Day of life at collection of second stool sample	13 (IQR:12 15; range: 12 19)	13 (IQR:12 15; range: 10 17)	0.621

Data are given as mean ± SD or median (IQR; range) or number (%).
^aPositive blood culture on a sample collected 48 h after admission to the NICU.

Alpha diversity. Alpha diversity in the study samples was measured using three different measures: Shannon, Chao1 and abundance-based coverage estimators (ACE) (Fig. 1b–d).

Week 1 samples (CGISCs vs. HIs): All alpha diversity measures showed no statistically significant differences between neonates with CGISCs and HIs at week 1 Shannon index (2.33 vs. 1.96; $p = 0.14$).

Week 2 samples (CGISCs vs. HIs): All alpha diversity indices showed no statistically significant differences between neonates with CGISCs and HIs at week 2 (Shannon index: 2.91 vs. 2.00; $p = 0.94$).

Alpha diversity decreased significantly in CGISCs from week 1 to week 2 ($p = 0.014$), but not in HIs ($p = 0.998$).

Beta diversity. Weighted Unifrac and Bray–Curtis distances were used to assess the beta diversity between groups.

Week 1 samples (CGISCs vs. HIs): The microbial community structures of neonates with CGISCs were comparable to HI on both weighted Unifrac and Bray–Curtis measures ($p = 0.415$ and 0.241, respectively) (Fig. 2a, c).

Week 2 samples (CGISCs vs. HIs): The microbial community structures of neonates with CGISCs were significantly different

from HI on both weighted Unifrac and Bray–Curtis measures (both $p = 0.003$) (Fig. 2b, d)

Relative abundance of bacteria at the phylum level. In both neonates with CGISCs and HIs, bacteria belonging to the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria accounted for more than 99.5% of all bacteria. Cyanobacteria, Deinococcus-Thermus, Fusobacteria, Planctomycetes and Verrucomicrobia accounted for the remaining 0.5%.

Comparison of relative abundance of major phyla on week 1 samples (CGISCs vs. HI): The levels of the four major phyla were comparable between CGISC and HI groups at week 1 (all $p > 0.05$; Fig. 3a).

Comparison of relative abundance of phyla on week 2 samples (CGISCs vs. HIs): CGISC group had significantly less Actinobacteria and Bacteroidetes ($p < 0.0001$ and < 0.001 , respectively) than HIs. CGISCs and HIs had comparable levels of Firmicutes. The stools of neonates with CGISCs had significantly more Proteobacteria compared to HIs ($p = 0.003$) (Fig. 3b).

Relative abundance at the genus level

Comparison of relative abundance of major genera on week 1 samples (CGISCs vs. HIs): CGISC infants had significantly more

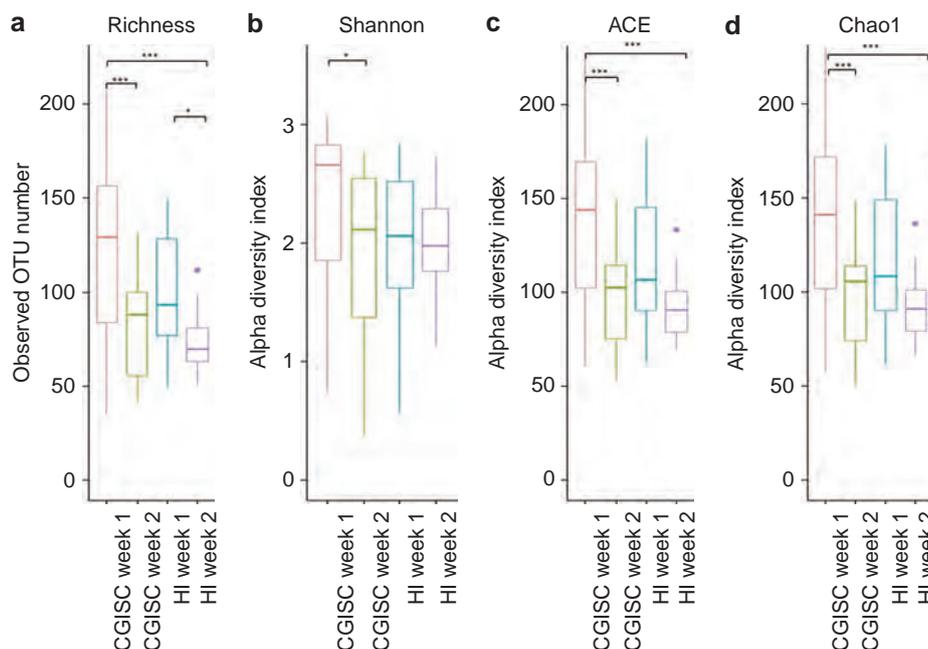


Fig. 1 Richness and alpha diversity measures of faecal microbiota in the study infants. The faecal microbiota of CGISC infants demonstrated significant decrease in bacterial richness and alpha diversity shown by Shannon index, ACE and Chao1 from week 1 to week 2 ($p < 0.05$), while the HI infants exhibited significant decrease in only bacterial richness ($p < 0.05$) (* $p < 0.05$; *** $p < 0.001$).

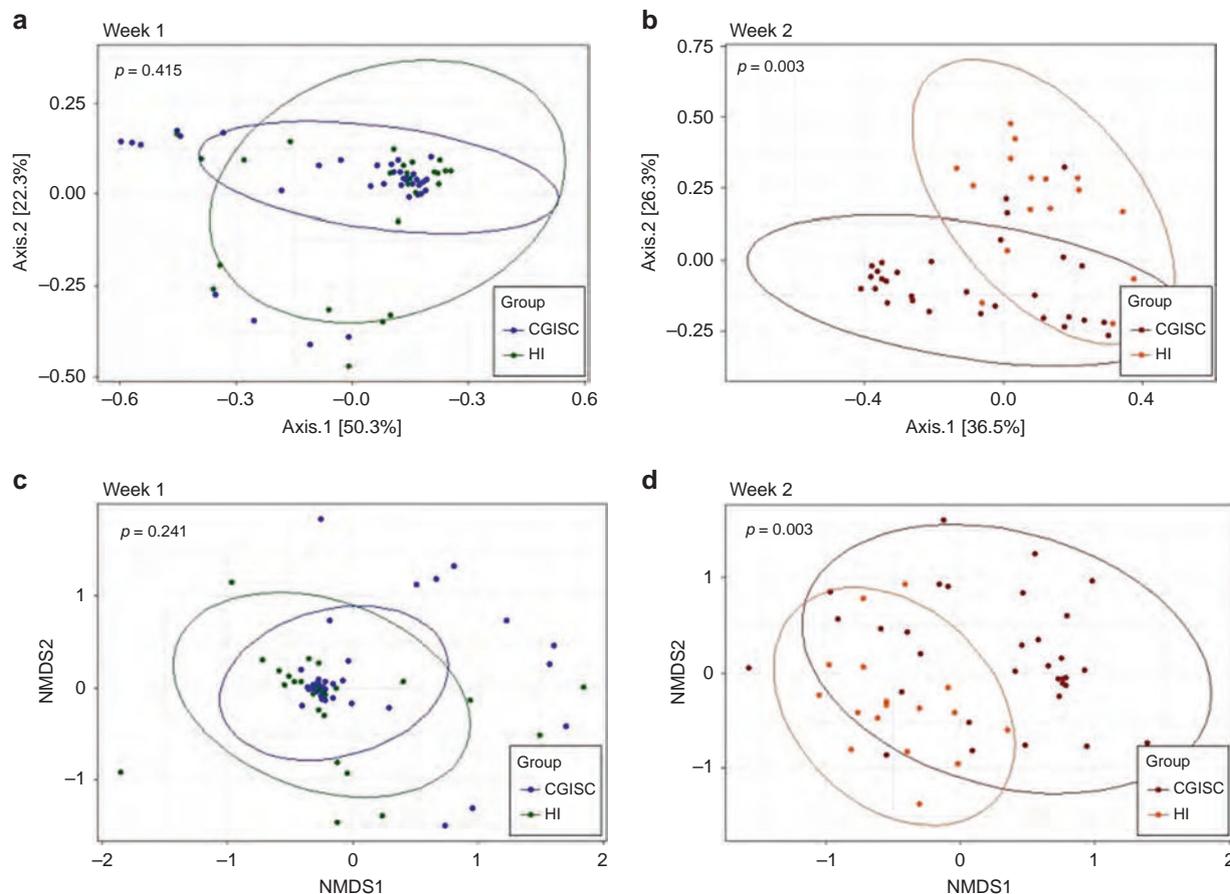


Fig. 2 Beta diversity measures in the study infants. Principal coordinate analysis plots of weighted Unifrac distance of the infants at week 1 (a) and week 2 (b). NMDS plots on Bray-Curtis dissimilarity at week 1 (c) and week 2 (d) of the infants. At week 1, HI and CGISC infants had similar community structures (a, c). However, at week 2, HI had significantly different community structure compared to CGISC infants (b, d).

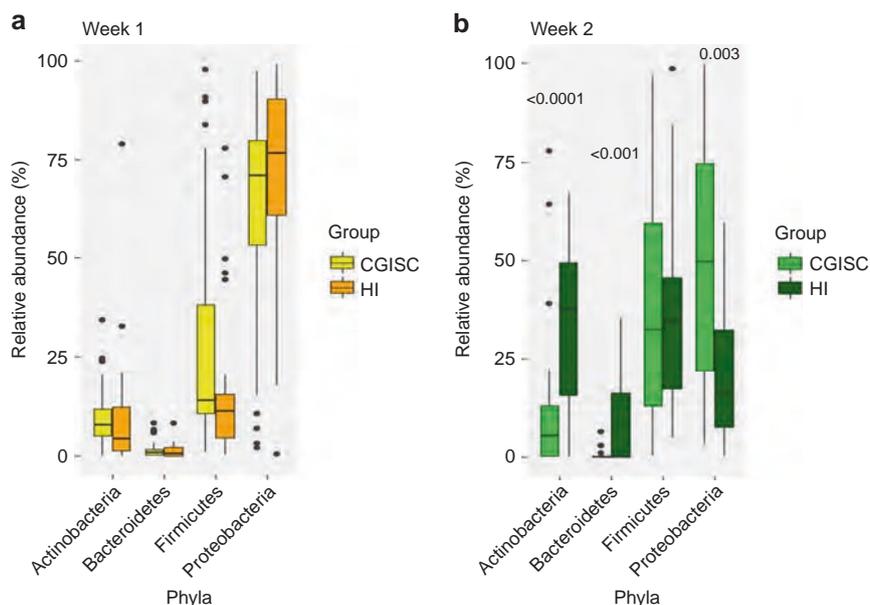


Fig. 3 Relative abundance of the top four phyla in the study infants. Both CGISC and HI infants have similar levels of the four phyla at week 1. However, at week 2, CGISC infants are significantly enriched for Proteobacteria and lower in abundance for Actinobacteria and Bacteroides.

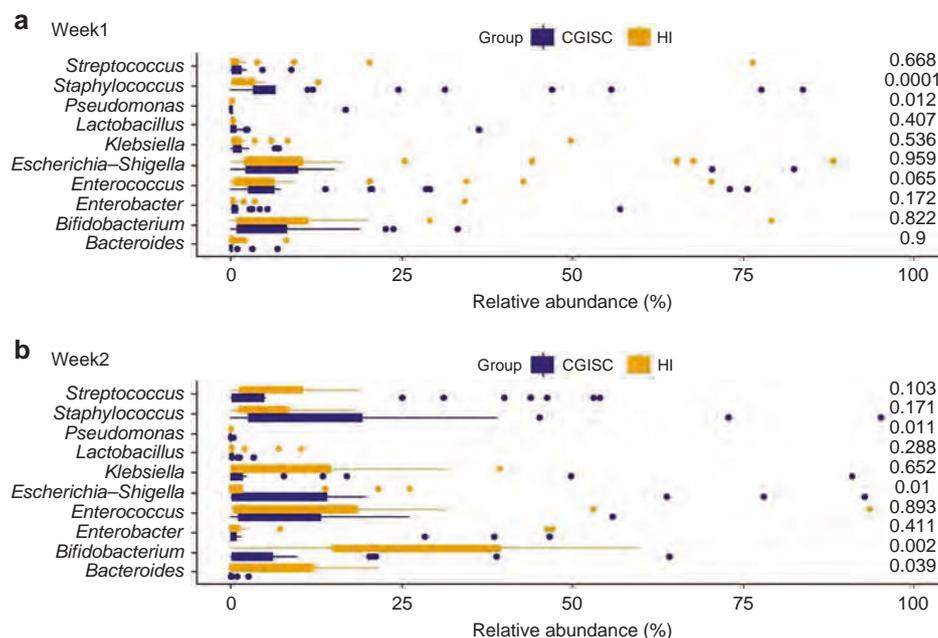


Fig. 4 Comparison of various genera in study infants. CGISC infants have significantly increased levels of *Staphylococcus* and *Pseudomonas* in week 1 compared to HI infants. At week 2, CGISC infants have significantly increased *Pseudomonas* and *Escherichia Shigella*, while HI infants are significantly enriched for *Bifidobacterium* and *Bacteroides*.

Staphylococcus ($p = 0.001$) and *Pseudomonas* ($p = 0.012$) than HI infants at week 1 (Fig. 4 and Supplementary Table S1). There were no significant differences between the groups for the other important bacterial genera (Fig. 4 and Supplementary Table S1).

Comparison of relative abundance of major genera on week 2 samples (CGISCs vs. HI): CGISC infants had significantly lower abundance of *Bifidobacterium* ($p = 0.002$) and *Bacteroides* ($p = 0.039$) and significantly higher abundance of *Escherichia-Shigella* ($p = 0.01$) and *Pseudomonas* ($p = 0.011$) than HIs (Fig. 4 and Supplementary Table S1). There were no significant differences

between the two groups for other genera such as *Staphylococcus*, *Enterococcus*, *Enterobacter*, *Klebsiella* and *Streptococcus* (Fig. 4 and Supplementary Table S1).

Results of SCFA analysis

The total SCFA levels were significantly lower in the CGISC group at week 1 (median 407.7, range: 302.2–696.1 $\mu\text{g/g}$ of wet faeces vs. 1208.2, range: 1036.5–6846.9, $p < 0.0001$) as well as at week 2 (median 410.2, range: 300.1–664.1 vs. 1750.6, range: 1046.8–7781.7, $p < 0.0001$) (Fig. 5). Analysis of individual SCFAs found that there were lower levels of acetic acid, isobutyric acid,

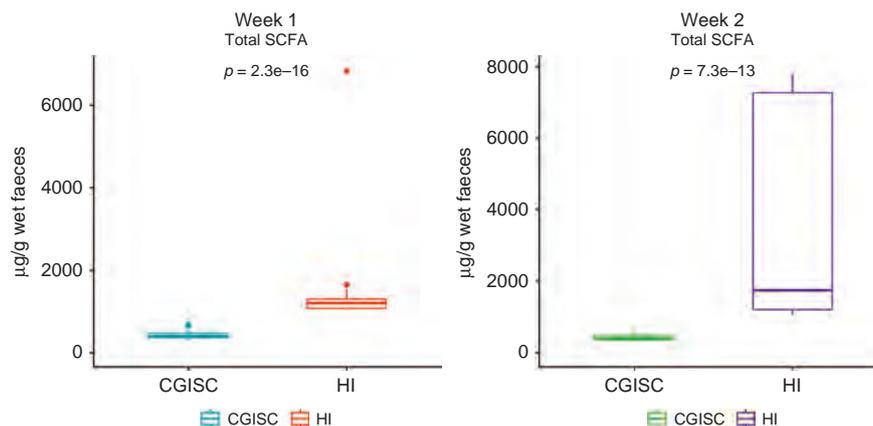


Fig. 5 Stool SCFA levels in study infants. At week 1, CGISC infants have significantly lower amounts of total short-chain fatty acid levels and remain so at week 2.

isovaleric acid, valeric acid, hexanoic acid, and heptanoic acid in the CGISC group as compared to HI at week 1 (Supplementary Table S2). There were no significant differences in concentrations of propionic acid and butyric acid between the groups at week 1 (Supplementary Table S2). At week 2, the concentrations of acetic acid, butyric acid, propionic acid and all other SCFAs were lower in the CGISC group (Supplementary Table S2). Among the CGISC group, there were no significant differences in SCFA levels between week 1 and week 2 samples (all $p > 0.05$). On the other hand, HI group showed significant increases in SCFA levels at week 2 compared to week 1 (all $p < 0.01$ except valeric acid and heptanoic acid where $p > 0.05$).

DISCUSSION

This prospective observational study found that by 10–14 days of life, term neonates with CGISCs developed significant dysbiosis with deficiency of the genera *Bacteroides* and *Bifidobacterium* and increased abundance of the genus *Escherichia-Shigella* and *Pseudomonas*. It also found significant deficiency of SCFAs in the stools of neonates with CGISCs.

Bifidobacterium was the predominant genus during the second week in HIs because they were predominantly breastfed and at home compared to CGISCs, who were still in hospital and had issues, such as feed intolerance, parenteral nutrition and intravenous antibiotics, all of which would have interfered with colonization by *Bifidobacteria*.^{18,19} Normally, HMOs are utilized by *Bifidobacteria*, which enables them to grow and enrich the gut.¹⁸ In the absence of adequate intake of breastmilk, there can be delay in the gut colonization by *Bifidobacteria*. In our study, the median age at the collection of second sample was 13 days, whereas the median age at full feeds was 15.5 days in infants with CGISCs. Many infants with CGISCs were still on parenteral nutrition and were being graded up on milk feeds when the second stool sample was collected.

Bifidobacteria are among the first microbial colonizers of the intestines of newborn infants and play key roles in the development of their physiology, including maturation of the immune system.^{20,21} Hence, their deficiency in surgical infants is of concern and may contribute to the morbidities faced by these vulnerable infants. A vicious cycle may arise wherein clinical morbidities lead to gut dysbiosis, which in turn worsens clinical morbidities.

In our cohort of HIs, richness was significantly lower at week 2 compared to week 1 in HIs. While the drop in alpha diversity was not discernible on Shannon index ($p = 0.998$), ACE and Chao1 indices suggested a trend towards lower alpha diversity at week 2 compared to week 1 even in HIs (ACE, $p = 0.075$; Chao1, $p = 0.084$, Fig. 1). These findings are similar to that of Chi et al.,²² who found

that alpha diversity drops significantly from week 1 to week 2 of birth in low birth weight as well as healthy term newborn infants, before stabilizing.

The richness and alpha diversity decreased markedly from week 1 to week 2 in surgical infants; in addition to the normal drop that occurs in HIs as observed in our study and the study by Chi et al.,²² the other contributing factor could be the use of antibiotics during that period. Bokulich et al.²³ reported that antibiotic use significantly diminishes the phylogenetic diversity and richness in the early newborn period.

The genera *Pseudomonas* and *Escherichia-Shigella* were significantly higher in CGISCs at week 2, compared to HIs. There were no significant differences in the relative abundances of other clinically important genera such as *Enterococcus*, *Enterobacter*, *Klebsiella*, *Staphylococcus* and *Streptococcus* between the surgical and HIs. Probable reason could be the extensive use of antibiotics in surgical infants, which might have suppressed these bacteria, and the short duration of follow-up; it takes some weeks before *Bifidobacteria* become well established at higher levels in healthy breastfed infants, replacing the facultative anaerobes.^{24,25} Newborn infants have an aerobic intestine at birth.²⁶ The high level of oxygen in the newborn GI tract favours the appearance of facultative anaerobes (e.g. *Enterobacter*, *Enterococcus*, *Streptococcus*, *Staphylococci*, *Escherichia coli* and *Klebsiella*). These early colonizers gradually create a reduced, anaerobic environment within the GI tract by consuming the available oxygen, consequently facilitating the establishment of obligate anaerobes such as *Bifidobacterium* and *Bacteroides*.

Evidence is mounting that gut microbial metabolites have a major influence on host physiology. SCFAs are volatile fatty acids produced by the gut microbiota in the large bowel as fermentation products from food components that are unabsorbed/undigested in the small intestine.²⁷ Acetic acid, propionic acid and butyric acid are the most abundant, representing 90–95% of the SCFAs present in the colon. SCFAs result in reduction in the luminal pH in the gut, which inhibits pathogenic microorganisms.²⁸ Acetate produced by *Bifidobacteria* and other commensals improves intestinal defence mediated by epithelial cells and thereby protects the host against lethal infections by enteropathogens. Butyrate is an important fuel of intestinal epithelial cells and stimulates the mitogen-activated protein kinase signalling pathway in intestinal cells, which is positively correlated with gut defences.²⁹ Butyrate also enhances the intestinal barrier by regulating the assembly of tight junctions. In addition, SCFAs are known to have anti-inflammatory properties.⁷

Bacteroides are known to increase the intestinal concentrations of acetate as well as propionate,³⁰ whereas Firmicutes are predominant contributors of butyrate. While *Bifidobacteria* are not butyrogenic by themselves, acetate and other organic acids

produced by them are converted to butyrate by other colonic bacteria via cross-feeding interactions.³¹

It is concerning that SCFAs were lower in CGISCs compared to HIs. The deficiency of SCFAs in neonates with CGISCs has the potential to increase the risk of sepsis due to weakened gut barrier function and other adverse outcomes.

Given that neonates with CGISCs suffer from gut dysbiosis, probiotic supplementation may improve the dysbiosis, SCFA levels and clinical outcomes of these infants. Probiotics are known to inhibit gut colonization with pathogenic bacteria enhance gut barrier function, facilitate colonization with healthy commensals, protect from enteropathogenic infection through production of acetate, reduce antimicrobial resistance, enhance innate immunity and increase maturation of the enteric nervous system and promote gut peristalsis.³² Through these mechanisms, probiotics have the potential to decrease the risk of sepsis, improve feed tolerance and minimize parenteral nutrition-associated cholestasis in infants with CGISCs.³²

Meta-analyses of RCTs in preterm infants (non-surgical) have shown probiotic supplementation to be beneficial in decreasing mortality, necrotizing enterocolitis (NEC), late-onset sepsis and improving feed tolerance.³³ Majority of the RCTs included in those meta-analyses used *Bifidobacteria* as the sole or one of the components of probiotic supplements. Recent meta-analyses that focussed on bifidobacterial supplementation found a significant reduction in the incidence of mortality and NEC in preterm infants.³⁴ In a RCT that included 24 neonates with gastroschisis (probiotics: 12; placebo: 12),³⁵ significant dysbiosis was noted, and it was partially attenuated by the administration of *Bifidobacterium longum* subsp. *infantis*.³⁵ The authors stated that their pilot study was not powered to look at clinical outcomes and that further studies are indicated.

In a small RCT by Murakami et al.³⁶ eight surgical infants were included (*Bifidobacterium*: 4; no *Bifidobacterium*: 4); they reported that unexpectedly there were significantly more Bifidobacteriaceae in the samples from those who did not receive probiotics ($p < 0.05$). Since the sample size was very small, the results may need to be interpreted with caution. The authors concluded that surgical stress appears to affect intestinal microbiota and that probiotic administration requires further clarification.

A meta-analysis that included 198 infants with HD (two RCTs, three observational studies) reported that the incidence of Hirschsprung-associated enterocolitis was 22.6% in the probiotic group vs. 30.5% in the controls, but the difference was not statistically significant (odds ratio 0.72; 95% confidence interval: 0.37–1.39; $p = 0.33$).³⁷ In an RCT of 30 children (<15 years) undergoing various surgeries, Okazaki et al.³⁸ reported that supplementation with *Bifidobacterium breve* BBG-001 was well tolerated without adverse effects, and postoperative infectious complications were significantly decreased. Faecal analysis showed increased levels of *Bifidobacterium* and decreased abundances of Enterobacteriaceae, *Clostridium difficile* and *Pseudomonas*.³⁸

Evidence is emerging from adult studies regarding the beneficial effects of probiotics in GI surgery.^{39,40} The meta-analysis by Lytvyn et al.,³⁹ which included 20 RCTs ($N = 1374$), concluded that probiotic/symbiotic supplementation decreases the risk of surgical site and urinary tract infections in patients undergoing abdominal surgery. Another meta-analysis by Yang et al.⁴⁰ that included 28 RCTs ($n = 2511$) involving adult patients undergoing GI surgery came to similar conclusions. The durations of hospital stay and antibiotic therapy were shorter in the probiotics/symbiotic group vs. controls.

Hence, there seems to be adequate rationale for conducting RCTs of probiotic supplementation, especially the one that contains *Bifidobacteria* in neonates with CGISCs.

Currently, two RCTs of probiotic supplementation in neonates undergoing GI surgery are underway (Howlette et al., Canada,

<https://ichgcp.net/clinical-trials-registry/NCT03266315>); Rao et al., Australia, ACTRN12617001401347).

While our study found dysbiosis in infants with CGISCs, it does not address if the dysbiosis is related to the underlying surgical condition or factors, such as surgical stress, delayed introduction of breastmilk, usage of antibiotics and being in the NICU ecosystem. Infants with CGISCs are probably at a higher risk of dysbiosis because they have more prolonged feed intolerance due to the underlying GI pathology. Additionally, gut is the place where these organisms are predominantly located and hence CGISCs are probably more prone to dysbiosis than other types of surgical conditions. Future studies need to compare the gut microbiota of neonates with CGISCs vs. other surgical conditions to address this issue.

The main strength of our study is that it is probably the first study comparing gut microbiota in neonates with CGISCs vs. healthy term infants using culture independent techniques. The only other study was the RCT by Powell et al.,³⁵ which demonstrated dysbiosis in neonates with gastroschisis; however, the gut microbiota of HIs was not investigated in that study. A limitation of our study is the short duration of follow-up (14 days) and that only two samples were collected from study infants (weeks 1 and 2). The reasons for this approach were logistic feasibility and funding.

We were concerned that there would be significant drop-out rates if a later postnatal age for the second sample collection was chosen (e.g. day 21). As experienced in the present study, even at week 2, there were significant drop-out rates (50%) from HIs. Drop outs would also have occurred in surgical infants because nearly 25% of our surgical infants were discharged home by day 17 and 50% by day 22. It is difficult for busy parents at home to collect samples on a weekly basis. Future studies should allocate adequate resources to have longer duration of follow-up and test multiple stool samples (e.g. once a week for 4–6 weeks).

The other limitation was the fact that HIs were more mature by 1 week compared to the CGISC group. Since the gut microbiota evolves rapidly in the neonatal period, the influence of this 1-week difference cannot be ruled out. Another limitation was that the week 1 samples were collected at an earlier postnatal age (median 2 day) compared to surgical infants (median 4 days). Surgical infants usually have delayed passage of meconium and infrequent passage of subsequent stools because of underlying gut anomaly, administration of narcotic analgesics, delayed commencement of enteral feeds and postoperative ileus.

In summary, during hospitalization, neonates with CGISC develop gut dysbiosis with depletion of the genera *Bacteroidetes* and *Bifidobacterium* and increased abundance of *Pseudomonas* and *Escherichia-Shigella*. They also have deficiency of biologically important SCFAs in their stools. Similar studies with larger sample size and longer duration of follow-up are essential.

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AUTHOR CONTRIBUTIONS

S.C.R.: Conception and design, acquisition of data, analysis and interpretation of data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published. M.E.: Analysis and interpretation of data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published. S.K.P.: Conception and design, interpretation of data; revising the article critically for important intellectual content;

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REVIEW ARTICLE



Probiotic supplementation in neonates with major gastrointestinal surgical conditions: a systematic review

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ABSTRACT

Objectives: Neonates with major gastrointestinal surgical conditions frequently suffer from prolonged feed intolerance, infections, and need multiple courses of antibiotics. All these put them at risk of gut dysbiosis. Probiotic supplementation has the potential to minimise dysbiosis and improve clinical outcomes in such infants. Hence, we aimed to conduct a systematic review of probiotics in neonates with major surgical conditions of the gut.

Methods: Medline, Embase, the Cochrane Central Register of Controlled Trials (CENTRAL), and other databases were searched in September 2016.

Results: Two randomised controlled trials (RCTs) were included; the first was conducted in 24 neonates with gastroschisis, the second in eight neonates with various surgical conditions. In the first study, the overall microbial communities were not significantly different between groups, though analysis of the final specimens demonstrated higher Bifidobacteriaceae, lower Clostridiaceae, and trends toward lower Enterobacteriaceae, Enterococcaceae, Staphylococcaceae, and Streptococcaceae in the probiotic group. In the second study, there were significantly more Streptococcaceae in the faecal samples in the probiotic group and significantly more Bifidobacteriaceae in the no probiotic group ($p < .05$).

Conclusions: There is limited evidence regarding the role of probiotics in neonates with gastrointestinal surgical conditions. Adequately powered RCTs are needed to address this issue.

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Probiotic; dysbiosis;
 newborn infant; surgery;
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Introduction

The major neonatal gastrointestinal surgical conditions include gastroschisis, exomphalos, duodenal atresia, intestinal atresia, congenital diaphragmatic hernia (CDH), tracheo-oesophageal fistula (TEF), short bowel syndrome (SBS), malrotation and volvulus, meconium ileus, hypoplastic left colon, meconium peritonitis, Hirschsprung disease (HD), and anorectal malformations [1–12]. Common morbidity in all these conditions includes feed intolerance, prolonged parenteral nutrition, increased risk of healthcare-associated blood stream infections (HABSI), and the need for multiple courses of antibiotics.

Recurrent administration of antibiotics can destroy the normal commensal gut bacteria [13]. In addition, delayed commencement of enteral feeds, living in the neonatal intensive care unit (NICU) and lack of exposure to mother's skin and breast milk microbiota can lead to intestinal dysbiosis [14–19]. Intestinal dysbiosis has been implicated as a cause or association for

many adverse outcomes in the neonatal, paediatric, and adult population [20–27]. A cohort study of 208 neonates with surgical conditions found that in majority of cases septicaemia was mainly a gut-derived phenomenon due to translocation of gut organisms, and hence suggested novel strategies for prevention of HABSI [28].

Probiotic supplementation has the potential to minimise/prevent dysbiosis, thereby improving the clinical outcomes of surgical neonates. Recent Meta analyses of randomised controlled trials (RCTs) in non-surgical preterm infants have concluded that probiotics enhance feed tolerance [29] and decrease the risk of necrotising enterocolitis [30] and late onset sepsis [31,32]. Similar beneficial effects are plausible in neonates with major surgical conditions of the gut. To our knowledge, there are no systematic reviews on this topic. Hence, we aimed to conduct a systematic review to evaluate the efficacy and safety of probiotic supplementation in newborn infants with major gastrointestinal surgical conditions.

Materials and methods

Guidelines from the Cochrane Neonatal Review Group (<http://neonatal.cochrane.org/resourcesreview-authors>) [33], Centre for Reviews and Dissemination (<http://www.york.ac.uk/crd/guidance/>), and the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statements [34] were followed for undertaking and reporting this systematic review. Ethics approval was not required.

Eligibility criteria

Types of studies: Only RCTs were included in the review. Observational studies, narrative reviews, systematic reviews, case reports, letters, editorials, and commentaries were excluded, but ready to identify potential additional studies.

Types of participants: Full-term and preterm neonates with major gastrointestinal surgical conditions.

Types of interventions

Studies comparing probiotics versus placebo or no probiotics were eligible for inclusion. Probiotics could be of any type, dose, and duration.

Types of outcome measures

Primary outcomes

Incidence of HABS, defined as positive culture on blood sample drawn 48 h after admission to the NICU and before discharge home.

Secondary outcomes

Average number of episodes of HABS per 1000 bed days, number of infants with at least one episode of (a) HABS due to coagulase negative staphylococcus (CONS), (b) candida, (c) Gram-negative bacteria, (d) probiotic organism; surgical wound infections, urinary tract infection, hospital acquired pneumonia, number of courses of antibiotics, duration of antibiotic therapy (days), C-reactive protein (CRP) levels (mg/dl), mortality during initial hospitalisation, time to full enteral feeds (days), duration of hospital stay (days), z scores for weight, length, and head circumference at the time of discharge.

Search methods for identification of studies

We used standard search methods of the Cochrane Neonatal Review Group. The following databases were searched to identify relevant studies: MEDLINE (Ovid),

MEDLINE (In Process & Other Non-Indexed Citations), Embase (Ovid), CINAHL (EBSCO), and Cochrane Central Register of Controlled Trials (CENTRAL), the Cochrane Library. Databases were searched using the following terms: (Probiotics OR Probiotic OR Lactobacillus OR Bifidobacterium OR Saccharomyces) AND ((Infant, Newborn OR Newborn OR Neonate OR Neonatal OR Infant* or Neonat*) AND (Surgery)). The word Surgery was replaced by individual conditions i.e. Gastroschisis OR Exomphalos OR Duodenal atresia OR Intestinal Atresia OR Congenital Diaphragmatic Hernia (CDH) OR Tracheo-Oesophageal Fistula (TOF), OR Short Bowel Syndrome (SBS), OR Malrotation OR Volvulus, OR Meconium Ileus OR Meconium Peritonitis OR Hypoplastic left colon OR Microcolon OR Meconium peritonitis OR Hirschsprung Disease (HD) OR Anorectal Malformations. Search was repeated using the term Paediatric Surgery OR Pediatric Surgery. The clinical trial registries ClinicalTrials.gov and International Clinical Trials Registry Platform (ICTRP, <http://www.who.int/ictcp/en/>) were searched using appropriate terminology. We did not apply any language restrictions.

Searching other resources

We searched the e-abstracts of the relevant perinatal meetings [including Pediatric Academy Society (PAS), Perinatal Society of Australia and New Zealand (PSANZ) and European Society of Paediatrics]. Grey literature was searched through the national technical information services (<http://www.ntis.gov/>), Open Grey (<http://www.opengrey.eu/>), and Trove (<http://trove.nla.gov.au/>). The reference lists of eligible studies and review articles were searched to identify additional studies.

Data collection and analysis

Data collection forms were compiled and completed independently by two of the reviewers (SR and SP). Reviewers SR and SP independently reviewed the results of the search and selected studies for inclusion. Disagreements were resolved by discussion among all reviewers.

Data extraction and management

Two review authors (SR and SP) separately extracted, assessed, and coded all data for each study using a form that was designed specifically for this review. For each included study, information regarding the primary surgical condition, sample size, gestational age, birth weight, time of commencement of probiotics, type/dose and duration of probiotic and other

clinically relevant information were collected. Discrepancies were resolved by discussion. Authors of the included RCTs were contacted for additional information from their study.

Assessment of risk of bias (ROB) in included studies: We assessed ROB by using the Cochrane "Risk of Bias Assessment Tool." Authors SR and SP independently assessed the ROB in all domains including random number generation, allocation concealment, blinding of intervention and outcome assessors, completeness of follow-up, selectivity of reporting, and other potential sources of bias. For each domain, the ROB was assessed as low, high, or unclear risk based on the Cochrane Collaboration guidelines. Where it was unclear, the authors of the included RCTs were contacted by email requesting clarification.

Data synthesis: Meta-analysis was planned to be conducted using Review Manager 5.3 (Cochrane Collaboration, Nordic Cochrane Centre, Copenhagen, Denmark) using fixed-effects model (FEM) (Mantel-Haenszel method). Analysis using random effects model (REM) was planned to ensure that the results and conclusions were not influenced by the type of model used for meta-analysis. Effect size was planned to be expressed as risk ratio (RR) and 95% confidence interval (CI) for dichotomous outcomes and mean difference and 95%CI for continuous outcomes. Statistical heterogeneity was planned to be assessed with the χ^2 test and I^2 statistic and by visual inspection of the forest plot (overlap of CIs). A p values $<.1$ on the χ^2 statistic was considered to indicate heterogeneity. I^2 statistic values were interpreted according to the guidelines of Cochrane Handbook as follows: 0% to 40% might not be important; 30% to 60% may represent moderate heterogeneity; 50% to 90% may represent substantial heterogeneity; 75% to 100%, considerable heterogeneity. The risk of publication bias was assessed by visual inspection of the funnel plot [35].

Subgroup analysis: Subgroup analyses were planned based on the type of surgical condition and gestation (full-term versus preterm).

Quality of evidence and summary of findings: The key information about the quality of evidence, the magnitude of effect of the intervention, and the sum of available data on the main outcome was planned to be presented in the summary of findings table according to the Grades of Recommendation, Assessment, Development and Evaluation (GRADE) guidelines [36].

Sensitivity analysis: We planned to conduct sensitivity analyses by excluding studies with high ROB on

random sequence generation and/or allocation concealment.

Results

The literature search retrieved 4889 citations, of which 4887 were excluded and two RCTs included [37,38]. The flow diagram of the study selection process is given in Figure 1. The study by Powell et al. [38] enrolled 24 infants with gastroschisis born at >34 weeks' gestation. Enrolled infants were randomly assigned to receive either *B. infantis* ATCC 15697 10^9 CFU (colony forming units) or placebo twice daily for 6 weeks or until hospital discharge (whichever came first). The supplementation with probiotic or placebo began following surgery as soon as consent was obtained. The supplement was instilled into the nasogastric (Replogle) tube which was then clamped for 1 h before returning to routine gastric decompression. The supplementation doses were given orally when the Replogle tube was removed. All caregivers and parents were blinded to the study group assignment. The primary outcome was faecal microbiota composition and the secondary outcome was length of hospital stay. Administration of the probiotic or placebo was well tolerated. The authors reported that overall microbial communities were not significantly different between groups, though analysis of the final specimens demonstrated higher Bifidobacteriaceae, lower Clostridiaceae, and trends toward lower Enterobacteriaceae, Enterococcaceae, Staphylococcaceae, and Streptococcaceae in the probiotic group. The median (IQR) duration of the supplementation was 19.5 (17.5, 29.5) versus 23 (IQR: 20.2, 36.7) days in the placebo versus probiotic group respectively. The duration (Placebo versus Probiotic) of TPN [22.5 (17.5, 29.2) versus 21 (19, 32.2); $p=.82$], antibiotic therapy [7 (5.7, 8) versus 6 (5, 7); $p=.25$], and length of hospital stay [(26.5 (21, 32.7) versus 27 (26, 42.5); $p=.44$] was similar between the two groups. There were no deaths and no cases of sepsis in both the groups [39].

The study by Mukarami et al. [37] evaluated the effect of probiotic supplementation on intestinal microbiota in neonates undergoing surgery within 3 days of birth. The probiotic strain used was *Bifidobacterium animalis* subsp. lactis LKM512 (LKM). Four infants were given and another four not given probiotics. Three healthy infants served as controls. Stool specimens (20 mg) were collected at five times (after birth, and on days 3, 7, 10, and 14 after surgery in surgical cases, and after birth, and on days 4, 8, 11, and 15 of life in controls). Among the probiotic group infants, all four had colostomies for anorectal

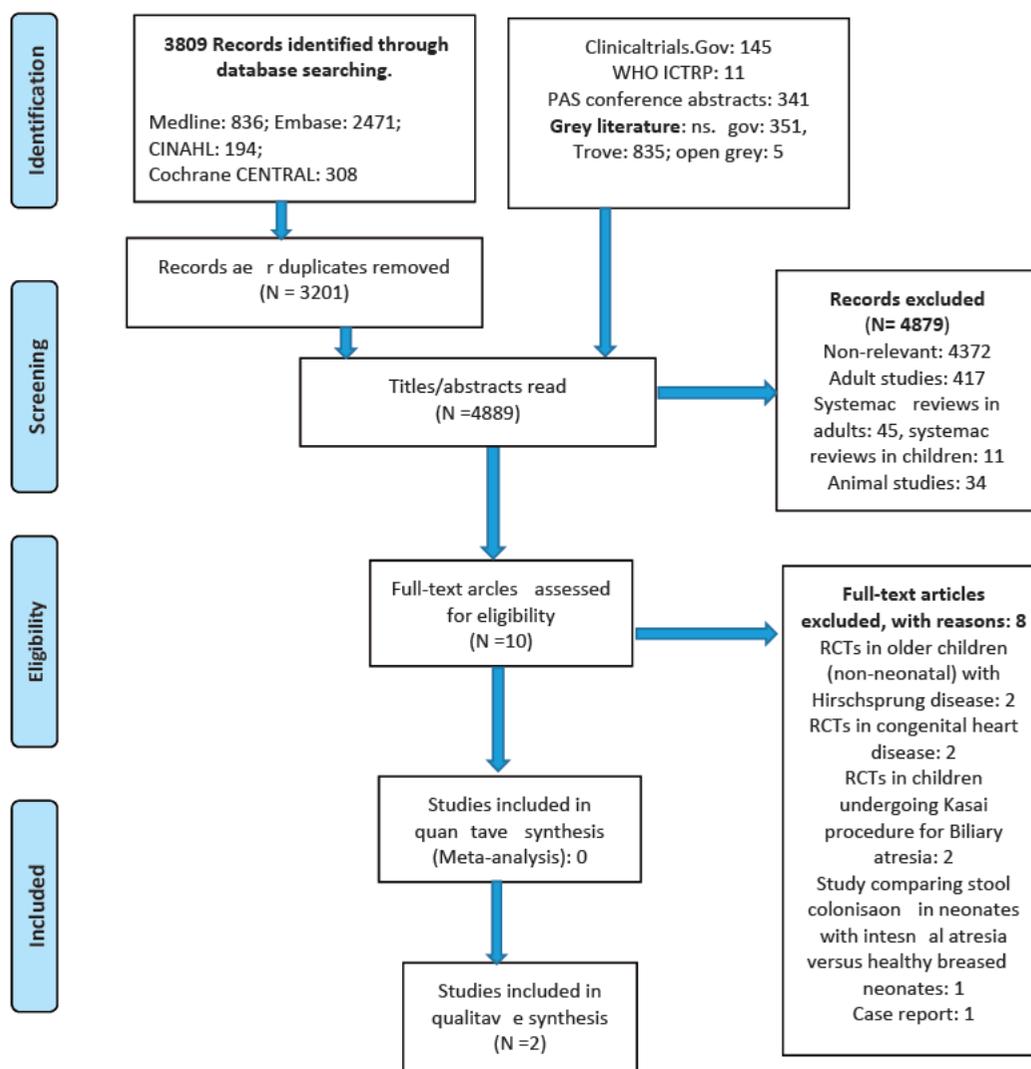


Figure 1. PRISMA 2009 flow diagram of study selection probiotic supplementation in neonates with major gastrointestinal surgical conditions: a systematic review.

malformation; among the no probiotic group infants, one had duodeno-duodenostomy for duodenal atresia and one had anorectal malformation and one had a lymphangioma requiring OK432 Injection. All surgical infants were prescribed ampicillin as a postoperative antibiotic according to the standard postoperative management protocol. Stool microbiota was analysed using 16S rRNA gene sequencing after DNA extraction. One surgical infant was a preterm infant weighing 2930g at birth and others were full-term weighing from 2426 to 3300g. Two were delivered by caesarean and the others were delivered vaginally. They observed major changes in gut microbiota in surgical infants in both the probiotic and non-probiotic group. There were significantly more Streptococcaceae in the faecal samples from those who received probiotics ($p < .05$) and unexpectedly there were significantly more Bifidobacteriaceae in the samples from those who did not receive probiotics ($p < .05$). In the

probiotic group, two cases developed egg allergy and one case developed generalised colitis at the age of ~ 12 months. In the no probiotic group, two cases developed colitis. No significant differences in weight gain were observed. They concluded that surgical stress appears to affect intestinal microbiota considerably.

ROB of included studies: Details of the ROB analysis are given in Table 1. The methods used to generate random sequence numbers and how allocation concealment was achieved was not available in both the included RCTs. Powell et al. used placebo and hence there was low risk of detection bias. Mukarami et al. did not use placebo. Follow up was complete in both the RCTs.

Results of meta-analysis: Meta-analysis could not be done due to the lack of data suitable for pooling.

GRADE evidence: The level of evidence was deemed low, mainly because of the small sample size and

Table 1. Risk of bias of the included studies.

Domains of ROB assessment	Powell 2015	Mukarami 2016
Random sequence generation (selection bias)	Unclear risk	Unclear risk
Allocation concealment (selection bias)	Unclear risk	Unclear risk
Blinding (performance bias and detection bias)	Low risk (placebo used)	Unclear (placebo not used)
Completeness of follow up (attrition bias)	Low risk	Low risk
Selective reporting (reporting bias)	Low risk	Low risk
Other bias	Low risk	Low risk

Table 2. Summary of findings (SOF) and GRADE evidence.

Outcomes	Probiotics	Placebo	Statistical analysis (e.g. relative risk, <i>p</i> value)	Number of participants	Quality of evidence (GRADE)	Comments
Mortality	0/12	0/12	Relative risk not estimable	24	Low	See footnotes
Acquired sepsis	0/12	0/12	Relative risk not estimable	24	Low	See footnotes
Duration of antibiotic therapy (days, median, and IQR)	6 (5, 7)	7 (5.7, 8)	<i>p</i> .25	24	Low	See footnotes
Duration of parenteral nutrition (days, median, and IQR)	21 (19, 32.2)	22.5 (17.5, 29.2)	<i>p</i> .82	24	Low	See footnotes
Length of hospital stay (days, median, and IQR)	27 (26, 42.5)	26.5 (21, 32.7)	<i>p</i> .44	24	Low	See footnotes

Probiotic supplementation compared with placebo in neonates with major surgical conditions of the gut.

*The evidence was deemed low in view of the very small sample size and unclear risk of bias in the domains of random sequence numbers and allocation concealment.

unclear ROB in some of the domains of assessment (Table 2).

Discussion

This first systematic review identified only two RCTs ($N=32$) that evaluated probiotic supplementation in infants undergoing gastrointestinal surgery. There is increasing number of probiotic RCTs in adult population undergoing gastrointestinal surgery. Recently three independent meta-analyses of data from adult studies have concluded that probiotic/symbiotic supplementation decreases the risk of post-operative sepsis in adults undergoing gastrointestinal surgeries [40–42]. The sample size in the included meta-analyses ranged from 1200–2500 (15–28 RCTs). There are two RCTs of probiotic supplementation in children (<18 years) with Hirschsprung disease [43,44]. While one of them showed beneficial effects of supplementation [43], the other did not [44]. In another RCT in 30 children (<15 years) with various surgical (majority gastrointestinal) conditions *Bifidobacterium Breve* (BBG-01) or placebo was administered daily from 7 days prior to surgery until discharge from hospital. Administration of the probiotic strain BBG-01 in the perioperative period was found to be safe and improved the gut flora, increased the faecal short-chain fatty acid (acetic acid) concentration, and decreased the risk of septicaemia [45].

A recent survey of 86 clinicians (70 institutions in Japan) from departments of paediatrics, newborn medicine, obstetrics and gynaecology and paediatric surgery reported that adverse events (functional ileus) occurred in two extremely preterm infants, and

B. breve bacteraemia in two surgical neonates. No serious adverse events with a poor outcome were observed [46]. The total number of patients treated with probiotics exceeded 23,000, with 169 being paediatric surgical cases [46]. This data provides additional reassurance that probiotic supplementation may be safe in infants with gastrointestinal surgical conditions.

Intestinal dysbiosis is known to be severe in critically ill patients [47,48]. Infants with gastrointestinal surgical condition are critically ill and hence are expected to have severe dysbiosis. Animal studies and *in vitro* studies have shown that probiotics improve gut barrier function [49], decrease gut bacterial translocation [50], inhibit gut colonisation with pathogenic bacteria [51], improve colonisation with healthy commensals [52] and protect from enteropathogenic infection through acetate production [53], enhance innate immunity [54], and increase maturation of the enteric nervous system [55], all of which have the potential to improve the outcomes of infants with surgical conditions of the gut. Hence, it is important to conduct adequately powered RCT to evaluate this potential therapy in this high-risk population of infants.

The strengths of our systematic review are the use of Cochrane methodology, and thorough literature search including the grey literature. The weaknesses are the lack of adequate number of RCTs and the extremely small sample size.

Conclusions

There is limited evidence regarding the role of probiotics in infants with gastrointestinal surgical conditions.

Given the biological plausibility for benefit and encouraging results from adult studies, adequately powered neonatal RCTs are needed to address this issue.

Disclosure statement

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Opinion

Probiotic research in neonates with congenital gastrointestinal surgical conditions – Now is the time

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The major congenital gastrointestinal surgical conditions (CGISC) include oesophageal atresia, gastroschisis, exomphalos, malrotation and volvulus, duodenal atresia, intestinal atresia, meconium ileus, hypoplastic colon, meconium peritonitis, intestinal stenosis, congenital short bowel syndrome, Hirschsprung disease (HD), anorectal malformations and others. In addition to surgical repair, strategies for managing such conditions include early commencement of enteral feeds, standardization of feeding advancement, strict hand hygiene and aseptic precautions for indwelling catheters (Graham, 2010; Lauriti *et al.*, 2014; Savoie *et al.*, 2016; Dama *et al.*, 2017). Despite such best practices and advances in surgical techniques, morbidities including feed intolerance, healthcare-associated infections, cholestatic jaundice, growth failure and neurodevelopmental disabilities continue to impose significant health burden on this cohort (Willis *et al.*, 2010; Bishay *et al.*, 2012; Wang *et al.*, 2014; Dwyer *et al.*, 2016; Hong *et al.*, 2018). Additional strategies are hence required to improve their outcomes.

Gut dysbiosis in infants with CGISC

Neonatal gut microbiota develops rapidly after birth and achieves an adult-like composition and stability by 2–3 years of age (Arrieta *et al.*, 2014). The evolution of gut microbiome is affected in infants with CGISC admitted in intensive care units (ICUs). These infants receive

parenteral nutrition (PN), get exposed to multiple courses of antibiotics, do not receive early enteral feeding and optimal maternal skin to skin contact. Decontamination of the skin for surgery, exposure to gastric acid suppressants, breakdown of natural barriers due to invasive procedures and indwelling tubes and catheters, colonization of the ICU room surfaces and hands of the healthcare providers also contribute to the risk of gut dysbiosis in infants with CGISC (Donnell *et al.*, 2002; van Saene *et al.*, 2003; Hussey *et al.*, 2011; Fouhy *et al.*, 2012; Ralls *et al.*, 2016; Rogers *et al.*, 2016; Kitsios *et al.*, 2017).

- (i) *PN and gut dysbiosis*: The role of PN in gut dysbiosis deserves attention as it is often the main/only source of nutrition in infants with CGISC. Lavalley *et al.* (2017) randomized neonatal piglets to receive total parenteral nutrition (TPN) or sow feeds (SF) for 14 days. Ileal segments and mucosal scrapings were used to assess the microbiota composition by 16S rRNA gene sequencing. Significant dysbiosis was noted in the TPN group, especially in those which received soy-based lipids. In another study, using a mouse model, Ralls *et al.* (2016) reported permeation of TPN-derived nutrients into the gut lumen, where they were preferentially utilized by Enterobacteriaceae, which then flourished.
- (ii) *Antibiotics and gut dysbiosis*: Fouhy *et al.* (2012) compared the gut microbiota of nine newborn infants treated with parenteral ampicillin and gentamicin, with that of nine matched healthy infants. Gut microbiota of the antibiotic-treated infants showed significantly higher proportions of Proteobacteria and lower proportions of Actinobacteria and the associated genus Bifidobacterium, as well as the genus Lactobacillus compared with the untreated controls 4 weeks after the cessation of treatment. Even by week 8, Proteobacteria levels remained significantly higher in the treated infants (Fouhy *et al.*, 2012). Increased abundance of Proteobacteria is a concern because it is considered as a potential diagnostic signature of dysbiosis and risk of disease (Shin *et al.*, 2015).
- (iii) *The ICU ecosystem and gut dysbiosis*: In a study in adult ICU patients, McDonald *et al.* (2016) showed

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evidence of extreme dysbiosis. The phylogenetic diversity at discharge was significantly lower than at admission. Faecal samples tended to have a lower relative abundance of Firmicutes and Bacteroidetes and an increased relative abundance of Proteobacteria and well-recognized pathogens such as *Enterobacter* and *Staphylococcus* (McDonald *et al.*, 2016). In a study in paediatric ICUs, Rogers *et al.* (2016) reported taxonomic alterations in the gut microbiota. These included enrichments of gut pathogens such as *Enterococcus* and *Staphylococcus* at multiple body sites and depletion of commensals such as *Faecalibacterium* and *Ruminococcus* from stool samples. Alpha and beta diversity were unstable over time (Rogers *et al.*, 2016).

Studies have shown an association between gut dysbiosis and morbidities such as hospital-acquired infections in neonates with surgical conditions (Donnell *et al.*, 2002; van Saene *et al.*, 2003) and Hirschsprung-associated enterocolitis (HAEC) (Li *et al.*, 2016).

Probiotics for CGISC

Given that gut dysbiosis occurs and is associated with morbidities in infants with CGISC, optimization of gut microbiota by probiotics is a potentially beneficial strategy to improve their outcomes.

Probiotics are defined as live microorganisms that when administered in adequate amounts confer health benefits on people with specific illnesses (Hill *et al.*, 2014). Probiotics inhibit gut colonization with pathogenic bacteria (Sassone-Corsi and Raffatellu, 2015), enhance gut barrier function (Bron *et al.*, 2017), facilitate colonization with healthy commensals (Garrido *et al.*, 2012), protect from enteropathogenic infection through production of acetate (Fukuda *et al.*, 2011), reduce antimicrobial resistance (Taft *et al.*, 2018), enhance innate immunity (Giorgetti *et al.*, 2015) and increase maturation of the enteric nervous system and promote gut peristalsis (Hyland and Cryan, 2016; De Vadder *et al.*, 2018). Through these mechanisms, probiotics have the potential to decrease the risk of sepsis, improve feed tolerance and minimize parenteral nutrition-associated cholestasis in infants with CGISC.

(i) *Evidence from studies in adult patients:* A recent meta-analysis of 20 RCTs ($N = 1374$) concluded that probiotic/symbiotic supplementation decreases the risk of surgical site and urinary tract infections in patients undergoing abdominal surgery (Lytvyn *et al.*, 2016). Another meta-analysis that included 28 RCTs ($n = 2511$) involving adult patients undergoing gastrointestinal surgery came to similar conclusions (Yang *et al.*, 2017). The durations of hospital stay and

antibiotic therapy were shorter in the probiotics/symbiotic group vs controls (Yang *et al.*, 2017). The need for caution in interpreting the results was emphasized considering the high risk of bias in included studies (Lytvyn *et al.*, 2016; Yang *et al.*, 2017).

(ii) *Evidence from studies in paediatric patients:* In a RCT, 30 children (<15 years) with various surgical (majority gastrointestinal) conditions were supplemented with probiotic *Bifidobacterium breve* BBG-01 or placebo daily from 7 days before the surgery until discharge. Probiotic supplementation was safe. It improved the gut flora, increased the concentration of faecal acetic acid and decreased the risk of septicaemia (Okazaki *et al.*, 2016). A recent meta-analysis that included 198 infants with HD (two RCTs, three observational studies) reported that the incidence of HAEC 22.6% in the probiotic group vs. 30.5% in the controls, but the difference was not statistically significant (OR 0.72; 95% CI 0.37–1.39; $P = 0.33$; Nakamura *et al.*, 2018). Majority of the infants in the included studies were outside the neonatal period.

(iii) *Evidence from studies in neonates:* A systematic review (Rao *et al.*, 2018) that focussed on CGISC exclusively in the neonatal population found only two small RCTs (Murakami *et al.*, 2016; Powell *et al.*, 2016). The Powell *et al.* (2016) RCT included 24 neonates with gastroschisis (Probiotics: 12, Placebo: 12). The probiotic supplement was administered for 6 weeks or until hospital discharge, whichever came first. Significant dysbiosis was noted in the study infants, and it was partially attenuated by administration of *Bifidobacterium longum* subsp. *infantis* (Powell *et al.*, 2016). In the RCT by Murakami *et al.* (2016), four surgical neonates (duodenal atresia, anorectal malformations) received probiotics, four received no probiotics. Bifidobacteriaceae was more abundant in neonates who had not received probiotics. It was concluded that surgical stress appeared to affect the intestinal microbiota considerably. The need for further RCTs in this area was emphasized.

Safety of probiotics

Evidence from over 35 RCTs with a total sample size of nearly 12 000 and observational studies with over 14 000 participants show that probiotics are beneficial and safe in preterm non-surgical infants (Olsen *et al.*, 2016; Rao *et al.*, 2016; Sawh *et al.*, 2016; Dermyshe *et al.*, 2017). Even a large RCT that did not show benefits of probiotic supplementation acknowledged that short-term safety of probiotics was good in preterm infants (Costeloe *et al.*, 2016). Recent meta-analyses have shown that probiotics do not increase or decrease the risk of intraventricular

haemorrhage, chronic lung disease, retinopathy of prematurity and neurodevelopmental outcomes in preterm non-surgical infants (Cavallaro *et al.*, 2017; Villamor-Martinez *et al.*, 2017; Upadhyay *et al.*, 2018). These findings provide reassurance regarding medium-term safety of probiotics in preterm infants. However, there are few case reports of sepsis due to probiotic organisms (Ohishi *et al.*, 2010; Vallabhaneni *et al.*, 2015; Brecht *et al.*, 2016). Hence, constant vigilance and quality assurance of the product while conducting RCTs of probiotic supplementation in infants with CGISC are warranted.

Ongoing RCTs of probiotics in infants with CGISC

To our knowledge, currently, there are two ongoing RCTs evaluating the role of probiotics in this area. One trial is being conducted in Calgary (Canada) and aims to recruit 88 infants born between 23 and 42 weeks of gestation who require gastrointestinal surgery (Mugarab-Samedi *et al.*, 2017). The probiotic supplement is FloraBabyTM (Renew Life Canada, Oakville, ON, Canada). Each sachet (1 g) will have 4 billion colony-forming units (CFU) of probiotics, consisting of *Bifidobacterium breve* (HA-129), *Lactobacillus rhamnosus* (HA111), *Bifidobacterium bifidum* (HA-132), *Bifidobacterium longum* subsp. *infantis* (HA-116) and *Bifidobacterium longum* subsp. *longum* (HA-135). Placebo is maltodextrin. The primary outcome of interest is length of hospital stay. Stool microbial analysis using culture independent 16S rRNA studies will be undertaken.

The other study (ours) is being conducted in Western Australia (Rao *et al.*, 2017). Sixty infants (≥ 35 weeks' gestation) with major CGISC will be recruited. The probiotic group will receive 3×10^9 CFU/day (i.e. 3 billion organisms) in 1.5 ml of the expressed breast milk or sterile water, given as a single daily dose via the orogastric/nasogastric feeding tube or orally. The probiotic sachet (Morinaga Industries, Tokyo, Japan) will contain a mixture of three strains (*B. breve* M-16V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536 (1×10^9 CFU of each strain per 1 g sachet)). Placebo is maltodextrin. Supplementation will be commenced as soon as possible after admission once the baseline stool samples are collected and will be continued until discharge. Primary outcome will be gut microbiota (using 16 s ribosomal RNA Pyrosequencing studies for phylogenetic profiling) on stool samples. Secondary outcomes will be stool short-chain fatty acids and relevant clinical outcomes.

Conclusions

In summary, probiotic supplementation has the potential to minimize gut dysbiosis and improve clinical outcomes

of neonates with CGISC. Though small, the completed and ongoing RCTs will provide important data and confidence to embark on adequately powered large RCTs in this exciting area.

Conflict of interest

None declared.

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CLINICAL RESEARCH ARTICLE



Probiotic supplementation in neonates with congenital gastrointestinal surgical conditions: a pilot randomised controlled trial

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OBJECTIVE: To evaluate whether probiotic supplementation attenuates gut-dysbiosis in neonates with congenital gastrointestinal surgical conditions (CGISC).

METHODS: Sixty-one neonates (≥ 35 weeks gestation) with CGISC were randomised to receive daily supplementation with a triple-strain bifidobacterial probiotic ($n = 30$) or placebo ($n = 31$) until discharge. Stool microbiota was analysed using 16S ribosomal RNA gene sequencing on samples collected before (T1), 1 week (T2), and 2 weeks (T3) after supplementation and before discharge (T4). The primary outcome was the sum of the relative abundance of potentially pathogenic families of Clostridiaceae, Enterobacteriaceae, Enterococcaceae, Pseudomonaceae, Staphylococcaceae, Streptococcaceae, and Yersiniaceae at T3.

RESULTS: The median gestational age [38 weeks (IQR: 37.1–38.9)] was similar in both groups. The probiotic group had lower rates of caesarean deliveries (40% versus 70%, $p = 0.02$). The relative abundance of potentially pathogenic families was lower in the probiotic group compared to placebo at T3 [(median: 50.4 (IQR: 26.6–67.6) versus 67.1 (IQR: 50.9–96.2); $p = 0.04$). Relative abundance of Bifidobacteriaceae was higher in the probiotic group at T3 [(median: 39.8 (IQR: 24.9–52.1) versus 0.03 (IQR 0.02–2.1); $p < 0.001$). Stratified analysis continued to show a higher abundance of Bifidobacteriaceae in the probiotic group, irrespective of the mode of delivery.

CONCLUSIONS: Probiotic supplementation attenuated gut dysbiosis in neonates with CGISC.

TRIAL REGISTRATION: <http://www.anzctr.org.au> (ACTRN12617001401347).

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IMPACT:

- Probiotic supplementation attenuates gut dysbiosis and improves stool short-chain fatty acid levels in neonates with congenital gastrointestinal surgical conditions.
- This is the second pilot RCT of probiotic supplementation in neonates with congenital gastrointestinal conditions.
- These findings will pave the way for conducting multicentre RCTs in this area.

INTRODUCTION

The common morbidities in neonates with congenital gastrointestinal surgical conditions (CGISC) are feed intolerance and healthcare-associated infections (HAI).^{1–9} Recurrent administration of antibiotics, delayed enteral feeds, use of parenteral nutrition (PN), and delayed exposure to the mother's skin and breast milk microbiota can lead to intestinal dysbiosis in these infants.^{10–15} Our previous prospective study found that neonates with CGISC develop gut dysbiosis during their stay in the neonatal intensive care unit.¹³

Experimental studies have shown that probiotic supplementation attenuates gut dysbiosis, strengthens the gut barrier, prevents enteropathogenic infections, reduces antimicrobial resistance, enhances immunity, and promotes gut peristalsis.¹⁶ Through these mechanisms, probiotics have the potential to improve the outcomes of neonates with CGISC.¹⁶ Many beneficial biological functions of probiotics are mediated via short-chain fatty acids (SCFAs).¹⁷ The major SCFAs (80–95%) in the gut are acetate, propionate, and butyrate.^{18,19}

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In infants, SCFAs are produced by the fermentation of milk oligosaccharides by gut bacteria, and the type of milk has a significant effect on the composition of the SCFA.²⁰

Our previous prospective study also found that neonates with CGISC have lower faecal SCFA levels.¹³

Systematic reviews from adult studies have concluded that probiotic/synbiotic supplementation is safe and reduces the risk of postoperative infections.²¹ A pilot randomised controlled trial (RCT) ($n = 24$) in neonates with gastroschisis reported that gut dysbiosis was partially attenuated by the probiotic *Bifidobacterium longum* subsp. *infantis*.²² Our systematic review found limited evidence regarding the role of probiotics in neonates with CGISC and recommended the conduct of RCTs.²³ Hence, we conducted this pilot RCT to evaluate the efficacy and safety of probiotics in attenuating dysbiosis and improving SCFA levels in term and near-term infants with CGISC.

METHODS

Hypothesis

Probiotic supplementation attenuates gut dysbiosis and increases stool SCFA levels in term and near term neonates with CGISC.

Design and setting

Double blind RCT in the neonatal intensive care unit of Perth Children's Hospital, Western Australia. Approval was obtained from the Institutional Human Research Ethics Committee (HREC Ref Number RGS0000002554). It was registered with the Australia and New Zealand Clinical Trials Registry (ACTRN12617001401347). The protocol is available in Supplement File 3. Infants were recruited between November 2017 and March 2020.

Eligibility criteria included

Neonates (≥ 35 weeks' gestation) with intestinal atresia, malrotation, congenital diaphragmatic hernia (CDH), tracheoesophageal fistula (TOF), gastroschisis, exomphalos, Hirschsprung disease, imperforate anus, short bowel syndrome, and other surgical conditions requiring stomas (e.g., severe meconium ileus, microcolon). Preterm infants less than 35 weeks of gestation were excluded to reduce the confounding effect of prematurity on the gut microbiota.

Intervention

A mixture of three strains (*Bifidobacterium breve* M 16V, *Bifidobacterium longum* subsp. *infantis* M 63, and *Bifidobacterium longum* subsp. *longum* BB536; 1×10^9 colony forming units (CFU) of each strain per 1 g sachet; Morinaga Milk Industry Co., Ltd., Japan]. Dose was 1 sachet per day, which provided a total of 3×10^9 (i.e., 3 billion) CFU per day. *Placebo*: Maltodextrin. The trial supplements were stored in a refrigerator at $2-8^\circ\text{C}$.

Group assignments were allocated using a computer generated sequence in randomly ordered block sizes of 2 and 4. These sequences were generated by our institutional trial pharmacist, without the involvement of the research team. Allocation concealment was optimised through pharmacy controlled allocation; the clinical trial pharmacist generated random numbers and used them to prepare sequentially numbered individual boxes, each containing 30 sachets of trial supplements. The sachets containing probiotics or placebo were of identical design, weight, and volume; the boxes containing the sachets were also of similar design. The contents of the sachets (probiotic or placebo) were similar in texture, smell, and taste. All these steps were undertaken to ensure adequate blinding of healthcare providers and parents. Once parental consent was obtained, the chief investigator or his delegate allocated the next box of study supplement to the infant without knowledge of the contents of the box (probiotic or placebo).

In the postoperative period, once baseline stool samples were collected, infants were given trial supplements once daily until discharge. Trial supplements were dissolved in 1.5 mL of expressed breast milk (EBM) or sterile water (if EBM was not available) and administered via the feeding tube or mouth. Supplementation was continued even when the infants did not receive enteral feeds. If an infant was having continuous or intermittent gastric suctioning, once the trial supplements were given, suctioning was stopped for 3-4 h prior to recommencing.

Feeding regimen of study infants

The standard policy of our unit is to commence PN within 48-72 h of admission for surgical infants who are unable to tolerate enteral feeds. Enteral feeding with expressed breast milk were commenced as soon as possible in the postoperative period (usually on postoperative day 2 and advanced as tolerated), depending on the underlying surgical condition and consensus opinion of neonatologists and surgeons. Infant formulae or hydrolysed formulae are rarely used in our unit for surgical infants. None of our study infants received donor breast milk because it was reserved for preterm infants less than 32 weeks of gestation in our unit.

Stool sample collection, storage, and analysis

Stool samples were collected at four time points: (a) as soon as possible after admission, but before commencing trial supplements (T1); (b) 1 week (T2), and (c) 2 weeks after commencing supplements (T3); and (d) prior to discharge (T4).

The samples were collected from the nappies into sterile microvials and stored in the NICU at 20°C for 3-4 days and subsequently at 80°C . At the completion of full recruitment, samples were shipped on dry ice to the University of New South Wales (Sydney, Australia), where microbial analysis was undertaken. Acidified samples were frozen at -20°C and shipped to the School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore, where SCFA analysis was performed.

The stool microbiota was assessed using the 16S ribosomal RNA gene sequencing method (Supplementary File 1). Stool short chain fatty acid assay was performed according to our previously described method¹³ with slight modifications (Supplementary File 1). The scientists who conducted microbial and SCFA assays and their respective statistical analyses were blinded to the randomisation groups. They conducted statistical analyses of data from stool samples as group 1 versus group 2. The trial pharmacist disclosed the groupings only after receiving all the results through e mail. This ensured adequate blinding of clinicians, research teams, lab scientists, and statisticians throughout all stages of the trial.

Primary outcome

The primary outcome was the sum of the relative abundance of potentially pathogenic families of Clostridiaceae, Enterobacteriaceae, Enterococcaceae, Pseudomonaceae, Staphylococcaceae, Streptococcaceae, and Yersiniaceae at T3 using 16S ribosomal RNA gene sequencing methods. T3 was chosen as the main time point of interest because 2 weeks is a reasonable duration of supplementation to enable colonisation by the probiotic strains, and many study infants will still be in the hospital.

Secondary outcomes

(1) Stool microbiota at T1, T2, and T4 time points; (2) SCFA levels at all time points; (3) short term clinical outcomes during initial stay in the NICU, such as incidence of mortality, HAI, duration of antibiotics, PN, hospital stay, time to reach full feeds after surgery, and physical growth. The z scores for weight, length, and head circumference at birth were calculated using the Fenton growth charts²⁴ and at discharge using the WHO charts²⁵ through the publicly accessible PediTools website of clinical calculators.²⁶

Statistical considerations

Sample size estimation. In a recent RCT, the sum of the relative abundance of potentially pathogenic families such as Enterobacteriaceae, Staphylococcaceae, Enterococcaceae, Clostridiaceae, and Streptococcaceae was approximately 76% in the stools of infants with gastroschisis who received placebo.²² Hence, we calculated that a total sample size of 60 infants (probiotic: 30, placebo: 30) would be required to demonstrate a 50% reduction of potentially pathogenic bacterial families from 76 to 38% after 2 weeks of supplementation with probiotics with an alpha error of 0.05, and a power of 80%.

Statistical analysis of clinical data. Continuous data with normal distribution were summarised using mean and standard deviations (SD) and compared using the two sample t test. Median, interquartile range (IQR), and range were used to summarise data with skewed distribution and compared using the Wilcoxon rank sum test. Binary outcomes were compared using the Fisher's exact test. To compare the z scores of physical growth parameters at discharge versus birth, the matched pairs t test was used. For all analyses, a p value less than 0.05 was considered significant.

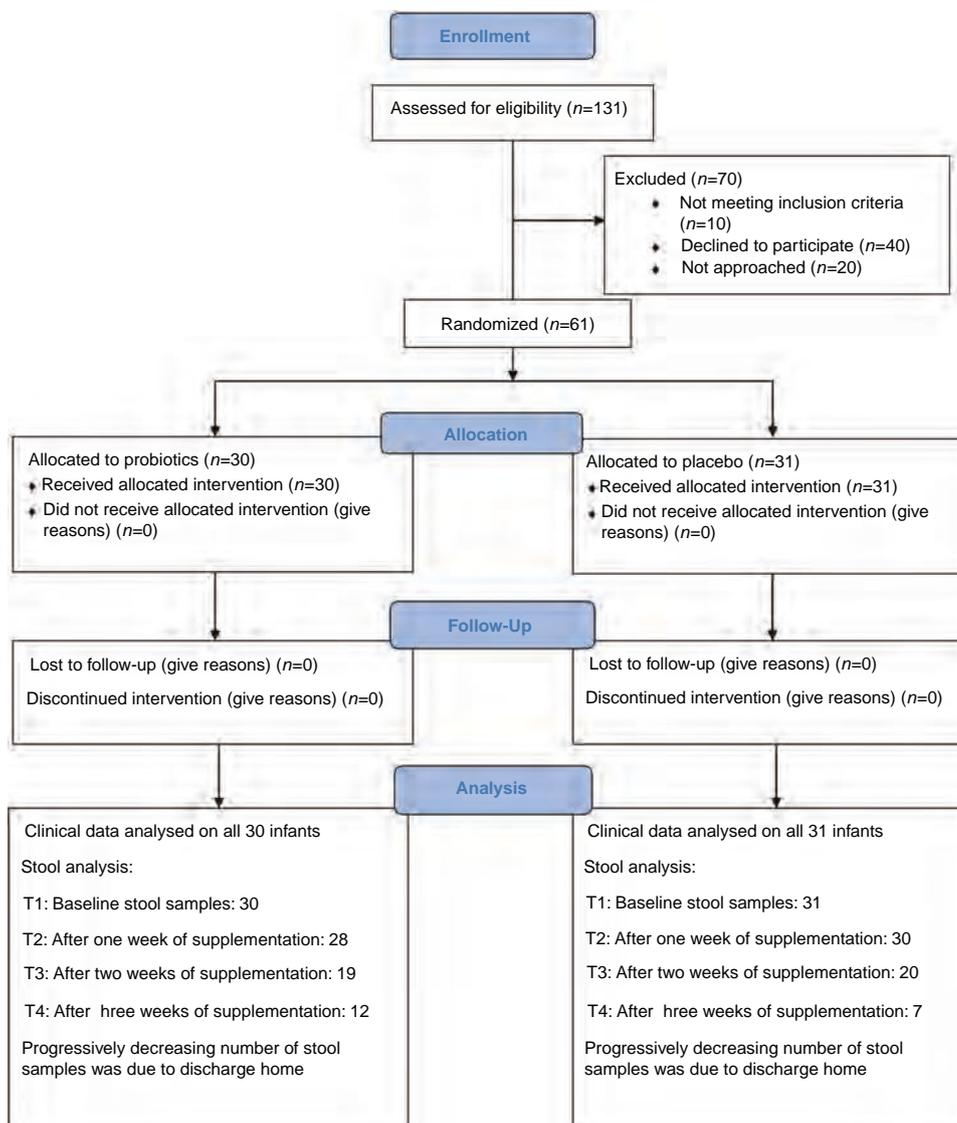


Fig. 1 CONSORT flow diagram showing participant flow through the trial.

Statistical analysis for microbiological data. Analyses were conducted with R version 3.6.0. For microbial richness, linear mixed model effects (LME) test (MASS, lme4, and lmerTest packages) was used to identify if there were significant differences between the groups over time. In our LME model, Subject ID was a random factor, while time and treatment were used as fixed factors. Post hoc pairwise comparisons were performed using Tukey's HSD method to adjust for multiple comparisons. Wilcoxon rank sum test with Benjamini Hochberg correction to adjust for multiple testing was used to identify differences between the groups at the various time points.

For beta diversity, PERMANOVA was used to check if community structures differed between the groups at the various time points followed by pairwise adonis test (<https://github.com/bwemheu/pairwise.adonis>) for pairwise comparisons between the groups. *P* values were adjusted for multiple testing using the Benjamini Hochberg correction.

Taxa significantly different between the groups were identified at the phylum, class family, and genus levels using analysis of the composition of the microbiome (ANCOM; v2.0).²⁷ ANCOM compares the log ratio abundance of each taxon among multiple groups to all the remaining taxa. It uses the Kruskal Wallis test to assess significant differences, with multiple testing corrected by the Benjamini Hochberg (BH) false discovery rate. For this study, we accepted the *W* statistic cutoff of 0.7 from the ANCOM output to define taxa as significantly different. Pairwise comparisons in the groups over time was then determined using our LME model. For pairwise comparison of the taxa at T3, Wilcoxon rank sum test with BH correction was

used. For all microbiota and SCFA analysis an adjusted *p* value of <0.05 was considered significant.

Statistical analysis of SCFA data. Wilcoxon's rank analysis with BH correction was performed to compare SCFA concentrations between the two groups (probiotics and placebo) at the different time points.

An independent data safety and monitoring committee (DSMC) reviewed the data at 50% of recruitments and advised to continue the study. The safety issues that arose during the early stages of the trial are described in Supplementary File 1. The CONSORT checklist (Supplementary File 2) was used to report the results of this RCT.²⁸

RESULTS

The study recruitment was completed in March 2020 after achieving full sample size. A total of 61 infants were randomised to receive either probiotics ($n = 30$) or placebo ($n = 31$). All infants received the trial supplements as per original randomisation without any crossovers. All infants were analysed as per the original assigned groups. Figure 1 shows a CONSORT flow diagram. Table 1 presents the clinical details of the study infants. The median gestational age, birth weight, Apgar scores, cord pH, and lactate levels were similar between the two groups. The incidence of maternal pregnancy-

Table 1. Clinical characteristics of study infants.

	Probiotic (N 30)	Placebo (N 31)	P value
Gestation (weeks)	38.1 (IQR: 37.1–39) (range: 35.1–41)	37.7 (IQR: 37.1–38.9) (range: 35.6–41.8)	0.675
Birth weight (g)	2960 (IQR: 2570–3688) (range: 1885–4130)	2985 (IQR: 2570–3270) (range: 2000–3730)	0.531
Birth length (cm)	50 (IQR: 47–51) (range: 40–57)	49 (IQR: 47–50) (range: 43–56)	0.623
Birth head circumference (cm)	33.7 (IQR: 32.5–35) (range: 31–37)	34 (IQR: 33–35) (range: 31–37.5)	0.820
Maternal PIH	0	1 (3%)	1.000
Maternal APH	7 (23.3%)	0	0.005
Maternal diabetes	1 (3.3%)	5 (16%)	0.20
Maternal chorioamnionitis	0	0	NE
Maternal intrapartum antibiotics	18 (60%)	20 (64%)	0.934
Caesarean	12 (40%)	22 (71%)	0.021
Apgar scores at 5 min	9 (IQR: 9–9) (range: 7–10)	9 (IQR: 8–9) (range: 6–10)	0.768
Cord pH	7.27 (IQR: 7.23–7.31) (range: 7.15–7.40)	7.28 (IQR: 7.23–7.33) (range: 7.1–7.36)	0.862
Cord lactates (mmol/L)	3.3 (IQR: 2.1–4.3) (range: 2–9.4)	3.8 (IQR: 2.4–5) (range: 1–8)	0.632
Major gastrointestinal conditions	CDH: 7, gastroschisis: 7; OA with TOF: 5, imperforate anus: 1; CDO: 2; exomphalos: 1; ileal atresia: 2; JIA: 2; malrotation: 1, meconium ileus: 0; colon perforation: 1; HD: 1, duplication cyst: 0	CDH: 1, gastroschisis: 5; OA with TOF: 5, imperforate anus: 6; CDO: 4, exomphalos: 1; ileal atresia: 1; JIA: 2; malrotation: 0, meconium ileus with CF: 2; colon perforation: 0; HD: 3, duplication cyst: 1	0.166
CDH	7	1	0.026
Day of life, first stool sample	6 (IQR: 5–6) (range: 2–17), N 30	6 (IQR: 3–9) (range: 1–25), N 31	0.425
Day of life, second stool sample	15.5 (IQR: 13–17.5) (range: 11–27), N 28	13 (IQR: 12–18) (range: 9–34), N 30	0.121
Day of life, third stool sample	23 (IQR: 20–25) (range: 16–30), N 19	20 (IQR: 20–26) (range: 13–42), N 19	0.463
Day of life, fourth stool sample	38.5 (IQR: 29.5–42) (range: 26–46), N 8	34 (IQR: 32–38) (range: 27–53), N 6	0.824
Day of life consent was given	5 (IQR: 4–7) (range: 2–18)	5 (IQR: 3–7) (range: 2–25)	0.535
Day of life supplements were commenced	6.5 (IQR: 5–9) (range: 4–18)	7 (IQR: 5–11) (range: 3–26)	0.820
Duration of supplementation (days)	18.5 (IQR: 11–27) (range: 3–54)	16 (IQR: 10–22) (range: 4–58)	0.337

Bold values indicate statistical significance $p < 0.05$.

SVD spontaneous vaginal delivery, AVD assisted vaginal delivery, CS caesarean section, APH antepartum haemorrhage, PIH pregnancy induced hypertension, NE not estimable, IQR interquartile range, OA oesophageal atresia, JIA jejuno ileal atresia, CF cystic fibrosis, HD Hirschsprung disease, IQR interquartile range.

induced hypertension (PIH), chorioamnionitis, and peripartum antibiotics was similar between the groups. The incidence of antepartum haemorrhage (APH) was higher, and caesarean delivery rates were lower in the probiotic group. The probiotic group had more infants with congenital diaphragmatic hernia than the placebo group. The median age at surgery was 2 days in both the groups. The median age at commencement of supplementation was approximately 7 days in both groups, and the duration of supplementation was 16–18 days.

Results of microbial analysis

Of the 61 recruited infants, stool samples were available for 61, 58, 39, and 29 infants at time points T1, T2, T3, and T4, respectively. The attrition in sample size was because 22 infants were discharged home before completing 2 weeks of supplementation (i.e., before reaching T3). We could not extend the trial beyond March 2020 to recruit additional infants because of severe restrictions that were implemented due to the COVID-19 pandemic during that period.

Alpha diversity (i.e., Richness and Shannon diversity index) was comparable between the two groups at all time points (all $p > 0.05$; Fig. 2a). Similar results were obtained on Chao1 and ACE estimations (data not shown). Observed OTUs increased in both groups over time and the probiotic group displayed significantly increased bacterial richness at T4 compared to T1 ($p = 0.015$) and

T2 ($p < 0.01$; Fig. 2a). Beta diversity analysis revealed that both groups had similar community structures at T1 ($p = 0.807$; Fig. 2b). However, at T2, T3, and T4, the community structures of the probiotic group were significantly different from those of the placebo group (all $p < 0.05$; Fig. 2b).

Comparisons at the bacterial phylum level. Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Proteobacteria were the most common phyla in the stool samples (e-Fig. 1A in Supplementary File 1). The median relative abundance of Actinobacteria was significantly higher in the probiotic group than in the placebo group at T3 (40.1% versus 0.1%; $p < 0.0001$) and at T2 and T4 (both $p < 0.01$; e-Fig. 1B in Supplementary File 1). Although the relative abundance of Proteobacteria was similar between the two groups at T3 (22.7% in probiotic versus 50.3% in placebo; $p = 0.27$) and at other time points (e-Fig. 2 in Supplementary File 1), the levels in the probiotic group decreased over time, with T2 and T4 being significantly different from T1 ($p = 0.048$ and $p = 0.046$, respectively).

Comparisons at the bacterial class level. The relative abundance of Gammaproteobacteria between the two groups was similar at all time points (e-Fig. 3 in Supplementary File 1).

Comparisons at the bacterial family level. The relative abundance of the sum of potentially pathogenic families of Clostridiaceae,

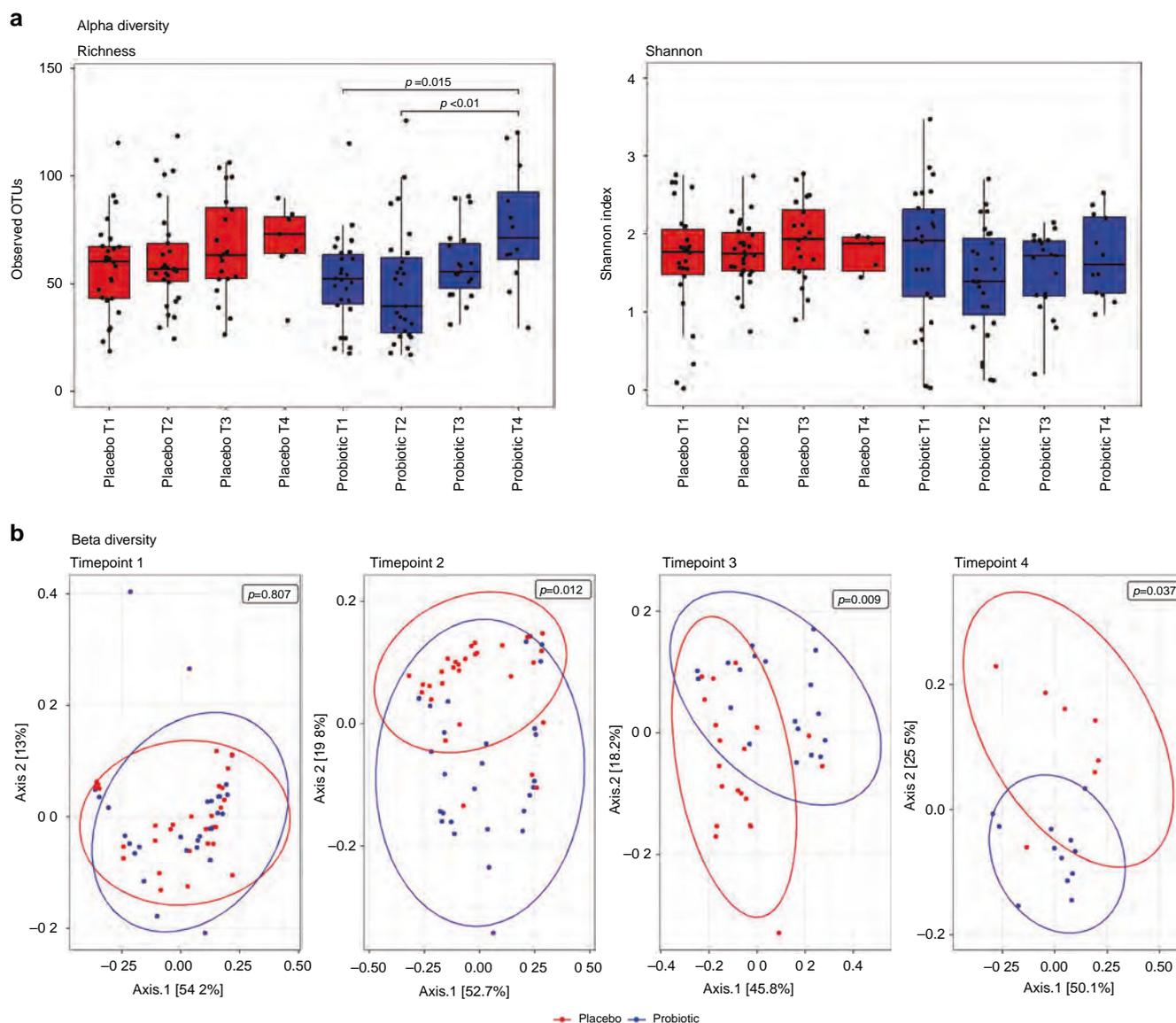


Fig. 2 Alpha and Beta diversity at various time points in the stool samples of study infants. **a** Alpha diversity. Richness and the Shannon diversity index were similar between the probiotic and placebo groups at all time points (all $p > 0.05$). **b** Beta diversity as measured by weighted unifrac by time point. At baseline, infants in the probiotic group had similar community structures to the placebo group ($p = 0.807$). However, at subsequent time points T2, T3, and T4, the community structures of the probiotic group were significantly different from the placebo group (all $p < 0.05$).

Enterobacteriaceae, Enterococcaceae, Pseudomonadaceae, Staphylococcaceae, Streptococcaceae, and Yersiniaceae were significantly lower in the probiotic group compared to placebo at T3 [(median: 50.4 (IQR: 26.6–67.6) versus 67.1 (IQR 50.9–96.2); $p = 0.044$) and at T2 and T4 ($p = 0.006$ and $p = 0.014$, respectively; Fig. 3a). Since there was a slight imbalance in the number of stool samples between the probiotic ($n = 19$) and placebo ($n = 20$) groups at T3, one sample from the placebo group was randomly removed and the data were reanalysed. The results continued to remain similar to the original analysis ($p = 0.036$).

The relative abundance of the family Bifidobacteriaceae was significantly higher in the probiotic group at T3 [(median: 39.8 (IQR: 24.9–52.1) versus 0.03 (IQR 0.02–2.1); $p < 0.001$) and at T2 and T4 (both $p < 0.001$; Fig. 3b).

Comparisons at the bacterial genus level. The relative abundance of the genus *Bifidobacterium* was significantly higher in the

probiotic group at T2, T3, and T4 (all $p < 0.001$; e-Fig. 4 in Supplementary File 1).

Stool SCFAs: The total SCFA levels were higher in the probiotic group than in the placebo group at T3 ($p = 0.008$; Fig. 4). Acetate levels were higher in the probiotic group (e-Fig. 5 in Supplementary File 1). The butyrate levels were similar between the groups at all time points except T2, when they were lower in the probiotic group (e-Fig. 6 in Supplementary File 1). The propionate levels were similar between the two groups at all time points (e-Fig. 7 in Supplementary File 1).

Post hoc subgroup analysis based on the mode of delivery

Since the probiotic group had significantly lower rates of caesarean section, we conducted a post hoc analysis separately for infants born via caesarean section and through the vaginal route. The results showed a significantly higher abundance of Bifidobacteriaceae in the

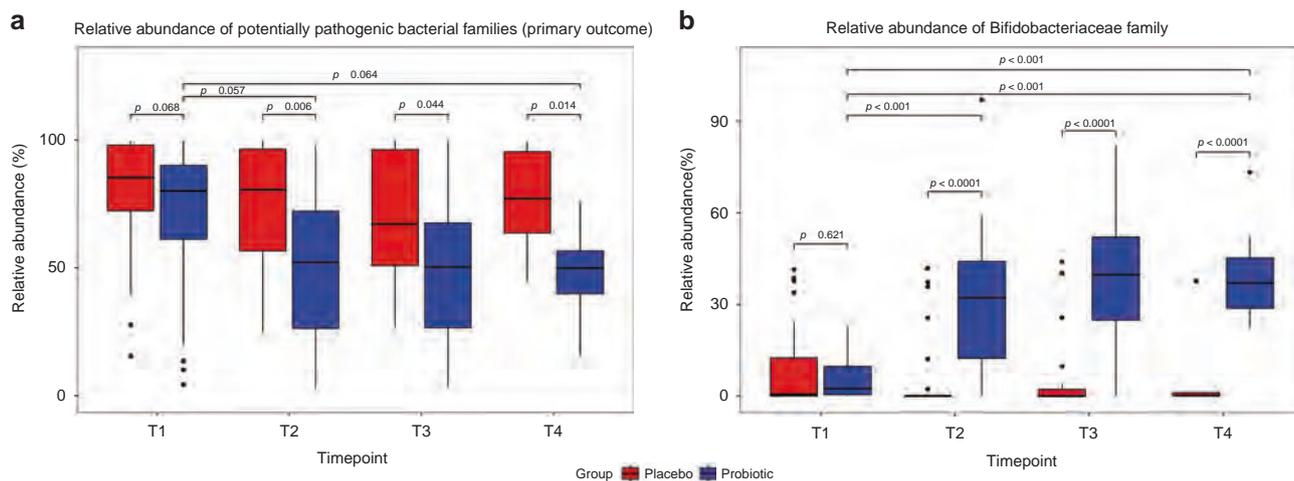


Fig. 3 Comparisons of relative abundances of bacterial families in the stool samples at various time points. **a** Comparison of relative abundance of potentially pathogenic families (sum-total of Clostridiaceae, Enterobacteriaceae, Enterococcaceae, Pseudomonadaceae, Staphylococcaceae, Streptococcaceae and Yersiniaceae) between probiotic and placebo at various time points. At the bacterial family level, the relative abundance of the sum-total of potentially pathogenic families of Clostridiaceae, Enterobacteriaceae, Enterococcaceae, Pseudomonadaceae, Staphylococcaceae, Streptococcaceae & Yersiniaceae were significantly lower in the probiotic group compared to placebo at time points T2, T3, and T4 ($p = 0.002$, 0.033 , and 0.007 respectively). **b** Comparison of relative abundance of family Bifidobacteriaceae between probiotic and placebo at various time points. The relative abundance of the family Bifidobacteriaceae was significantly higher in the probiotic group at T2, T3, and T4 (all $p < 0.001$).

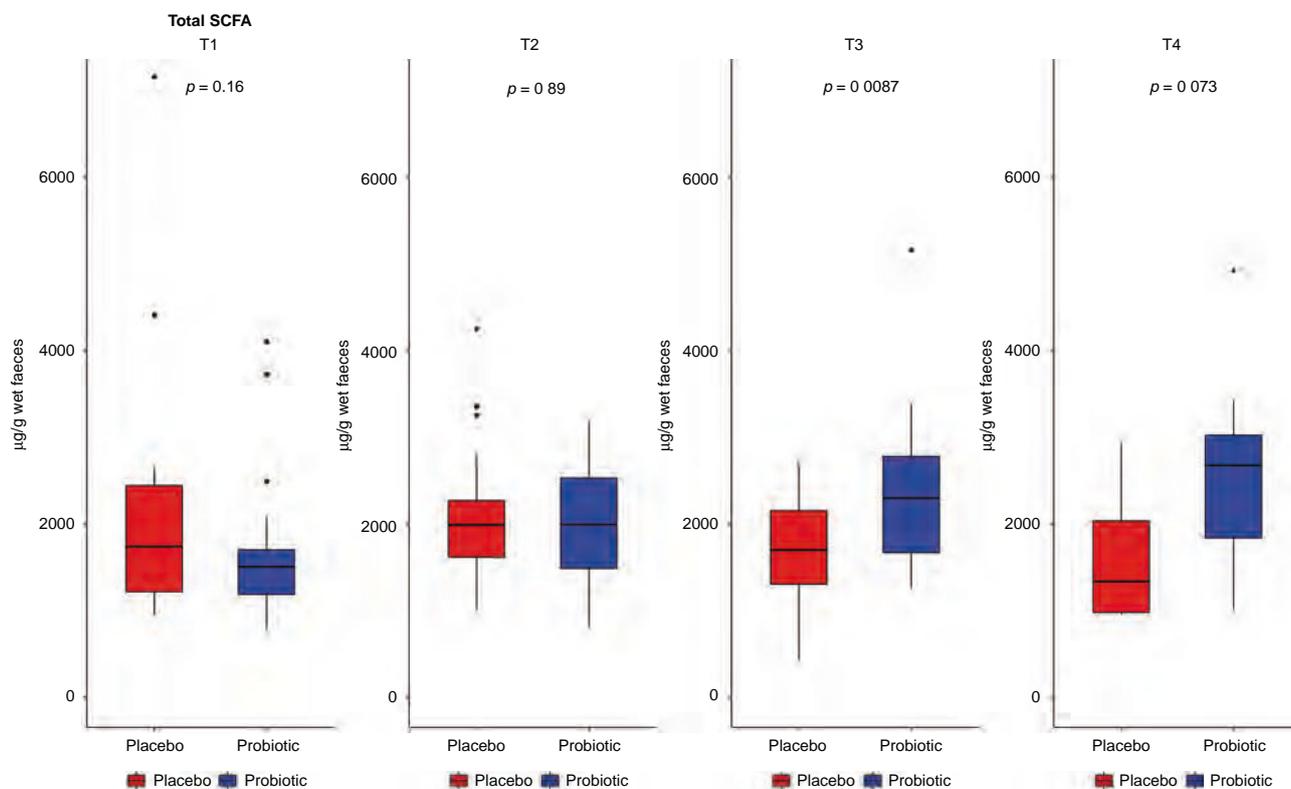


Fig. 4 Total SCFA levels in stools between probiotic and placebo groups at various time points. Total SCFA levels in stools were significantly higher in the probiotic group at time point T3 ($p = 0.008$). At other time points, the differences between the two groups were not statistically significant.

probiotic group, irrespective of the mode of delivery. However, the beneficial effects of supplementation on SCFA levels were significant only in infants born by caesarean section. The relative abundance of pathogenic families was not statistically significant in either mode of delivery, even though the trend was in favour of probiotics (Table 2).

Clinical outcomes

All participating infants survived. There were no significant differences between the groups in the incidence of HAI, duration of antibiotics, PN, hospital stay, and time to reach full feeds after surgery (Table 3). All routine clinical specimens (blood, urine, CSF,

Table 2. Effect of probiotics supplementation at T3, based on route of delivery. Effect of probiotics supplementation based on route of delivery

	Vaginal delivery			Caesarean section		
	Probiotic Median (IQR, range) N = 9	Placebo Median (IQR, range) N = 6	P value	Probiotic Median (IQR, range) N = 9	Placebo Median (IQR, range) N = 12	P value
Sum of relative abundance of pathogenic families at T3	49.1 (25.9–67.4)	74.2 (49.6–89.8)	0.37	52.5 (44.2–67.7)	58.4 (51.2–95.9)	0.39
Relative abundance of Bifidobacteriaceae at T3	42.8 (23.7–55.2)	0.05 (0.03–12.9)	0.049	33.7 (28.9–45.4)	0.03 (0.01–1.51)	0.001
Total SCFA levels at T3 (µg per gram of wet faeces)	2254 (QR: 1915–2668); range: 1243–5152	1529 (QR: 1349–2208); range: 723–2647	0.18	2598 (QR: 1588–2786); range: 1399–3388	1725 (QR: 1092–2135); range: 426–2723	0.034

Bo d va ues indicate statistica significance $p < 0.05$. IQR interquartile range, SCFA short-chain fatty acid, T3 two weeks after commencing supplementation.

endotracheal secretions, wound swabs) from study infants were analysed using aerobic and anaerobic culture methods. There were no cases of infections due to the administered probiotics.

Physical growth outcomes

The z-scores for weight were significantly lower at discharge than at birth in the probiotic and placebo groups (both $p < 0.0001$; e-Fig. 8 in Supplementary File 1). The degree of postnatal growth restriction for weight was similar between the two groups (0.93 in the probiotic and 0.79 in the placebo; $p = 0.486$; Table 1, e-Fig. 9 in Supplementary File 1). The z-scores for head circumference were lower at discharge than at birth in both the probiotic and placebo groups (both $p < 0.0001$; e-Fig. 10 in Supplementary File 1). However, postnatal growth restriction for head circumference was less severe in the probiotic group ($p = 0.013$; Table 1 and Fig. 5). The z-scores for length at discharge were similar to those at birth in both the probiotic and placebo groups (e-Fig. 11). The degree of postnatal growth restriction for length was similar between the two groups (e-Fig. 12).

DISCUSSION

This pilot RCT found that after 2 weeks of supplementation with the three-strain bifidobacterial probiotic, neonates with CGISC had a lower relative abundance of potentially pathogenic bacterial families, higher abundance of bifidobacteria, and higher SCFA levels in their stools compared to placebo. Postnatal growth restriction for head circumference was less severe in the probiotic group than in the placebo group. Other clinical outcomes were similar between the two groups. No infections related to the administered probiotic organisms were observed. These results provide reassurance regarding the use of this probiotic in neonates with CGISC.

The only other RCT in neonates with surgical conditions was by Powell et al.,²² in which 24 infants with gastroschisis were supplemented with *Bifidobacterium longum* subsp. *infantis* ATCC 15697 or placebo. In their study, the daily dose was 2×10^9 CFU compared with 3×10^9 CFU in our study. Similar to their study, the majority of our infants received breast milk as the sole source of diet, trial supplements were commenced in the postoperative period, and given for a median duration of 3 weeks. Similar to their study, the relative abundance of Bifidobacteriaceae increased to 40% after commencing supplements in the probiotic group and remained at 3% in the placebo group.

In line with the recent literature, our study found that neonates with CGISC develop postnatal growth restriction for weight and head circumference.^{29,30} Studies in preterm infants have reported an association between postnatal growth restriction of the head circumference and adverse developmental outcomes.^{31–33} Hence, it was reassuring that postnatal growth restriction for head circumference was less severe in the probiotic group in our RCT. Similar beneficial effects of probiotics on head circumference were observed in extremely low birth weight infants in recent RCTs.^{34,35}

In our current RCT, SCFA levels were significantly higher in the probiotic group at T3 than in the placebo group, mainly due to elevated acetates. Even though the levels were also higher at T2 and T4, they were not statistically significant, probably because infants would have received only 1 week of supplementation by T2, whereas by T4, the sample size was very small. Bifidobacteria are known to ferment HMOs and produce acetate as a by-product.³⁶ Bifidobacteria are not butyrogenic, and hence butyrate levels were not elevated in the probiotic group in our study. While it has been suggested that acetate and lactate produced by bifidobacteria can be used as a substrate by other commensal bacteria (cross-feeding)³⁷ to produce butyrate, thereby increasing its levels, this was not observed in our study.

The relative abundance of Proteobacteria, considered to be the microbial signature of gut dysbiosis,³⁸ was lower in the probiotic

Table 3. Clinical course and outcomes of study infants.

	Probiotics	Placebo	P values
Mortality	0	0	NE
Healthcare associated infections (HABSI or UTI or SSI or VAP or pleural infection or peritonitis or meningitis or viral infection)	4 (13.3%)	6 (19.3%)	0.731
HABSI before commencing trial supplements	0	1 (3%)	1.000
HABSI after commencing trial supplements	0	2 (6.5%)	0.492
Duration of antibiotics before commencement of trial supplements (days)	5 (IQR: 4 7) (range: 2 10)	5 (IQR: 3 6) (range: 1 11)	0.336
Duration of antibiotics after commencement of trial supplements (days)	3 (IQR: 0 6) (range: 0 31)	2 (IQR: 0 5) (range: 0 13)	0.305
Total duration of antibiotics (days)	9 (IQR: 4 13) (range: 2 34)	6 (IQR: 5 10) (range: 1 22)	0.312
Cholestatic jaundice	1 (3.3%)	1 (3.2%)	1.000
Sepsis due to the administered probiotic organism	0	0	NE
Duration of PN (days)	13.9 (IQR: 9 24) (range: 0 46)	10.1 (IQR: 6.9 21.3) (range: 0 58.7)	0.246
EBM to commence feeds	30 (100%)	28 (93.3%)	0.492
Time to commence feeds after surgery (days)	3 (IQR: 2 5) (range: 0 10)	2 (IQR: 1 4) (range: 0 9)	0.023
Time to full enteral feeds after surgery (days)	12 (IQR: 8 16) (range: 0 46)	9 (IQR: 6 15) (range: 1 52)	0.306
Exclusive EBM at discharge	24 (80%)	22 (71%)	0.554
Use of formula milk	6 (20%)	9 (29%)	0.554
Duration of hospital stay (days)	27.5 (IQR: 16 37) (range: 10 102)	20 (IQR: 13 31) (range: 10 67)	0.094
Z scores for weight at discharge minus at birth	0.93 (SD 0.52)	0.79 (SD 1.00)	0.486
Z scores for head circumference at discharge minus at birth	0.20 (SD 0.63)	0.70 (SD 0.84)	0.011
Z scores for length at discharge minus at birth	0.40 (SD 1.02)	0.14 (SD 1.20)	0.455

Bold values indicate statistical significance $p < 0.05$.

EBM expressed breast milk, NE not estimable, HABSI healthcare associated bloodstream infections, UTI urinary tract infection, SSI surgical site infection, VAP ventilator associated pneumonia, EBM expressed breast milk, IQR interquartile range, NE not estimable, SD standard deviation.

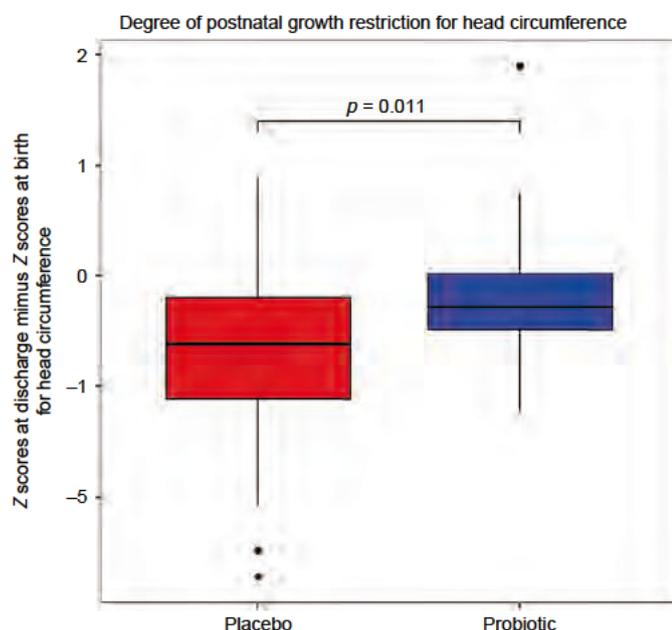


Fig. 5 Comparison of the degree of postnatal growth restriction for head circumference in study infants. The degree of postnatal growth restriction for head circumference was less severe in the probiotic group than in the placebo group.

group than in the placebo group (22.7% versus 50.3%), but the differences were not statistically significant. Studies with larger sample sizes are needed to determine if this difference is real.

Although not statistically significant, hospital stay was longer in the probiotic group (27 versus 20 days, $p = 0.095$). We speculate that this was because the probiotic group had more infants with complex conditions such as CDH and gastroschisis, and fewer infants with imperforate anus.

It was reassuring that none of the infants developed infection due to the administered bifidobacterial strains. There are case reports of bifidobacterial sepsis in surgical infants and preterm infants without surgical conditions.^{39,40} Hence, ongoing microbiological surveillance is important when conducting probiotic RCTs.

In our study infants, the use of exclusive breast milk dropped to 70–80% by discharge compared to 93–100% at birth. However, the use of formula milk was similar between the two groups (20% in the probiotic group versus 29% in the placebo group; $p = 0.554$). The common reasons for the use of formula milk were inadequate production of breast milk and mother's choice. Some studies have suggested that probiotics are more effective in infants who are breastfed rather than formula.^{41–43} Given such findings and overall benefits of breast milk, every effort should be made to encourage breastfeeding and to express breast milk during hospital stay and after discharge in these infants.

While our study did not specifically address the issue of cross-contamination (aka cross-colonisation),⁴⁴ the relative abundance of the genus *Bifidobacterium* in the placebo group was only 5% at all time points T2–T4, versus 35–45% in the probiotic group. Hence, even if there was cross-contamination, the load was not

enough to allow them to colonise adequately in the placebo group and, hence, unlikely to be clinically significant.

An important limitation of our study was the higher rates of caesarean delivery in the placebo group. Infants born by caesarean delivery are known to have a lower abundance of bifidobacteria in the first few months of life.⁴⁵ In this context, it was reassuring to know that even in subgroup analysis, the probiotic supplemented group had higher bifidobacterial counts. Our findings are similar to Frese et al.⁴⁶ who reported that supplementation resulted in higher colonisation with bifidobacteria, which persisted even after supplementation was ceased, whether the infants were born vaginally or by caesarean section.

Since many factors such as gestational diabetes,⁴⁷ mode of delivery,⁴⁵ intrapartum antibiotics^{48–50} and maternal probiotics^{51–53} can affect neonatal gut microbiota, it is difficult to achieve a balance between the two groups for all such variables in small RCTs. Future studies should consider using the technique of “allocation by minimisation”⁵⁴ to ensure adequate balance. In addition to maternal factors, important neonatal variables to be balanced include gestational age⁵⁵ and underlying surgical conditions. Since the type of milk used (breast milk versus cow’s milk based versus hydrolysed formula) can influence gut microbiota,⁵⁶ standardising their feeding regimen is desirable, but may not be feasible, given that multiple factors can influence milk production and its content. One option is to use pasteurised human donor milk, but the quantity required will be enormous because majority of these infants are born at term or near term, rather than at an extremely preterm gestation, in whom the quantity required is small. RCTs with large sample size have the potential to minimise the risk of imbalance in two groups regarding the type of milk used and other variables.

Another limitation of our study was that 16S rRNA gene sequencing allowed the allocation of reads only up to the genus level. Hence, it was not possible to confirm whether the increase in the relative abundance of *Bifidobacterium* in the probiotic group was due to the administered strains. A whole-metagenome approach that provides species- and strain-level information needs to be incorporated in future studies. Another limitation of the study was that stool samples from only 39 of the 61 study infants were available for analysis at T3, because others were discharged home by then.

While surgical conditions included in our RCT were heterogeneous, all of them had common issues such as feed intolerance, need for PN, and risk of infections. Hence, we decided to include all types of gastrointestinal surgical conditions in this pilot RCT. Another reason was because it would have taken at least 7–9 years to achieve the sample size of 60 if we had conducted it in a single surgical condition (for example, our unit admits only 10 cases of gastroschisis per year).

There is some observational evidence from adult literature that maltodextrin may lead to adverse health outcomes secondary to alterations in gut microbiota.⁵⁷ In our RCT, the probiotic supplement contained probiotic organisms and maltodextrin, whereas placebo was only maltodextrin. Hence, any effect of maltodextrin on gut microbiota would have occurred in both placebo and probiotic groups. To evaluate the effects of maltodextrin (at the small dose of 1 gram per day as used in our trial) on gut microbiota, prospective cohort studies comparing gut microbiota in healthy breastfed infants receiving maltodextrin versus healthy breastfed infants are needed.

The important strengths of our study are as follows: (a) It is the first RCT to evaluate the efficacy and safety of the three-strain bifidobacterial supplementation in CGISC in neonates and (b) all study infants had baseline stool samples prior to commencing supplements.

The current pilot RCT found that the degree of postnatal growth restriction of head circumference was less severe in the probiotic supplemented group than placebo. Hence, one could hypothesise that probiotic supplemented group will have better

neurodevelopmental outcomes. Our recent retrospective study³⁰ with a sample size of 400 found the incidence of suboptimal neurodevelopmental outcomes (SNDO) to be 16% in term and near-term neonates with CGISC. A sample size of 516 infants (258 in each arm) will be required to have 80% power at the two-sided 5% significance level to detect a 50% difference in the primary outcome (16% in controls and 8% in the probiotic group). Since nearly 30% of them are expected to be discharged home even before completing 2 weeks of supplementation, the sample size should be increased by another 154, and hence the final sample size will be around 670 infants. With such a large sample size, baseline frequencies of potential confounders such as gestational age, maternal antibiotics, maternal probiotic usage, mode of delivery, severity of illness and type of milk feeds are expected to be balanced between the two groups. Involvement of multiple centres will be crucial to achieve this sample size within a reasonable time period of 24–36 months.

In summary, this pilot RCT found that after 2 weeks of supplementation with the three-strain *Bifidobacterium*, neonates with CGISC had a lower relative abundance of potentially pathogenic bacterial families, higher abundance of *Bifidobacterium*, and higher SCFA levels in their stools. Larger studies with clinical endpoints and long-term follow-ups are necessary.

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AUTHOR CONTRIBUTIONS

Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, and final approval of the version to be published: S.C.R., M.E., L.C., A.D.K., I.J.G., K.N.S., B.W., P.L.C., and S.K.P.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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REVIEW ARTICLE **OPEN**


Probiotic supplementation for neonates with congenital gastrointestinal surgical conditions: guidelines for future research

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Our pilot RCT found that probiotic supplementation with the three-strain bifidobacterial product (*B. breve* M-16V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536) attenuates gut dysbiosis, increases stool short-chain fatty acid (SCFA) levels and improves the growth of head circumference in neonates with congenital gastrointestinal surgical conditions (CGISC). In this article, we have provided guidelines for designing future multicentre RCTs based on the experience gained from our pilot RCT. The recommendations include advice about sample size, potential confounders, outcomes of interest, probiotic strain selection, storage, dose, duration and microbial quality assurance, collection of stool samples, storage and analysis and reporting. Following these guidelines will increase the validity of future RCTs in this area and hence confidence in their results.

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IMPACT:

- Probiotic supplementation attenuates gut dysbiosis, increases stool short-chain fatty acid (SCFA) levels and improves the growth of head circumference in neonates with congenital gastrointestinal surgical conditions.
- The current review provides evidence-based guidelines to conduct adequately powered RCTs in this field.

BACKGROUND

Our pilot randomised controlled trial (RCT)¹ found that supplementation with the three-strain probiotic containing *Bifidobacterium breve* M-16V, *Bifidobacterium longum* subsp. *infantis* M-63 and *Bifidobacterium longum* subsp. *longum* BB536 attenuates gut dysbiosis and increases faecal short-chain fatty acid (SCFA) levels in neonates with congenital gastrointestinal surgical conditions (CGISCs). The head circumference growth was better in the probiotic group. While the results were in favour of probiotics, there were areas of uncertainty due to the following reasons:

- Caesarean section^{2,3} rates were higher in the placebo group (70 versus 40%), whereas the incidence of congenital diaphragmatic hernia was higher in the probiotic group (23 versus 3%).
- The relative abundance of proteobacteria, considered the signature of dysbiosis,⁴ was not significantly different between the two groups after 2 weeks of supplementation (22.7% in probiotic versus 50.3% in placebo; $p = 0.27$).
- Being a pilot RCT, the study was not powered to identify clinically significant differences between the groups.

Based on the experience gained from our pilot RCT,¹ we make the following suggestions for designing future RCTs to overcome the above uncertainties. Some of the suggestions are general and could be used for probiotic RCTs in other population such as preterm infants for prevention of necrotising enterocolitis (NEC).

SAMPLE SIZE

Our pilot RCT found that the head circumference growth was better in the probiotic group.¹ Hence, one could hypothesise that the probiotic supplemented group will have better neurodevelopmental outcomes. Our retrospective study⁵ with a sample size of 400 found the incidence of suboptimal neurodevelopmental outcomes to be 16% in term and near-term infants with CGISC. A sample size of 516 infants (258 in each arm) will be required to have 80% power at the two-sided 5% significance level to detect a 50% difference in the primary outcome (16% in controls and 8% in the probiotic group). Since nearly 30% of infants are expected to be discharged home before completing 2 weeks of supplementation, the sample size should be increased by another 154, and hence the final size will be 670 infants. The involvement of

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multiple centres will be crucial to achieving this sample size within a reasonable time period.

The results of our pilot RCT¹ also provide the data for estimating the sample sizes for RCTs primarily aimed to compare the stool bacterial community structures between the probiotic and placebo groups. The SIMR package⁶ allows users to calculate sample sizes and conduct power analysis for longitudinal studies. For studies comparing beta-diversity, micropower package⁷ can be used as it calculates sample sizes using pairwise distance and permutational multivariate analysis of variance. It is essential to involve bioinformaticians to ensure appropriate methods for power and sample size calculations and plan the statistical techniques for analyses based on the study hypothesis and design.^{8–10}

INCLUSION CRITERIA

Our pilot RCT¹ focussed on late preterm and term infants with CGISC to avoid the confounding effect of extreme prematurity on gut microbiota. Future RCTs, if of adequate sample size, could include even very preterm (<32 weeks) and extremely preterm (<28 weeks) infants with CGISC. With a large sample size, baseline characters, including gestational age, are expected to be similar between the study groups. Probiotic supplementation is safe and beneficial even in extremely preterm infants without surgical conditions and reduces the risk of NEC, feed intolerance, and late-onset sepsis.^{11–15}

RANDOMISATION

Since our pilot RCT¹ had a small sample size of 60 infants, we used computer-generated random sequence numbers in random blocks of two and four to ensure that an equal number of infants receive probiotics or a placebo. However, this method did not minimise the chance of unequal distribution of essential confounders (e.g. mode of delivery and severity of the surgical condition), which can affect the gut microbiota.

Therefore, future studies should consider using “treatment allocation by minimisation”¹⁶ or “rank minimisation”¹⁷ to achieve balance, especially if the sample size is small. The involvement of clinical epidemiologists at a very early stage of trial protocol is crucial to achieving the ideal design for the trial, including the randomisation method.

Factors such as gestational diabetes,¹⁸ mode of delivery,³ intrapartum antibiotics^{19–21} and maternal probiotics,^{22–24} gestational age²⁵ and underlying surgical conditions that can affect the neonatal gut microbiota need to be balanced between the probiotic and placebo groups. Since the type of milk used (breast milk versus cow’s milk-based versus hydrolysed formula) can influence gut microbiota,²⁶ standardising their feeding regimen is desirable but unlikely to be feasible, given that multiple factors can affect milk production and mother’s choice and clinicians’ opinions.

SELECTION OF PROBIOTIC PRODUCT

So far, only two pilot RCTs have evaluated probiotics in neonates with CGSC. The first study was by Powell et al., who reported that daily administration of the single-strain probiotic *B. longum* subsp. *infantis* (2×10^9 colony-forming unit (CFU) per day) resulted in partial attenuation of gut dysbiosis in neonates with gastro-schisis.²⁷ The second RCT was ours,¹ in which the multi-strain probiotic product, a mixture of *B. breve* M-16V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536 (1×10^9 of each strain per sachet; Morinaga Milk Industry Co, Japan) was safe and effective in improving gut microbiota and SCFA levels. We found the study sachets (probiotics and placebo) free of harmful bacteria by conducting regular microbial analyses on random sachets.

Future RCTs in neonates with CGISC could consider using this product or the strain used by Powell et al.²⁷ In the other small RCT currently being conducted in Canada (ClinicalTrials.gov identifier NCT03266315), 20 newborn infants with CGSCs will be randomised to receive the multi-strain product FloraBaby™, a mixture of bifidobacteria and lactobacilli strains. It is essential to wait for the results of that study before conducting multicentre RCTs using that product in neonates with CGISC.

While many other probiotic products are safe and effective in preterm infants without surgical conditions, it is essential to test them in pilot RCTs in neonates with CGISCs before considering them in large multicentre RCTs. This is because, unlike preterm infants without CGISCs, neonates with CGISC undergo contrast dye studies, laparotomies, get exposed to general anaesthetics and some of them receive bowel enemas/suppositories, all of which could influence the effects of the administered probiotics.

Another factor to consider is the safety of supplemented probiotics, especially the potential for translocation and causing sepsis. Unlike preterm infants without surgical problems, neonates with CGISC undergo laparotomy. Hence, there is a risk that the administered live probiotic bacteria could spill over into the peritoneal cavity and systemic circulation resulting in sepsis and seeding in various organs. While infants in our pilot RCT¹ or Powell et al.²⁷ did not experience such a complication, a large sample size study would be needed to ensure safety.

In that context, it is also reassuring that infection due to administered probiotic organisms is infrequent even among extremely preterm infants and can be treated with antibiotics. Sakurai et al.²⁸ reported that bifidobacterial bacteraemia occurred in 6 out of 298 neonates (i.e. 2%), but none had severe illnesses due to bacteraemia. They speculated that the reason behind the high incidence of *B. breve* bacteraemia in their cohort was rigorous laboratory methods.

Alternatives to probiotics such as prebiotics^{29,30} and paraprobiotics (dead probiotic bacteria)³¹ that are unlikely to carry the risk of probiotic sepsis should also be tested in future RCTs.

DOSE OF PROBIOTIC SUPPLEMENTS

To our knowledge, there are no dose-finding studies in neonates with surgical conditions. There are two studies evaluating the dose response^{32,33} and one study on dose interval³⁴ of probiotics on colonisation rates in preterm infants.

Dutta et al.³² randomly allocated 149 preterm infants to groups A–D (received 12-hourly probiotic supplements of 10^{10} cells for 21 days, 10^{10} cells for 14 days, 10^9 cells for 21 days and placebo, respectively). They reported that colonisation with *Lactobacillus* and *Bifidobacterium* by day 28 was significantly higher in groups A, B, and C versus placebo, respectively. They also reported that there were trends toward more CFU of *Lactobacillus* and *Bifidobacterium* per ml of stool in group A versus B and group B versus C.

Underwood et al.³³ randomly allocated 12 preterm infants receiving formula feedings to receive either *B. infantis* or *B. lactis* in increasing doses over a 5-week period. The dose was 5×10^7 , 1.5×10^8 , 4.5×10^8 , 1.4×10^9 , 4.2×10^9 , at weeks 1, 2, 3, 4 and 5, respectively. There was a greater increase in faecal bifidobacteria among infants receiving *B. infantis* than those receiving *B. lactis*. This difference was most marked at a dose of 1.4×10^9 CFU twice daily. Relative abundance of bifidobacteria declined with increasing dosage over time/dose in the *B. lactis* group and showed a statistically nonsignificant trend towards increase in the *B. infantis* group. It shows that the colonisation response is not only dependent on the dose but also on the strain.

Watkins et al.³⁴ investigated the appropriate dosing interval of a dual strain probiotic given daily ($n=8$), biweekly ($n=8$) and weekly ($n=10$) in preterm infants <32 weeks’ gestation. The control group consisted of 12 preterm infants who did not receive

the probiotic. *Bifidobacterium bifidum* (1×10^9 CFU) and *Lactobacillus acidophilus* (1×10^9 CFU), was administered (2×10^9 CFU of bacteria in total), until 34 weeks postmenstrual age (PMA). Stool samples were collected at 31, 34, 41 and 44 weeks PMA. At all ages assessed, colonisation levels of administered probiotic organisms were higher in the once daily group. They concluded that a daily dose is a suitable dosage for preterm infants.

Given that there is insufficient evidence regarding the dose of probiotics, dose-finding studies with the chosen strain/s need to be conducted prior to embarking on large RCTs.

Our study¹ used a total of 3 billion probiotic organisms per day (3×10^9 CFU), whereas Powell et al.²⁷ had used 2 billion probiotic organisms per day (2×10^9 CFU). Powell et al.²⁷ reported partial attenuation of gut dysbiosis after probiotic supplementation, whereas our study showed a higher attenuation level. Hence, it provides indirect evidence that a dose of at least 3×10^9 CFU per day could be used in future RCTs. Doses higher than 3×10^9 CFU per/day may offer further benefits but need to be tested in dose-finding studies initially (for example, 3×10^9 versus 4×10^9 versus 5×10^9 versus 6×10^9). Smaller quantities <1 billion (i.e. $<1 \times 10^9$) CFU may not be enough to colonise the gut adequately, limiting their effectiveness.^{35,36}

STORAGE OF PROBIOTICS/PLACEBO SACHETS

We stored the main stock of probiotic/placebo sachets in the trial pharmacy department in the refrigerator at 2–8 °C. To enable ease of access, at least five such sequentially numbered boxes were kept in the automated dispensing machine (ADM) within the neonatal intensive care unit (NICU) at refrigerator temperatures of 2–8 °C. Once parental consent was obtained, the box next in order was labelled with the Unique Medical Record Number sticker of that infant, thereby declaring that particular package belongs to that infant for the entire hospital stay. Each day, one sachet from that box was taken out from the ADM by the nurses and administered to that particular infant. A similar approach could be undertaken in future RCTs. Keeping the trial supplements at room temperatures may affect their viability and longevity.

TIMING OF STARTING THE TRIAL SUPPLEMENTS

In our study,¹ trial supplements were commenced predominantly in the immediate postoperative period. The main reason for the delay was difficulty collecting baseline stool samples for reasons discussed earlier. In our RCT and the RCT by Powell et al., probiotics were commenced in the immediate post-operative period and found it to attenuate dysbiosis. The majority of the studies involving adults who underwent gut surgeries found benefits of probiotics when administered in the preoperative period.³⁷ To obtain maximum benefit, the best time to commence probiotics is probably in the pre-operative period, but collection of stool samples before starting probiotics may not be feasible in all cases.

ADMINISTRATION OF PROBIOTICS WHILE INFANTS ARE FED NIL-ENTERALLY

We administered the study supplements even when the infants were fed nil-enterally and found no side effects.¹ Waiting until enteral feeds are commenced may decrease the efficacy of probiotics by delaying their gut colonisation. Considering the small dose volume and low osmolarity (320–350 mOsm/l) when reconstituted in expressed breast milk,³⁸ it is reasonable to start the supplement even if the infant is fed nil-enterally.

WHEN TO WITHHOLD SUPPLEMENTATION

In our study,¹ we continued supplementation even when infants were critically ill as long as there were no significant abdominal

symptoms. We suggest that supplements only be withheld if there is a gut perforation or suspicion of abdominal compartment syndrome with a distended, firm and tender abdomen to minimise the risk of gut translocation by the supplemented probiotic organisms.

CARE IMMEDIATELY AFTER ADMINISTERING THE TRIAL SUPPLEMENTS

We administered the trial medication as a single daily dose for convenience. Many infants with CGISC will have nasogastric tubes (NGTs) on free drainage or suction for gastric decompression. We clamped the NGT for at least 1 h and preferably 3–4 h to prevent the retrograde flow of the administered probiotics/placebo into the free-drain container.²⁷

HAND HYGIENE PRECAUTIONS

Rigorous hand hygiene needs to be followed to prevent the risk of cross-contamination. While our pilot RCT¹ did not specifically address the issue of cross-contamination (aka cross-colonisation),³⁹ we were reassured that the relative abundance of the genus *Bifidobacterium* in the placebo group was only about 5% at all time points T2–T4. In contrast, it was around 35–45% in the probiotic group. Hence, even if there was cross-contamination, the load was not enough to allow them to colonise adequately and, therefore, unlikely to be clinically significant.

The relative abundance of 5% for the genus *Bifidobacterium* in the placebo arm after 2 weeks of supplementation and also prior to discharge in our study¹ was lower than the Australian PROPREAMS trial in preterm infants, in which it was 17.5% (SD 27.4) in the placebo group and 36.4% (32.5) in the probiotic group.⁴⁰ The UK PIPS trial⁴¹ in preterm infants (non-surgical) reported high cross-colonisation rates because 49% of infants in the placebo group were colonised (culture positive) with the administered strain. However, they did not report on the relative abundances, and hence it is difficult to know if such cross-colonisation impacted the clinical outcomes of the trial.⁴¹ Future studies should report cross-colonisation rates and relative abundances from study infants.

NICU environmental contamination (refrigerator doors, telephone receivers, infant cots, monitors) with the administered probiotic organism is possible and may result in cross-colonisation of infants receiving placebo.^{39,42} Further research is needed to confirm if such risk may be lessened if the preparation is done off-site from the NICU environment.

Some researchers have recommended that future multicentre studies may have to adapt a cluster RCT design to overcome the issue of cross-contamination.⁴¹ Irrespective of whether the trial design is conventional or a cluster RCT and whether the supplements are prepared in the NICU are off-site, strict hand hygiene is essential while handling them and caring for neonates.

MEDICATION RECONCILIATION

Our method was to record doses administered, omitted and wasted sachets. It was matched against the number of unused sachets in the package after it was collected by the trial pharmacists when the infant had completed the intervention.

SAFETY OF PROBIOTICS

Researchers need to inform the parents and research ethics committees that probiotics are live bacterial organisms, and there are reports of sepsis due to the administered probiotic organism.^{43–49} However, researchers should also reassure parents that most cases of probiotic sepsis were successfully treated by antibiotics.^{28,43} In addition, it is essential to inform parents that 63

RCTs, 30 observational studies and many meta-analyses including the Cochrane review have found probiotics to be safe in preterm (non-surgical) infants.^{11–15,35,50–56} Our pilot RCT¹ and the RCT by Powell et al.²⁷ found probiotics to be safe in neonates with CGISC. The only published case report of mortality after probiotic supplementation in a preterm (non-surgical) infant was because of contamination of the product.⁵⁷ Independent assessment of the *product quality* is of paramount importance,⁵⁸ and probiotics should be free of contaminants and from companies with a high safety track record.

ONGOING MICROBIOLOGICAL QUALITY ASSURANCE

Having a well-resourced microbiology laboratory is essential for any centre planning to conduct RCT of probiotics.⁵⁸ It is crucial to conduct microbial analysis of random sachets of study supplements to rule out the presence of harmful pathogens and to check viability and colony counts of the probiotic strain. All routine clinical specimens (blood, urine, cerebrospinal fluid, endotracheal secretions, wound swabs) from study infants should be analysed using culture methods to enable diagnosis of infections due to supplemented probiotic organisms. As per our standard practice for routine clinical care, we used the Becton Dickinson BACTEC™ PEDS PLUS™/F Medium aerobic blood culture bottles with incubation monitored in the Bactec 9120 system.⁵⁹ No special culture bottles were used for the study. Our laboratory's automated blood culture system detects bifidobacteria (if present) within the standard 5-day (120 h) incubation period. While some studies have shown that, if incubation in aerobic culture bottles is ceased after 120 h, a few cases of bifidobacterial bacteraemia could be missed,²⁸ we decided to restrict to 120 h because going beyond that period would require larger capacity size incubators. The other issue if incubation goes beyond 120 h is the likely recovery of slow-growing contaminant organisms, which can affect the clinical interpretation of the results. On the other hand, incubation of <120 h will miss many cases and hence cannot be recommended.

COLLECTION OF STOOL SAMPLES

In our study,¹ we collected stool samples into 0.5 ml sterile micro-tubes (sarstedt.com). If the sample is collected in a different container and subsequently transferred to the micro-tubes, there is a risk of microbial contamination. We used sterile wooden spoons to scoop fresh samples from the nappies (diapers) of study infants. There were many challenges during the collection of stool samples. (a) Since the nappies are checked only once in 3–4 h, in many cases, stools had dried up by then. (b) Watery stools got absorbed into the nappies, so the collection was impossible. (c) Delayed passage of meconium and infrequent stooling in the pre-operative and immediate post-operative periods due to intestinal obstruction, postoperative ileus, and the use of morphine/fentanyl. (d) Use of radio-contrast enema or upper GI contrast for diagnostic purposes. If stool samples are collected after the contrast study, it will not represent the actual gut microbiota of the infant. (e) Missed opportunity to collect samples because most infants pass only one stool in the pre-operative period and none until 3–5 days in the post-operative period. Hence, extra vigilance and cooperation by the bedside nurses are essential to ensure the timely collection of stool samples.

A recent study found rectal swabs correlated well with the simultaneously collected faecal samples in neonates.⁶⁰ In contrast, a similar study in critically ill adults reported systematic differences in gut microbial profiles between simultaneously collected rectal swabs and stool samples.⁶¹ Further studies are needed to confirm the reliability of rectal swabs for microbial analysis. Such studies should also evaluate the effect of collection mode on stool SCFA levels.

LABELLING OF STOOL SAMPLES

Accurate labelling of the micro-tubes is essential to maintain the integrity of the data. They could be labelled the samples as follows if the total sample size is 100–999:

The first three digits to represent the study number of the infant, the second two digits refer to the sample number and subsequent alphabets represent the purpose of the sample.

Example: 003-01-DNA means study infant number 3, first stool sample (i.e. before commencing supplements), and the sample is for DNA sequencing.

003-01-SCFA means study infant number 3, stool sample before commencing supplements, and the sample is for SCFA analysis.

STORAGE OF STOOL SAMPLES

In our study,¹ we stored the samples in a 20 °C freezer immediately after collection and subsequently transferred them in a cold portable cooler for final storage at 80 °C within next 96 h. While rapid freezing to 80 °C is considered the best practice, it is not feasible even in the most resourceful settings. On the other hand, storing the samples at room temperatures is not recommended as it is known to lower Shannon diversity and evenness.⁶² It is suggested that the samples should be preserved at 20 °C within 15 min after collection and then transferred on dry ice within 24 h of collection and stored at 80 °C thereafter.¹⁰

Recent studies have shown that specific commercially available reagents allow for stool samples collection, preservation and storage at ambient temperatures for longer periods.⁶³ Many laboratories provide their vials to collect stool samples that have DNA-preservation agents in the vials. Hence, it is important to discuss with the laboratory at the protocol stage of the RCT. It is also essential to ensure that the sample preservation and storage methods are consistent across all samples to minimise variations in results.^{10,62,64}

SHIPPING OF STOOL SAMPLES

Given that stool samples are biological specimens, only accredited couriers should be used for shipping such samples. Maintaining a cold chain at 80 °C using dry ice is essential, especially while sending the samples that do not contain preservation media. Transportation logistics, including temperatures, need to be discussed with the receiving laboratory well in advance.

METHOD OF ANALYSING STOOL SAMPLES FOR GUT MICROBIAL DATA

There are excellent guidelines on the best approaches for analysing microbial data.⁶⁵ Briefly, the methods used in microbiome research include amplicon, metagenomic and metatranscriptomic sequencing. The amplicon sequencing involves the 16S rRNA gene sequencing for bacteria. It is relatively inexpensive, but the analysis is limited to genus-level taxonomic resolution. On the other hand, the metagenomic sequencing method sequences all microbial genomes (DNA) within a sample. It extends the taxonomic resolution to species or strain level. If there is adequate funding, metagenomic sequencing is preferred. Metatranscriptomics uses RNA sequencing to profile transcription in microbiomes, providing information on gene expression and the active functional output of the microbiome. It gives better insight into the functional activity of a microbial community. The pros and cons of each method are well described by Knight et al.⁶⁵ It is important to decide whether to limit to 16s ribosomal RNA gene sequencing or metagenomics during the early stages of protocol development in collaboration with the laboratory scientists. Even if the aim is to restrict to the former, it is helpful to collect additional stool samples and store them at 80 °C so that

metagenomic sequencing can be undertaken in future when funding becomes available.

METHOD OF ANALYSING STOOL SAMPLES FOR SCFA

SCFAs are produced mainly by intestinal microbiota and play an important role in many biological processes in humans. Gas chromatography–mass spectrometry^{66–68} is the commonly used method for SCFA assay. Alternative methods are high-performance liquid chromatography, nuclear magnetic resonance and capillary electrophoresis.⁶⁹

Since SCFAs are volatile, keeping the stool samples in appropriate conditions after collection is important. Samples are usually kept at 80 °C, although many researchers have successfully used 20 °C.⁶⁹ It is important to screw the lid tight and not open it until it reaches the laboratory for analysis. The stool samples for SCFA analysis should be collected in vials separate from those used for microbial analysis.

ENSURING BLINDING OF THE DATA

It is important to ensure blinding of the group allocation till the full results are available. Only the trial pharmacist or a similar professional with no vested interest in the project should know which sachets are probiotic and placebo. When the stool samples are sent to laboratories for microbial and SCFA analyses, they should be labelled as groups 1 and 2 to enable comparison without disclosing the groups. Clinical data also should be collected and compared as group 1 and group 2. Only in the end, unblinding should be done by the trial pharmacist.

Once the analysis comparing the microbiota of group 1 versus group 2 is completed, the bioinformatician may be able to guess group allocations (even when blinded) if the relative abundance of the supplemented bacteria is higher in one group. If researchers and statisticians assessing clinical outcomes become aware of those results, bias might be introduced. Hence statistical analysis of clinical data must be done by people who are blinded to the results of the microbial analysis and vice versa. This is especially important when the primary outcome of interest is clinical (sepsis, mortality, duration of hospital stay, time to full feeds, neurodevelopment).

DATA SAFETY AND MONITORING BOARD (DSMB)

Establishing a DSMB is essential before recruitment into the RCT.⁷⁰ The charter should have pre-defined stopping rules both for efficacy and safety. While *p* values around stopping rules are essential, they should not be the sole criteria while deciding whether the trial should continue or stop.

REPORTING

Reporting metagenomic analysis of stool samples should follow the recently published STROBE-Metagenomics guidelines.⁷¹ Given that the study design will be an RCT, CONSORT guidelines help report clinical outcomes.

CONCLUSIONS

In summary, following these guidelines will increase the validity of future RCTs in this area, hence confidence in their results.

DATA AVAILABILITY

Data sharing is not applicable to this article as no data sets were generated or analysed during the current study.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Since it is a review article, patient consent is not required.

ADDITIONAL INFORMATION

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Early neurodevelopmental outcomes of congenital gastrointestinal surgical conditions: a single-centre retrospective study

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ABSTRACT

Background Evidence is emerging that surgery in the neonatal period is associated with increased risk of suboptimal neurodevelopmental outcomes (SNDO). The aim of this study was to describe neurodevelopmental outcomes (at 1 year) of neonatal surgery for congenital gastrointestinal surgical conditions (CGSC) and to explore risk factors.

Methods Retrospective study (2005–2014) of infants born ≥ 34 weeks gestation with CGSC and admitted to the surgical neonatal intensive care unit of Perth Children's Hospital, Western Australia. Clinical details and 1-year developmental outcomes based on Griffiths Mental Developmental Assessment Scales were collated from the database and by reviewing the medical records of study infants. SNDO was defined as one or more of the following: a general quotient less than 88 (ie, >1 SD below mean), cerebral palsy, blindness or sensorineural deafness. Univariable and multivariable logistic regression analyses were carried out to explore risk factors for SNDO. A total of 413 infants were included, of which 13 died. Median gestation was 37.6 weeks (IQR: 36.4–39.1). Information on developmental outcomes was available from 262 out of 400 survivors. A total of 43/262 (16.4%) had SNDO. On univariable analysis, lower z scores for birth weight, prolonged duration of antibiotics, increased episodes of general anaesthesia and prolonged duration of hospital stay were associated with SNDO. On multivariable analysis, lower z scores for birth weight and prolonged hospital stay were associated with increased risk of SNDO.

Conclusions Late preterm and term infants undergoing neonatal surgery for CGSC may be at risk for SNDO. Studies with longer duration of follow-up are needed to further evaluate the role of potentially modifiable risk factors on their neurodevelopmental outcomes.

INTRODUCTION

Survival following neonatal surgery has improved in the recent years, but short-term and long-term complications continue to have significant effects on these infants and their families.¹ A recent population-based study that compared developmental outcomes of 124 neonates undergoing non-cardiac surgery versus 92 who underwent

What is known about the subject?

- ▶ Surgery in the neonatal period may have an adverse effect on neurodevelopment outcomes.
- ▶ Infection and excessive inflammation are harmful to the developing brain.

What this study adds?

- ▶ Nearly 16% of late preterm and term infants who underwent neonatal surgery for congenital gastrointestinal conditions had suboptimal neurodevelopment at one year of age.
- ▶ Lower z scores for birth weight and prolonged hospital stay were associated with increased risk of suboptimal neurodevelopmental outcomes.
- ▶ C reactive protein levels and infections were not associated with suboptimal neurodevelopmental outcomes at 1 year of age.

cardiac surgery and 162 healthy infants found that cardiac surgery carried the highest risk of developmental delay, but infants undergoing non-cardiac surgeries also had 7%–14% incidence of developmental delay.²

Factors associated with poor developmental outcomes in neonates undergoing surgery include low birth weight,³ chromosomal anomalies, growth restriction,⁴ prolonged hospital stay,⁵ need for Extracorporeal Membrane Oxygenation,^{6–7} chronic lung disease,⁸ increasing number of surgeries⁵ and low socioeconomic status.⁹ One factor that has not been adequately explored in neonates undergoing surgery is the influence of infection and inflammation. Exploring this area is important because infection and excessive inflammation are potentially harmful to the developing brain.^{10–14}

We conducted this retrospective study to evaluate 1-year developmental outcomes of late preterm and term infants who underwent



surgery for congenital gastrointestinal surgical conditions (CGSC) in our unit and to explore the potential risk factors. Another aim of the study was to analyse the impact of inflammation on neurodevelopmental outcomes of those infants.

METHODS

This was a retrospective cohort study of all late preterm and term infants born at $\geq 34^{0/7}$ weeks gestation between January 2005 and December 2014 with CGSC who underwent surgery in the neonatal period at the tertiary neonatal intensive care unit (NICU) of Perth Children's Hospital, Western Australia.

The following conditions were included in the study—gastroschisis, exomphalos, duodenal atresia, malrotation, jejunoileal atresia, large bowel atresia, meconium ileus, Hirschsprung disease, multiple gut anomalies, gut perforations/stenoses, short bowel syndrome, biliary atresia, anorectal anomalies and benign abdominal cysts. We included oesophageal atresia and congenital diaphragmatic hernia because they also involve the gastrointestinal tract and have long-term gastrointestinal complications.

Infants were identified by interrogating the departmental database. Infants with chromosomal anomalies and syndromes known to adversely affect developmental outcomes were excluded. Infants born at < 34 weeks gestation were excluded because they carry a higher risk of adverse developmental outcomes due to prematurity compared with late preterm and term infants.

Clinical characteristics of study infants were extracted from their medical records by one author (VB) and verified for accuracy by a second author (SR). Two neonatologists with expertise in developmental follow-up (DW and JKG) collated the results of 1-year outcomes based on Griffiths Mental Development Scales (GMDS-II) from the departmental database. The GMDS-II assesses development in five areas: locomotor, personal and social, hearing and speech, eye and hand coordination, and performance. The five subscales are assessed and scored separately and then combined to provide an overall general quotient (GQ) reflecting the child's developmental performance level relative to the general population. On these scales, a combined GQ of 100.2 (SD 12) is considered normal.¹⁵ The GMDS-II is a well-recognised tool for identifying neurosensory disability and is used widely.^{16 17}

Outcome of interest for this study was suboptimal neurodevelopmental outcomes (SNDO) at 1 year of age. SNDO was defined as one or more of the following: (1) a GQ of < 88 (ie, > 1 SD below mean) on GMDS-II,¹⁵ (2) cerebral palsy (based on assessment by neurologist or developmental paediatrician) (3) blindness (visual acuity of $< 6/60$ in the better eye) and (4) sensorineural deafness (based on audiometry assessment) requiring hearing aids.

Healthcare-associated infection (HAI)-included urinary tract infection (UTI) or healthcare-associated

blood stream infection (HABSI), meningitis or surgical site infection or any type of viral infection. HABSI was defined as positive blood culture on a sample taken 48 hours after admission to the NICU. UTI was defined as positive culture based on a sample collected from suprapubic sample or in-and-out catheter. Meningitis was diagnosed based on positive culture on cerebrospinal fluid (CSF) samples collected with aseptic precautions. The diagnosis of wound infection was based on the presence of erythema/oedema/induration at the surgical site and positive culture on the wound swab. Respiratory viral infection was diagnosed based on PCR on postnasal aspirate samples taken in infants who presented clinical symptoms of respiratory illness.

C reactive protein (CRP) was used as the marker of inflammation. We stratified the CRP levels based on the timing in relation to the surgical procedure. Empirically, a CRP done in the preoperative period was considered to be a surrogate marker of early onset sepsis, whereas CRP performed within 72 hours of surgery was considered to be related to the degree of surgical injury and CRP performed after 72 hours of surgery to indicate hospital acquired infection.

Statistical analysis was done using the STATA V.16 software (StataCorp). The summary statistics for normally distributed continuous variables were expressed as mean and SD; those with skewed distribution were expressed as median and IQR. Categorical variables were expressed as frequency and percentage. Univariable and multivariable random effect logistic regression models were carried out to derive unadjusted and adjusted odds ratios and 95% CIs. Random effect was included in the fitted model to minimise bias due to the presence of correlated data (ie, multiple measurements of CRP values from individual patients). One-sample t-test was used to compare the mean GQ scores to the population mean (100.2).¹⁵ For all analyses, a two-tailed $p < 0.05$ was considered statistically significant.

This retrospective study was approved by the institutional ethics committee as a quality assurance activity. All clinical variables and the results of developmental assessments (GMDS-II) collected for this study were retrospective in nature. Strengthening the Reporting of Observational Studies in Epidemiology guidelines were used to report this study.¹⁸

Patient and public involvement

The development of research question and outcome measures for this retrospective study were not informed by patients' priorities, experience and preferences. Patients were not involved in the design, in the recruitment to and conduct of the study. Patients were not invited to comment on the study design and were not consulted to develop patient relevant outcomes or interpret the results. Patients were not invited to contribute to the writing or editing of this document for readability or accuracy. There are no plans to disseminate the results of this study to study participants.

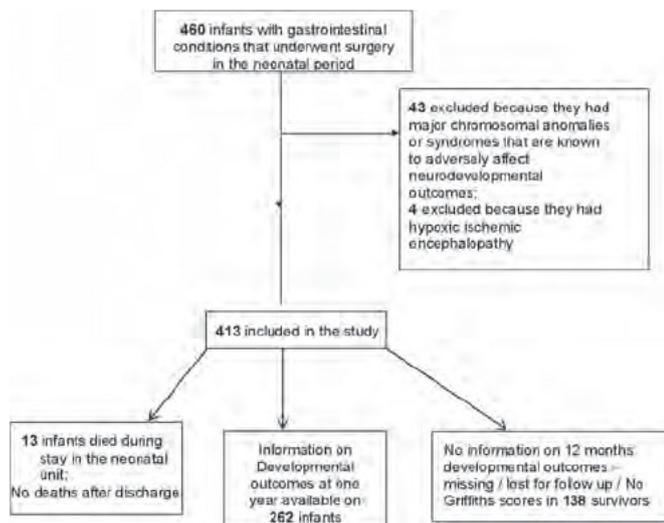


Figure 1 Study flow diagram.

RESULTS

A total of 460 neonates underwent surgery for CGSC during the study period, of which 43 were excluded because of chromosomal anomalies or syndromes that are known to adversely affect neurodevelopmental outcomes. Four infants were excluded because they had moderate to severe hypoxic ischaemic encephalopathy due to perinatal asphyxia. The remaining 413 infants were included in the study. Of them 13 died, and of the 400 surviving infants, full information on developmental outcomes was available for 262/400 (65%) surviving infants. The flow diagram of patient selection process is given in [figure 1](#).

The median gestation was 37.6 weeks (IQR: 36.4–39.1) and median birthweight 3000 grams (IQR: 2590–3405). The median duration of hospital stay was 18 days (IQR: 11–26 days, range: 1–153 days). There were 13 deaths, all of which were during initial hospital stay. There were no deaths after discharge from the hospital. [Table 1](#) summarises the clinical characteristics in detail.

The major surgical conditions were gastroschisis, malrotation, oesophageal atresia with or without tracheo-oesophageal fistula, Hirschsprung disease and congenital diaphragmatic hernia ([table 2](#)).

A total of 43/262 (16.4%) infants had SNDO, with nine infants having a GQ <76 (ie, more than 2 SD below the mean). One infant had deafness, one had cerebral palsy and none had blindness. The mean GQ was 96.3 (SD 10.3), which was significantly lower than the population mean of 100.2; $p < 0.001$. Infants with multiple gut anomalies, oesophageal atresia, Hirschsprung disease, exomphalos and congenital diaphragmatic hernia had highest rates of SNDO among survivors ([table 2](#)).

HABSI occurred in 27 infants (6.5%). A total of 51 (12.4%) infants developed at least one episode of HAI (UTI or HABSI or viral infection or surgical site infection). None of the infants had early-onset sepsis. Coagulase negative *Staphylococcus*, *Klebsiella* spp and *Escherichia Coli* were the most common pathogens isolated ([table 3](#)).

Association between neonatal risk factors and SNDO among survivors

On univariable analysis, lower birthweight z scores, prolonged duration of antibiotic therapy increasing episodes of general anaesthesia and prolonged duration of hospital stay were associated with higher odds of SNDO among survivors ([table 4](#)). On multivariable analysis, lower birthweight z scores and longer duration of hospital stay were associated with increased odds of SNDO among survivors ([table 4](#)).

DISCUSSION

Our study found an overall mortality rate of 3.1% and SNDO in 16.4% of neonates undergoing surgery for CGSC. These findings are similar to a recent study that reported an incidence of 7%–14% in various domains of assessment at 3 years among 124 children who underwent surgery for non-cardiac conditions in the neonatal period.² While the mean GQ of 96.3 in our cohort might not appear too low, it is important to note that the GMDS-II norms are based on population sample more than two decades ago. It is well known that developmental quotients and intelligence quotients in the general population increase by 2–3 points each decade (Flynn effect).¹⁹ If assessed using the GMDS-II tools, healthy 12-month-old infants during the study period of 2005–2014 would probably have scored a mean of 103 rather than 100.

Since the study spanned over 10 years (January 2005 to December 2014), advances in anaesthesia, surgical techniques, intensive care management, and changes to family and societal environment during that period could have influenced the in-hospital clinical outcomes and 1-year developmental outcomes of study infants. Contemporary multicentre studies with adequate sample size are needed to enhance knowledge in this area.

While many variables were found to be associated with increased risk of SNDO on univariable analysis, only lower birthweight z scores and longer duration of hospital stay were found to be having significant association on multivariable analysis. Lower birthweight z scores indicate fetal growth restriction and prolonged hospitalisation is usually related to the complex nature of the underlying surgical condition. Hence their association with adverse neurodevelopmental outcomes is not unexpected. The width of the CI for birthweight z-scores was very wide, ranging between a drop in the odds between 2% and 50%. The probable reason for this wide range could be related to the timing of intrauterine growth restriction (IUGR). For the same degree of IUGR, the one that starts early during pregnancy is known to have worse outcomes compared with late gestation IUGR.

Each additional day of stay in the hospital resulted in a change in the odds of SNDO by 3%. Many surgical infants stay for a protracted period of time in the hospital and hence these odds are likely to be clinically significant.



Table 1 Characteristics of study infants

Clinical characteristic	Median or no (percentage)	IQR	Range	N
Gestation (weeks)	37.6	36.4 to 39.1	34.1 to 41.5	413
Gender (male:female)	57%:43%	NA	NA	413
Birth weight (grams)	3000	2590 to 3405	1664 to 5060	413
Birthweight z scores	-0.23	-0.87 to 0.41	-2.81 to 4.78	413
Birth length (cm)	49	47 to 51	40 to 58	403
Birth length z scores	-0.03	-0.75 to 0.57	-3.38 to 4.23	403
Birth head circumference (cm)	34	32.5 to 35	28.5 to 47	409
Birth head circumference z scores	0.07	-0.62 to 0.78	-3.32 to 3.29	408
APGAR 5 min	9	9 to 9	4 to 10	409
Presurgery C reactive protein (CRP) levels (mg/dL)	7.5	5 to 20	1 to 188	933
CRP levels within 72 hours of initial surgery (mg/dL)	35.5	19 to 69	3 to 346	747
CRP levels after 72 hours of surgery (mg/dL)	15	7 to 29	1 to 325	2912
Healthcare-associated blood stream infection	27 (6.5%)	NA	NA	413
CSF culture positive	1 (0.25%)	NA	NA	413
Lumbar puncture done	14 (3.4%)	NA	NA	413
Viral infections (all respiratory)	17 (4.1%)	NA	NA	413
Urinary tract infections (UTI)	1 (0.24%)	NA	NA	413
Culture positive surgical site infections	14 (3.4%)	NA	NA	413
Any healthcare-associated infection (blood stream or CSF or viral or UTI or wound infection)	51 (12.4%)	NA	NA	413
No of antibiotic courses	2	1 to 2	1 to 14	406
Cumulative duration of antibiotics (days)	6	4 to 8	1 to 56	406
Surgery episodes under GA	1	1 to 2	1 to 5	413
No of episodes of hypoglycaemia (blood glucose <2.6 mmol/L)	0	0 to 0	0 to 15	413
Length of stay (days)	18	11 to 26	1 to 153	413
Post conception age at discharge (weeks)	41	39.4 to 42.4	35.4 to 60.2	413
Death before discharge	13 (3.1%)	NA	NA	413
Death before 1 year	13 (3.1%)	NA	NA	413
Corrected age at Griffiths assessment (months)	12	12 to 12.5	10 to 15.5	270
GQ scores at 12 months	96.5	92 to 102	49 to 131	270
SNDO	43/262 (16.4%)	NA	NA	262

GA, gestational age; GQ, general quotient; NA, not applicable; SGA, small for gestational age; SNDO, Suboptimal developmental outcomes.

The burden of HAI and HABS in neonates with CGSC has not been explored adequately. Donnell *et al* and van Saene *et al* conducted a prospective study of surgical infants <6 months to find infection rates.^{20 21} Thirty-two infants developed blood culture positive sepsis (15%); predominant micro-organisms (86%) were coagulase-negative staphylococci and enterococci. Other pathogens, including aerobic gram-negative bacilli, were responsible for the remainder. They suggested that gut translocation was the main factor behind sepsis in surgical infants rather than central lines and cautioned that prevention is unlikely to be successful if abnormal gut flora is ignored.²¹ Another study by Bishay *et al* reported that 31 out of 112 surgical infants (28%) had a total of 65 episodes of septicemia.²²

In very preterm infants, it is well established that neonatal sepsis is associated with higher risk of adverse neurodevelopmental outcomes. A recent systematic review by Cai *et al*²³ found that preterm infants with neonatal sepsis were at a higher risk of neurodevelopmental impairments such as cerebral palsy and neurosensory deficits, compared with infants without sepsis (OR 3.18; 95% CI 2.29 to 4.41).²³ Hence, we had expected similar findings in our cohort of surgical infants. However, in our study, HAI was not associated with increased risk of SNDO, either on univariable or multivariable analysis. Similarly, higher levels of CRPs were not associated with SNDO irrespective of the timing in relation to the surgeries. This could be related to the resilience of the brain of late preterm and term

**Table 2** Developmental outcomes of neonates with CGISC*

Major gastrointestinal anomaly	No	Mortality	SNDO among infants who were assessed	Median GQ
Gastroschisis	92 (22.3%)	3/92 (3.3%)	8/55 (14.5%)	98.5 (IQR:92.5–103) n=60
Malrotation	48 (11.6%)	3/48 (6.2%)	4/33 (12.1%)	96 (IQR:93–103) n=34
Oesophageal atresia	44 (10.6%)	1/44 (2.3%)	10/27 (37%)	93 (IQR:85–100) n=27
Hirschsprung disease	44 (10.6%)	1/44 (2.3%)	6/32 (18.7%)	98 (IQR:93–102) n=33
Congenital diaphragmatic hernia	42 (10.2%)	1/42 (2.4%)	5/34 (14.7%)	94.5 (IQR:92–105) n=34
Ano-rectal anomalies	39 (9.4%)	0/39 (0%)	3/21 (14.3%)	96 (IQR:90–101) n=22
Gut perforations and stenoses	19 (4.6%)	1/19 (5.3%)	1/12 (8.3%)	101.5 (IQR:94.5–106) n=12
Duodenal atresia	19 (4.6%)	0/19 (0%)	1/13 (7.7%)	99 (IQR:94–110) n=15
Jejuno-ileal atresia	16 (3.9%)	0/16 (0%)	1/9 (11.1%)	97.5 (IQR:95–102) n=10
Exomphalos	13 (3.1%)	0/13 (0%)	1/6 (16.7%)	99 (IQR:89–100) n=7
Meconium ileus	12 (2.9%)	0/12 (0%)	0/5 (0%)	98 (IQR:96–99) n=5
Multiple gut anomalies	10 (2.4%)	1/10 (10%)	3/7 (42.8%)	88 (IQR:84–100) n=7
Short bowel syndrome	5 (1.2%)	2/5 (40%)	0/1 (0%)	95 n=1
Large bowel atresia	5 (1.2%)	0/5 (0%)	0/1 (0%)	104 n=1
Benign abdominal cysts and tumours	4 (0.97%)	0/4 (0%)	0/1 (0%)	103 n=1
Biliary atresia	1 (0.24%)	0/1 (0%)	0/1 (0%)	92 n=1

*For all outcomes, infants who underwent at least one episode of surgery were included; infants who died prior to undergoing any surgery were excluded. The information on neurodevelopmental outcomes was available for 65% of survivors. CGISC, congenital gastrointestinal surgical conditions; GQ, general quotient; SNDO, suboptimal neurodevelopmental outcomes.

infants to the harmful effects of infection and inflammation, unlike the vulnerable extremely preterm infants. However, prolonged duration of antibiotic therapy, which could be a surrogate marker of clinically suspected infection, was associated with SNDO on univariable, but

not multivariable analysis. Further studies with larger sample size and a longer duration of follow-up beyond 1 year of age are needed to explore the role of infection and inflammation in late preterm and term infants undergoing neonatal surgery.

Table 3 Micro-organisms isolated from infants with healthcare-associated infections

Micro-organism	Blood	CSF	Urine	Viral infections	Wound/skin swab
CONS	16	1	–	–	3
<i>Escherichia coli</i>	4	–	–	–	3
<i>Klebsiella</i>	3	–	–	–	–
<i>Pseudomonas</i>	1	–	–	–	2
<i>Streptococcus mitis</i>	1	–	–	–	–
<i>Moraxella</i>	1	–	–	–	–
<i>Enterococcus</i>	1	–	–	–	1
<i>Candida albicans</i>	–	–	1	–	2
<i>Staphylococcus aureus</i>	–	–	–	–	2
<i>Enterobacter cloacae</i>	–	–	–	–	1
Rhino virus	–	–	–	12	–
RSV	–	–	–	2	–
Influenza A	–	–	–	2	–
Parainfluenza	–	–	–	1	–
Total	27	1	1	17	14

CONS, coagulase negative *Staphylococcus*; RSV, respiratory syncytial virus.



Table 4 Risk factors for SNDO

Variable	Unadjusted OR and 95% CI	P value	Adjusted OR and 95% CI	P value
Gestational age at birth (≥ 37 weeks)	1.07 (0.53 to 2.19)	0.840	1.54 (0.65 to 3.63)	0.321
Birthweight z scores	0.64 (0.47 to 0.89)	0.008*	0.69 (0.49 to 0.98)	0.038*
Female gender	0.71 (0.36 to 1.39)	0.313	0.53 (0.24 to 1.16)	0.112
No of episodes of hypoglycaemia (< 2.6 mmol/L)	1.06 (0.75 to 1.49)	0.730	0.98 (0.60 to 1.58)	0.923
General anaesthesia (> 3 episodes)	3.31 (1.29 to 8.50)	0.013*	0.77 (0.17 to 3.59)	0.745
Preoperative CRP levels	0.90 (0.59 to 1.37)	0.627	0.92 (0.60 to 1.40)	0.698
CRP levels within 72 hours of surgery	0.90 (0.63 to 1.28)	0.555	1.06 (0.69 to 1.62)	0.769
CRP levels after 72 hours of surgery	0.64 (0.41 to 1.01)	0.053	0.99 (0.63 to 1.56)	0.985
Any infection	1.20 (0.49 to 2.96)	0.683	0.44 (0.11 to 1.77)	0.247
Cumulative duration of antibiotics	1.05 (1.01 to 1.10)	0.043*	1.00 (0.91 to 1.10)	0.990
Degree of postnatal growth restriction	2.00 (0.59 to 6.81)	0.265	1.56 (0.62 to 3.97)	0.348
Length of stay	1.02 (1.00 to 1.03)	0.003*	1.03 (1.00 to 1.06)	0.034*

*Statistically significant associations
CRP, C reactive protein.

The harmful effect of exposures to general anaesthesia on developing brain is an area of debate and active research.^{24–25} While animal studies have consistently shown general anaesthesia to be toxic to the developing brain,²⁶ one recent large RCT²⁷ and a large prospective cohort study²⁸ found no significant association. Both these studies evaluated a single exposure to general anaesthesia, and hence do not address the issue of repeated exposures. A recent large data linkage study found that children exposed to general anaesthesia before 4 years have poorer development outcomes at school entry and school performance.²⁹ In another cohort study,³⁰ children who had multiple exposure to gestational age (GA) before 3 years of age scored 1.3 points (95% CI -3.8 to 1.2 ; $p=0.32$) less than unexposed children on intelligence tests; children who had one exposure to GA scored 0.5 points (95% CI -2.8 to 1.9 ; $p=0.70$) less than unexposed children. However, the parents of children who had multiple exposure to GA reported increased problems related to executive function, behaviour and reading.³⁰ In our cohort, increasing episodes of general anaesthesia were associated with higher risk of SNDO on univariable analysis, but not on multivariable analysis. Further studies with long duration of follow-up are needed in this area.

While we found lower birthweight z scores and prolonged hospital stay to be associated with increased risk of SNDO, one should not ignore the possibility that the underlying surgical condition in itself could be an important risk factor that drives other morbidities leading to SNDO. In our cohort, multiple gut anomalies and oesophageal atresia had the highest incidence of SNDO (42.8% and 37%, respectively), which is not unexpected because these infants have significant in-hospital and postdischarge morbidities, which puts them at a higher risk of SNDO.

One of the limitations of our study was the shorter duration of follow-up of 1 year and the findings may not track subsequently. In a recent study, Fairbairn *et al* reported that Bayley-III results for all domains at 1 year of age were a weak predictor of outcomes at 3 years of age in infants who had early major cardiac and non-cardiac surgery and healthy infants.³¹ Hence all infants, irrespective of the results of developmental assessments at 1 year should be followed with formal developmental assessments at least until 5 years of age. At the same time, infants identified as high risk based on the 1-year assessments could be provided early developmental interventions to optimise their outcomes. Only recently, we have commenced routine developmental follow-up until 2 years of age with Bayley Scales of Infant Development to all infants undergoing surgery in the neonatal period.

Surgical infants who need prolonged duration of mechanical ventilation are at higher risk of hypoxic episodes and hence worse developmental outcomes. At the same time, prolonged ventilation could be a marker of severity of the underlying anomaly. A limitation of our study was the lack of reliable information on the duration of mechanical ventilation among the study infants.

The other limitations of our study were: (1) retrospective design without healthy controls, (2) the indication for doing CRP levels was at the discretion of clinicians rather than based on a standardised protocol, (3) full information on developmental outcomes was missing from nearly 35% of survivors, (4) lack of information on sociodemographic status of family and (5) missing information about duration of general anaesthesia which can have significant influence on developmental outcomes. The data were from a single centre from a high-income country and hence the findings may not be generalisable. The main strength of the study is the large sample size



of surgical infants and the use of regression analyses to adjust for confounders.

CONCLUSIONS

Late preterm and term infants undergoing surgery for CGSC may be at risk for SNDO at 1 year of age. Studies with long-term follow-up are needed to further evaluate the influence of potentially modifiable risk factors on neurodevelopmental outcomes in such infants.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not required.

Ethics approval This retrospective study was approved by the institutional ethics committee as a quality assurance activity.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. The deidentified patient data are available from the correspondence author and will be provided on reasonable request.

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Appendix 6

Papers published by the student during PhD enrolment and cited in thesis chapters



Trans-anastomotic tube feeding in the management of congenital duodenal obstruction: a systematic review and meta-analysis

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Abstract

Background Feed intolerance is a common problem in neonates with congenital duodenal obstruction (CDO). Some surgeons insert trans-anastomotic tubes (TAT) to facilitate feed tolerance. We conducted a systematic review to evaluate the efficacy and safety of TATs in CDO.

Methods Medline, EmBase, CINAHL, and Cochrane Library were searched till July 2020. Risk of bias was assessed using ROBINS-I tool. Meta-analysis was conducted using Random Effects Model.

Results No randomized controlled trials addressing the question were identified. In the 6 included observational studies, 96 infants underwent intraoperative TAT placement and 117 did not. Four studies reported benefits of TAT such as early attainment of full feeds and decreased need for parenteral nutrition. Two studies reported better outcomes in the no-TAT group. Accidental removal of TAT without clinical harm was reported in three studies [5/37 (14%), 4/17 (23%), and 2/4 (50%)]. Overall meta-analysis found no differences between the groups on any outcome. However, sensitivity analysis after excluding two studies with high risk of bias found that TAT tubes are associated with shorter duration of PN and shorter time to full enteral feeds. GRADE of evidence was very low for all outcomes.

Conclusions Evidence is limited regarding the efficacy and safety of intraoperative TAT placement in neonates with CDO. Well-designed RCTs are needed to address the issue definitively.

Keywords Congenital duodenal obstruction · Feed intolerance · Trans anastomotic tube · Parenteral nutrition

Introduction

Congenital duodenal obstruction (CDO) is a common cause of intestinal obstruction in neonates [1]. Depending on the type of CDO, surgical management is with duodeno-duodenostomy, duodeno-jejunostomy or simple resection of the web [2]. Feed intolerance is common in infants with CDO [3], which necessitates the administration of parenteral nutrition (PN). Administration of PN is known to be associated with complications such as central line associated blood stream infections [4], cholestasis and other morbidities. Delayed

attainment of a full enteral feeds results in prolonged hospital stay and increases financial costs to family and the health care system. Some of the factors that contribute to feed intolerance in neonates with CDO are dilatation and dysmotility of the proximal duodenal pouch [5], prematurity, associated gut and cardiac anomalies [3]. To facilitate enteral feeds, some surgeons insert trans-anastomotic tubes (TAT) at the time of surgery. The theoretical advantage of TATs is that the feeds bypass the patulous and dysmotile proximal duodenal pouch and enter the jejunum directly, thereby increasing feed tolerance. Once full feeds through the TAT are achieved, gradual transition to the standard naso-gastric or oro-gastric tube is undertaken and subsequently, the TAT is removed. While this appears to be an attractive approach, naso-jejunal TATs have the potential to cause complications including bowel necrosis, dislodgement, migration and bowel perforation [6]. Hence, we conducted a systematic review to evaluate evidence on the efficacy and safety of TATs in neonates with CDO.

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Methods

A systematic search of the literature published up to July 2020 was performed by searching MEDLINE, EMBASE, Cochrane Library, and CINHAL. The following medical subject headings (MeSH) and keywords were used for the literature search: congenital duodenal obstruction, duodenal atresia, duodenal web, annular pancreas, newborn, infant, surgery, parenteral nutrition, post-operative care, trans-anastomotic tube and enteral feeding. Reports yielding from search results were manually screened as per the study criteria. Full text articles of the short-listed reports were read in detail to confirm suitability for inclusion. Ethics approval was not necessary for the conduct of this review.

Inclusion criteria

(1) Neonates with CDO (duodenal atresia, stenosis, web, annular pancreas, or duodenal duplication cysts causing obstruction) who underwent surgical correction. (2) Comparison of TAT and no-TAT groups in the same paper.

Exclusion criteria

Non-CDO cases, animal or laboratory studies, case reports, studies where there was no information on TAT versus no-TAT cohorts, or studies describing only either of the methods, not both.

Outcomes of interest

Time to full enteral feeds in the postoperative period, duration of PN, duration of hospital stay, mortality, sepsis, potential side effects of the TAT such as migration, intestinal perforation, and need for re-laparotomy.

Data extraction

Articles meeting the inclusion criteria were reviewed by two authors (NB and SR) and data were extracted onto a spreadsheet. Where necessary, correspondence authors were contacted with a request to provide additional data or clarify the data.

Risk of bias assessment

The ROBINS-I (Risk Of Bias in Non-randomized Studies—of Interventions) tool was used to assess the risk of bias in the included studies [7]. In addition, the reviewers assessed the overall risk of bias across the studies for each

outcome of interest and incorporated them into judgements about the ‘quality of evidence’.

Statistical analyses

Meta-analysis was conducted using a random-effects model (DerSimonian and Laird) using Review Manager Version 5.3 (Cochrane Collaboration, Nordic Cochrane Centre). Mean and standard deviation (SD) were estimated from median, IQR and range using the formula by Wan et al. [8] Summary effect sizes were expressed as relative risks (RRs) and 95% confidence intervals (CIs) for dichotomous outcomes. Continuous outcomes were expressed as mean differences (MDs) and 95% CIs. Statistical heterogeneity was assessed with the χ^2 test, I² statistic, and by visual inspection of the forest plot (overlap of CIs). Considering the importance of potential confounders in observational studies, sensitivity analyses were conducted by excluding studies with serious or critical risk of bias (ROB) in this domain.

Reporting

The results of this systematic review were reported using the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-analysis) [9] and MOOSE (Meta-analysis Of Observational Studies in Epidemiology) [10] guidelines.

Results

The summary of the selection process is presented in Fig. 1 using the PRISMA flow diagram [9].

No Randomized controlled trials (RCTs) comparing TAT versus no-TAT were identified. A total of six studies were included, of which five were retrospective, and one was a retrospective study with a prospective observational arm. In total, there were 213 CDO patients of which 96 were managed with TAT placement and 117 without TAT. Two authors responded to our request [11, 12], of which one [11] gave additional data from their study. Table 1 gives clinical details of individual studies.

Mooney et al. (1987)

In this retrospective study from St Louis, USA [13], the authors described their 10 years’ experience with CDO (1973 to 1983). It included 20 infants with a mean gestational age of 36.5w (range 32 to 43w) and a mean birth weight of 2360 g (range 1340 to 3800 g). One infant died before surgery due to prematurity and associated esophageal atresia. TAT was inserted in ten infants, whereas no TAT was inserted in the remaining nine. The authors mentioned that the two groups were comparable with regard to

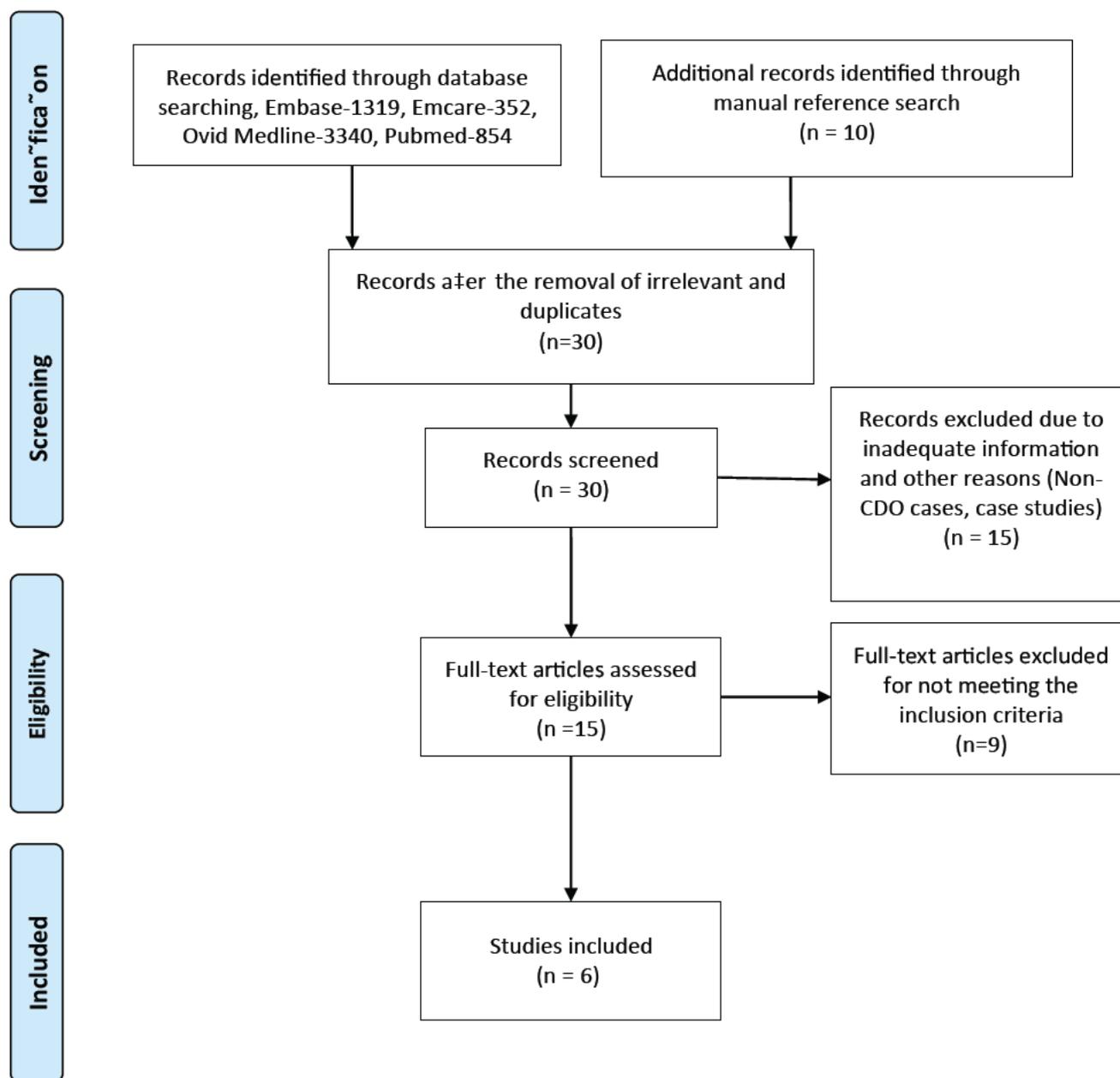


Fig. 1 PRISMA flow diagram of study selection

prematurity, other anomalies, and type of repair. The TAT group took longer time to reach full enteral feeds (mean post-op 15.7 days vs 5.3 days) and had longer hospital stay (mean 39.6 vs 21.5 days) than the no-TAT group. Hence, they concluded that TAT is not recommended.

Upadhyay et al. (1996)

In this retrospective study from Birmingham, UK [14] (1982–1993), authors compared the outcomes of 33 infants with CDO who received 3 different types of interventions.

Group A: duodeno-duodenostomy with gastrostomy and TAT ($n = 12$); group B: duodeno-duodenostomy with post-anastomotic jejunostomy feeding tube ($n = 12$); group C: duodeno-duodenostomy only ($n = 9$). Mean birth weight (2210–2610 g), gestational age (35–37w), and age at surgery (3–4 days) were similar between the groups, and there were no associated gastrointestinal anomalies in any of them. Infants in group C (i.e., no-TAT) attained full feeds earlier compared to group A and B (12 vs 22 vs 25 days; $p < 0.05$). The duration of hospital stay was significantly lower in group C (mean 15 days, range 12–20) than group

Table 1 Clinical characteristics of included studies

	Mooney et al	Upadhyay et al	Ruangtrakool et al	Arnbjornsson et al	Hall et al	Harwood et al
Sample size						
TAT	10	12	4	16	17	37
No-TAT	9	9	30	9	38	22
Birth weight (kg)						
TAT	NA	2.6	2.19	2.8	2.8	2.85
No-TAT	NA	2.6	2.2	2.17	2.6	2.64
Age at surgery (days)						
TAT	NA	3.3	NA	3	NA	4
No-TAT	NA	3	NA	1	NA	3.5
Type of CDO (DS:DA:DW)						
TAT	0:12:8 ^a	NA	1:19:7 ^a	NA	7:33:15 ^a	8:20:9
No-TAT						3:14:5
Associated anomalies (trisomy 21: cardiac: gastrointestinal)						
TAT	2:3:3 ^a	8: NA: 0	13:12:7 ^a	6:6:2	4:3:0	1 had Trisomy
No-TAT		2: NA: 0		2:2:2	9:8:0	
Type of surgery (DD:DJ: web excision and duodenoplasty)						
TAT	7:9:3 ^a	12:0:0	29:2:3 ^a	7:6:3	47:0:8 ^a	20:9:8
No-TAT		9:0:0		6:2:1		16:2:2
Type of TAT tube	NA	Silastic tube, size not given	NA	NA	6F soft feeding tube	6F Silicon tube
Type of feed	NA	NA	NA	NA	NA	EBM and formula

DS duodenal stenosis, DA duodenal atresia, DW duodenal web, DD Duodeno-duodenostomy, DJ duodenojejunostomy, NA Information not available, EBM Expressed breast milk

^aCombined data, separate TAT and no-TAT information not available

A (mean 26 days, range 17–55) or group B (mean 31, range 11–74, $p < 0.05$).

Ruangtrakool et al. (2001)

In this retrospective study from Bangkok, Thailand [15], authors compared the outcomes of various surgical techniques in 34 infants with CDO (1990–1999). One of the comparisons was TAT (4 infants) versus no-TAT (30 infants). The mean birth weight was similar between the two groups (2198 ± 242 g vs 2242 ± 564 g). TPN was required in 75% of infants in the TAT group (3/4) versus 96.7% of infants in the no-TAT group (29/30). Although the group with a TAT received earlier first feeding (4.5 days vs 7.8 days; $p = 0.09$), the post-operative onset of full feeding (15.5 ± 4.36 vs 15.46 ± 8.14 days) and the hospital stay (23.5 ± 10.08 vs 19.79 ± 13.11 days) were not different between the groups. A significant limitation of the study was the unequal distribution of infants between the two groups (4 vs 30).

Arnbjornsson et al. (2002)

In this Swedish bi-institutional retrospective study [11] (1993–1998), authors compared the outcomes of nine neonates with CDO from one institution where TAT was the standard practice versus nine from another institution where TAT was not performed. The demographic data, associated anomalies, and methods of treatment were similar between the groups. The mean gestational age was similar between the TAT and no TAT group [(36 (SD 2) vs 35 w (SD 3)] and mean birth weights were 2479 g vs 2172 g. They reported that infants with TAT needed significantly less time to achieve full enteral feeds ($p < 0.001$). The center which did not perform routine TATs subsequently introduced TAT as standard practice and reported that the new cohort (seven infants) took less time to reach full feeds (9 ± 3 days) compared to the old cohort (17 ± 4 days). Only one neonate in the new TAT cohort required parenteral nutrition compared to all nine from the old cohort from the same institution. No mechanical complications related to TAT such as migration, displacement, and perforation were noted. The authors concluded that the use of TAT leads to earlier attainment of full enteral feeds.

Hall et al. (2011)

In this retrospective study from Southampton, UK [12] (1999–2008), authors compared the outcomes of 17 neonates with CDO who were managed with TAT versus 38 without TAT. Median gestational age (38 vs 37 weeks) and birth weight (2800 g vs 2600 g) were similar between the groups. Enteral feeds post-operatively were commenced earlier in infants with a TAT compared to those without (2 days vs 3 days; $p=0.006$). Infants with a TAT achieved full feeds sooner after surgery than those without [6 days (2–12) vs 9 days (3–36); $p=0.005$]. The multivariable analysis also confirmed that the use of a TAT was associated with reduced time to full feeds by an average of 4 days (95% CI 0.9–7.1). The need for central venous catheters (CVC) and parenteral nutrition was lower in the TAT group (2/17 vs 28/38, $p=0.0001$). Four TATs got displaced and were removed before achieving full enteral feeds. One infant in the TAT group developed an anastomotic leak and jejunal perforation that required re-operation; he/she also had trisomy 21 and was subsequently diagnosed to have Hirschsprung disease. In the no-TAT group, there were six CVC-related complications (five infections, one PN extravasation). The authors concluded that TAT significantly decreases time to full enteral feeds and the need for central venous access and PN. It was the only study that used multivariate regression analyses to adjust for potential confounders.

Harwood et al. (2019)

In this retrospective study from Manchester, UK [16] (2004–2014), authors studied the impact of TAT on the cost of post-operative nutrition in neonates with CDO. Median gestational age and birth weight were similar between the TAT and no-TAT groups [38 vs 36.5 weeks and 2.85 kg versus 2.64 kg, respectively]. Of the 59 infants, 37 received intraoperative TAT, and 22 were managed without TAT. Baseline characters were similar between the two groups. The duration of postoperative parenteral nutrition was significantly shorter in the TAT group [6 (0–11) vs 12 (8–19) days, $p=0.006$], as were the cost of PN [£750 (0–1375) vs £1500 (1000–2375), $p=0.006$] and the total cost of nutrition [£765.26 (38.36–1404) vs £1387.52 (1008.23–2363.08), $p=0.015$]. Overall, there was a median cost saving of £622.26 per patient. TAT displacement occurred in 5/37 (14%) infants, but no other complications related to TAT occurred. They concluded that TAT is a safe and effective way to reduce the duration of PN in patients with CDO.

Risk of bias in the included studies

Summary of risk of bias assessment using the ROBINS-I tool is given in Table 2 and Fig. 2. Three studies carried a “serious” or “critical” risk of bias in the domain of “Bias due to confounding”. All studies carried a “moderate” risk of bias in the other domains given their retrospective nature.

Table 2 Risk of Bias in the included studies

Author (year)	Bias due to confounding	Bias in all other domains ^a
Mooney et al. (1987)	Serious: Six infants in the study received gastrostomies, not clear if they belonged to TAT or no-TAT group. Authors conducted a sensitivity analysis wherein TAT versus no TAT groups was compared after taking into consideration prematurity and serious anomalies. Hence there was an appropriate attempt at adjusting for potential confounders, but details of the results were not given	Moderate
Upadhyay et al. (1996)	Serious: The TAT group had received gastrostomy which could in itself result in morbidities Feeding gastrostomies are not standard practice in managing CDO	Moderate
Ruangtrakool et al. (2001)	Critical: Unequal distribution of infants between the two groups (4 versus 30)	Moderate
Arnbjornsson et al. (2002)	Moderate: Mix of Retrospective and prospective design; No significant difference across the groups with respect to demographic characteristics, associated malformations and method of treatment	Moderate
Hall et al. (2011)	Moderate: Baseline characters were similar between the two groups. Multivariable analysis was done to adjust for potential confounders	Moderate
Harwood et al. (2019)	Moderate: Baseline characters were similar between the two groups	Moderate

^aAll studies had moderate risk of bias in the domains of “Bias in selection of participants into the study”, “bias in classification of interventions”, “bias due to missing data”, bias in “measurement of outcomes” and bias in “selection of the reported result”

Results of meta-analysis

Mortality: There were no significant differences in the risk of mortality between the two groups (RR: 1.24; 95% CI 0.07, 22.22) (Fig. 3).

Sepsis: There were no significant differences in the risk of sepsis between the two groups (RR: 1.55; 95% CI 0.42, 5.73) (Fig. 4). The results were similar on sensitivity analysis (Fig. 5).

Time to reach full enteral feeds: On primary analysis, there were no significant differences in the time to reach full feeds between the groups (mean difference: -1.43 ; 95% CI -8.43 to 5.58) (Fig. 6). However, on sensitivity analysis, time to reach full feeds was significantly shorter in the TAT group (mean difference: -6.63 ; 95% CI -8.83 to -4.43) (Fig. 7). **Duration of parenteral nutrition:** On primary analysis, there were no significant differences in the duration of PN between the two groups (mean difference: -2.02 ; 95% CI -7.36 to 3.33) (Fig. 8). However,

Fig. 2 Summary risk of bias in the included studies

ROBINS-I GRAPHICAL PRESENTATION

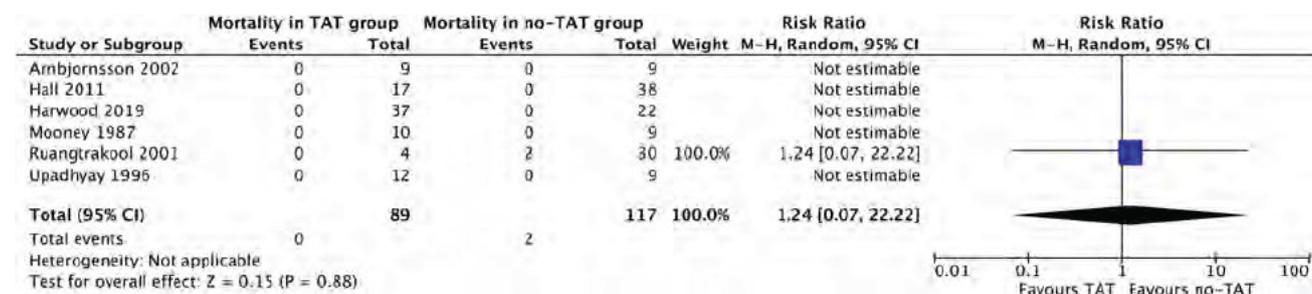
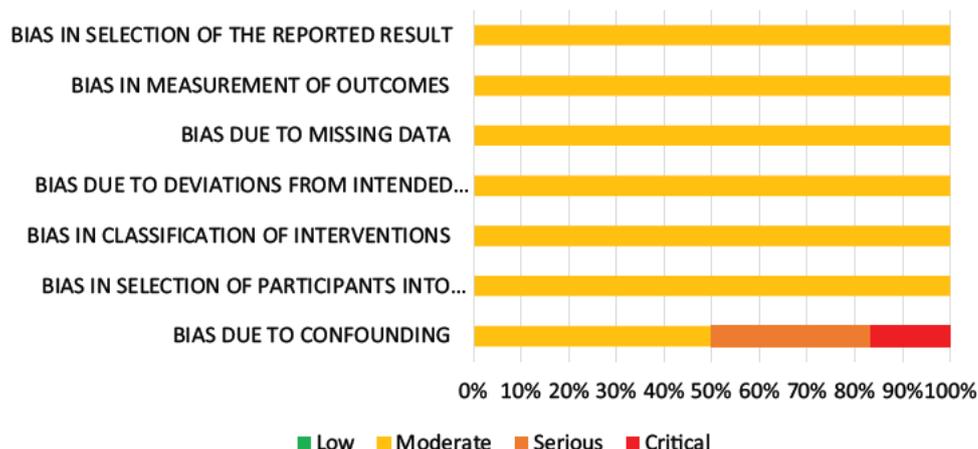


Fig. 3 Mortality

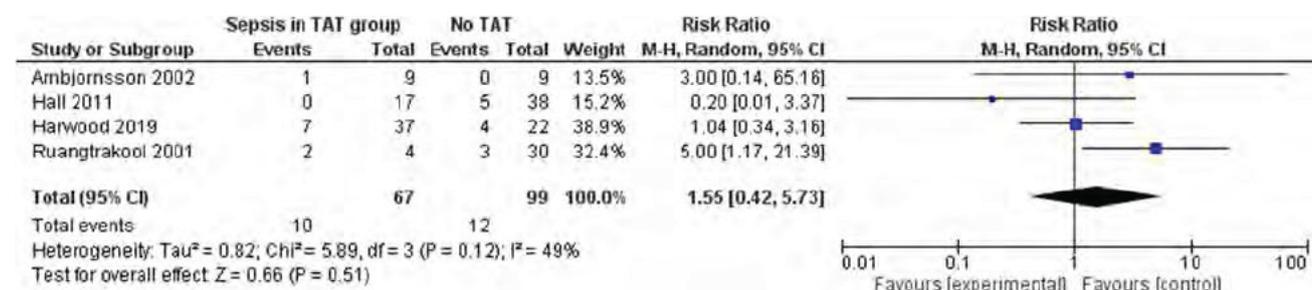


Fig. 4 Sepsis

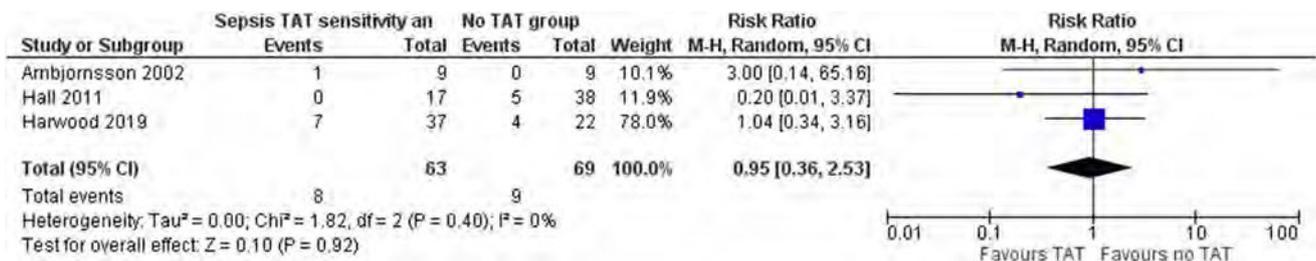


Fig. 5 Sepsis: sensitivity analysis after excluding studies with a high risk of bias

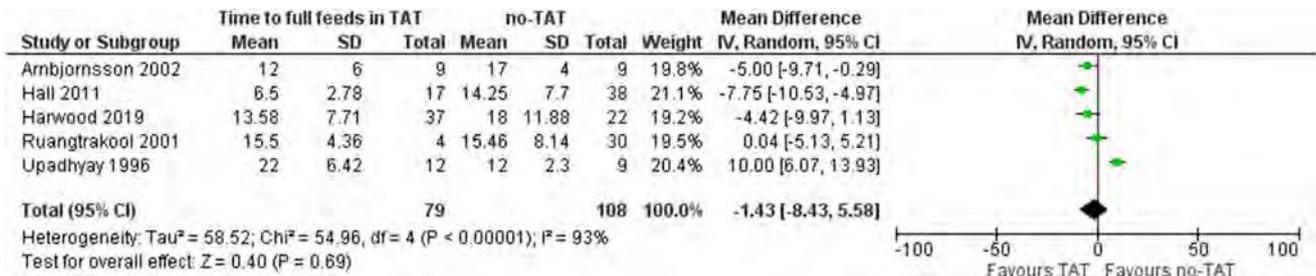


Fig. 6 Time to reach full feeds

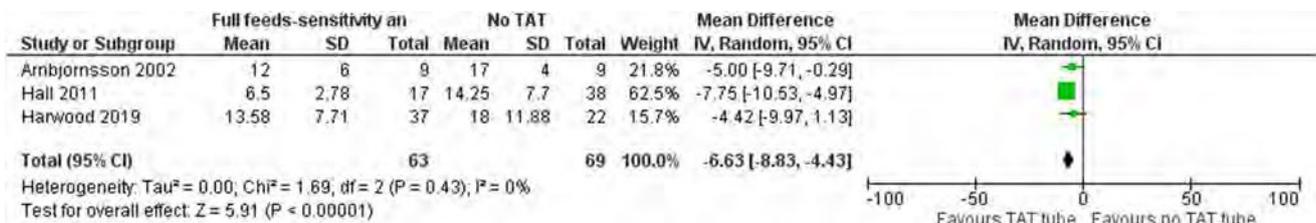


Fig. 7 Time to reach full feeds: Sensitivity analysis after excluding studies with a high risk of bias

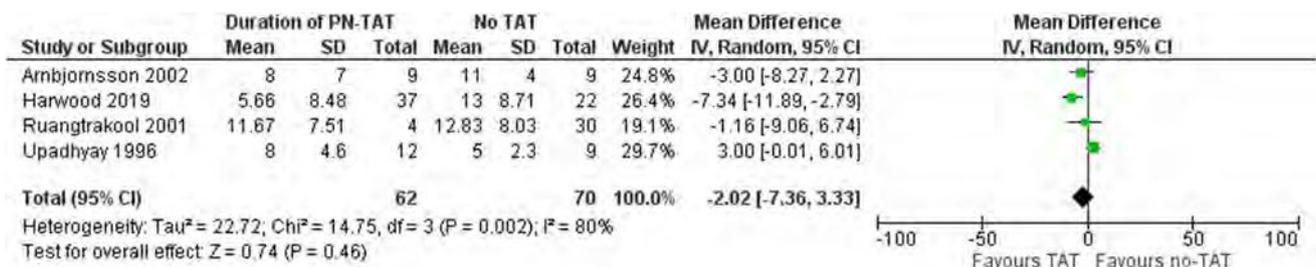


Fig. 8 Duration of parenteral nutrition

on sensitivity analysis, duration of PN was significantly shorter in the TAT group (mean difference: -5.38 ; 95% CI -9.61 to -1.15) (Fig. 9).

Duration of hospital stay: On primary analysis, there were no significant differences in the duration of hospital stay between the two groups (mean difference: 2.22 ; 95%

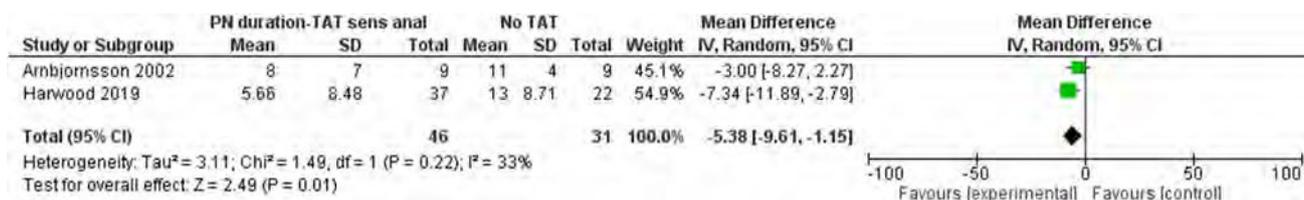


Fig. 9 Duration of parenteral nutrition: sensitivity analysis after excluding studies with high risk of bias

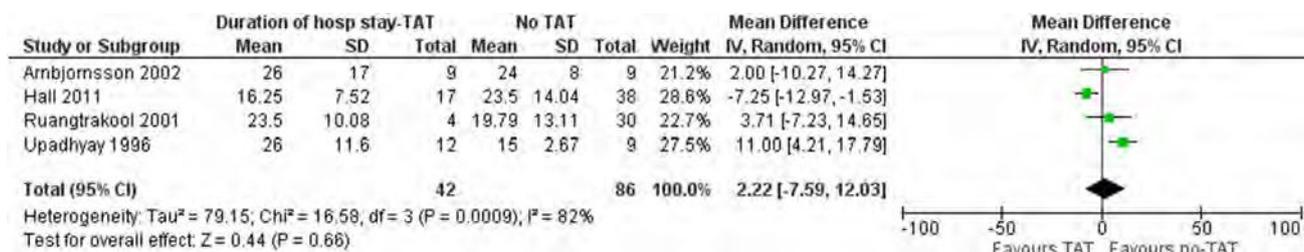


Fig. 10 Duration of hospital stay

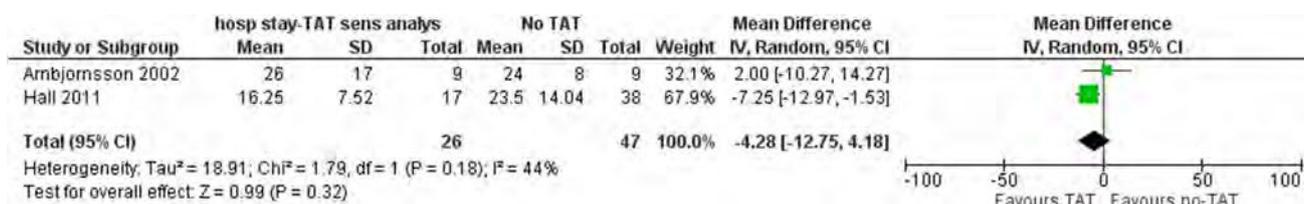


Fig. 11 Duration of hospital stay: sensitivity analysis after excluding studies with high risk of bias

CI – 7.59 to 12.03) (Fig. 10). The results were similar on sensitivity analysis (Fig. 11).

Overall quality of evidence: On GRADE analysis, the overall quality of evidence was considered very low since all included studies were non-RCTs, had small sample sizes and there was statistically significant heterogeneity on meta-analysis.

Discussion

In this systematic review that included 6 observational studies, there were 96 infants who underwent intraoperative TAT placement and 117 who did not. Four studies reported some benefits of TAT such as early commencement of enteral feeds, early attainment of full feeds, and decreased need for parenteral nutrition [11, 12, 15, 16]. On the other hand, two studies reported better outcomes in the no-TAT group [13, 14]. Accidental removal of TAT without clinical harm was reported in three studies [(5/37(14%), 4/17(23%), and 2/4(50%)] [16]. One study reported that one infant in the TAT group had jejunal perforation, but the infant also

had Hirschsprung disease and the site of perforation was away from the tip of the TAT [12]. Apart from the studies included in our systematic review, Bairdain et al. [3] studied their CDO series to identify the predictors of the need for PN. Among 87 infants studied, 13 received intraoperative TAT whereas the remaining 74 did not. While they did not directly compare TAT versus no-TAT groups, they reported that the presence of TAT was not associated with time to reach full enteral feeds on multivariable analysis. They also reported that gestational age ≤ 35 weeks, congenital heart disease, and concurrent malrotation were independently associated with the need for PN. While the median time to reach full enteral feeds in the TAT group was lower at 9 days (IQR: 7 to 22) compared to 12 days (9 to 17) in the entire cohort, it was not statistically significant ($p = 0.28$). TAT-associated complications were not observed in any of the 13 infants who received TAT [3].

Parenteral nutrition, while important in ensuring adequate growth and preventing nutritional deficiencies, can lead to morbidities such as sepsis, cholestasis, and mechanical complications of central venous catheters. Bishay et al. [4] in their retrospective study of 54 cases reported that while

infants with CDO can be managed without PN [4], a third of them subsequently required PN, lost weight, and had a higher rate of sepsis. They also reported that the ‘Never PN group’ ($n = 22$) had the least sepsis related complications (14%) and were discharged earlier (8 days) compared to ‘Initial PN’ ($n = 19$, 37%, 14 days) and ‘Delayed PN’ ($n = 13$, 46%, 25 days) groups. Hence, parenteral nutrition, while essential, needs to be used judiciously and to the shortest duration possible.

Prolonged fasting can lead to mucosal atrophy, decreased expression of intestinal enzymes, and altered peristalsis [17], leading to further intolerance of enteral feeds. Early commencement and advancement of enteral feeds in the postoperative period have the potential to improve the outcomes of neonates with surgical conditions [18–20]. Hence every effort should be made to facilitate enteral feeds early in the postoperative period. Insertion of TAT during surgery for CDO is one strategy, but our systematic review found that current evidence comes only from retrospective studies, and hence the evidence is inconclusive regarding its benefit and safety. Well-designed RCTs are needed in this area.

An important strength of our study is the comprehensive search of the literature and the assessment of the risk of bias using the ROBINS-I tool. To our knowledge, it is the first systematic review evaluating the usage of TATs in CDO. An important limitation was the lack of RCTs and the small sample size. In addition, none except one of the included studies used multivariate regression analyses to adjust for potential confounders. Other important limitation was the presence of heterogeneity or lack of information on the frequency of anomalies such as trisomy 21, esophageal atresia, congenital heart disease; duodenal stenosis vs duodenal atresia, type of anastomosis used (duodeno-duodenostomy vs duodenojejunostomy, diamond vs standard anastomosis), type of feed (breast milk vs formula), etc. Future RCTs should be designed in such a way to ensure balance between the two groups for such potential confounders.

In the RCTs of TAT versus no-TAT, since it is not possible to blind the investigators and healthcare professionals to the type of intervention, it is important to ensure rigorous methodology in generating random sequence numbers and to achieve thorough allocation concealment. The primary outcome of interest could be time to reach full enteral feeds in the postoperative period because theoretically, infants with TAT should be able to tolerate enteral feeds early because the TAT bypasses the patulous proximal duodenal segment. Establishing a standardized feeding regimen will ensure uniform practice among all trial participants and hence results will be more reliable. This step is very important to prevent bias by treating physicians given the unblinded nature of the RCT. Other important nutritional outcomes that should be measured are z scores for weight, length and head

circumference at discharge to assess the impact of intervention on postnatal growth restriction in these infants.

In a recent study by our group (to be published) that included 80 infants with CDO who were managed without TAT, the meantime to full feeds after surgery was 14.6 days (SD 12.9). To demonstrate that the use of TAT reduces the time to full feeds by 50% to 7.3 days, with an alpha error of 5% and power of 80%, a sample size of 50 infants in each group would be needed (total sample size: 100). It would need a multicenter trial to achieve this sample size within a reasonable period of time. The other clinical outcomes of interest such as sepsis, mortality, duration of TPN, duration of hospital stay, cholestasis, and potential complications of TAT such as migration, intestinal perforation, and the anastomotic leak should all be monitored in such an RCT.

Conclusions

No RCTs comparing TAT versus no-TAT were identified. The available data comprising predominantly retrospective studies show no clear advantages or disadvantages. The current evidence is limited regarding the efficacy and safety of intraoperative TAT placement in neonates with CDO. Well-designed RCTs are needed to address the issue definitively.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00383-021-04954-7>.

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Declarations

Conflicts of interests The author declare no conflict of interests.

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ORIGINAL ARTICLE

Does continuous positive airway pressure for extubation in congenital tracheoesophageal fistula increase the risk of anastomotic leak? A retrospective cohort study

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Aim: Immediate post-operative care of tracheoesophageal fistula (TEF) and oesophageal atresia (EA) requires mechanical ventilation. Early extubation is preferred, but subsequent respiratory distress may warrant re-intubation. Continuous positive airway pressure (CPAP) is a well-established modality to prevent extubation failures in preterm infants. However, it is not favoured in TEF/EA, because of the theoretical risk of oesophageal anastomotic leak (AL). The aim of this study was to find out if post-extubation CPAP is associated with increased risk of AL.

Methods: Retrospective cohort study (2007–2014).

Results: Fifty-one infants underwent primary repair in the newborn period. Median age at surgery was 24 h (interquartile range: 12, 24). In the post-extubation period, 10 received CPAP, whereas 41 did not. The median post-operative day at the commencement of CPAP was 2.5 days (interquartile range: 1, 6 days). Zero out of 10 in the CPAP group and 4/41 in the 'no CPAP' group developed AL on routine post-operative contrast studies ($P = 0.57$). Zero out of 10 in the CPAP group and 1/41 in the 'no CPAP group' developed recurrence of TEF necessitating re-surgery ($P = 1.00$). The neonate with recurrent fistula also had coarctation of aorta and needed protracted hospitalisation of 6 months, mainly because of the recurrence of TEF.

Conclusion: The use of CPAP in the immediate post-extubation period after corrective surgery for TEF/EA appears to be safe and may not be associated with increased risk of AL or recurrence of the fistula. Information from other centres, surveys and large databases is needed to define the benefits and risks of use of CPAP in these infants.

Key words: anastomotic leak; CPAP; oesophageal atresia; tracheoesophageal fistula.

What is already known on this topic

1 Anastomotic leak is a known complication after repair of tracheoesophageal fistula (TOF).

What this paper adds

1 Continuous positive airway pressure after extubation in the post-operative period for TOF may not increase the risk of anastomotic leak.

Post operative management of tracheoesophageal fistula (TEF)/oesophageal atresia (EA) includes provision of mechanical ventilation (MV).^{1–3} Because prolonged MV carries the risk of pneumonia, atelectasis and other morbidities,⁴ early extubation is preferable; but post extubation atelectasis or tracheomalacia might warrant re intubation.

Continuous positive airway pressure (CPAP) has been used successfully to prevent extubation failure in preterm infants⁵ and in

the post operative care of critically ill children with heart diseases.⁶ However, the role of CPAP in the post operative management of TEF/EA is not clear.

There are theoretical concerns that CPAP in the post operative period might result in increased distension of the oesophagus leading to anastomotic leak (AL). In a retrospective review of X rays of 57 preterm infants on CPAP, Walor *et al.* found significant distension of the hypopharynx and the cervical oesophagus.⁷

Oesophageal AL is an important post operative complication, with an incidence of 2–8%.^{8–14} While majority of the leaks are asymptomatic and heal spontaneously, they can also lead to mediastinitis, recurrence of the fistula¹⁵ and oesophageal strictures.¹⁶

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Conflict of interest: None declared.

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Table 1 Demographic characteristics

Female : Male	22 : 29
Birthweight (grams)	2783 (2325, 3142)
Gestational age (weeks)	38 (36.4, 39.5)
Antenatal known	9
Apgar at 5 min	9 (8, 9)
Resuscitation at birth	
PPV + CPAP	7
PPV + ventilation	2
Self ventilating	42
Type of TEF/EA†	
Type A	1
Type B	1
Type C	44
Type D	1
Type E (H type fistula)	4

†Gross, RE. The surgery of infancy and childhood. Philadelphia, WB Saunders; 1953. All discrete data presented as (n, %) and continuous data presented as median (IQR). CPAP, continuous positive airway pressure; EA, oesophageal atresia; PPV, positive pressure ventilation; TEF, tracheoesophageal fistula.

Considering that CPAP is a well established modality to prevent extubation failure in other conditions,^{5,6} but might carry a theoretical risk of AL, we aimed to find out whether the use of CPAP in the

immediate post extubation period is associated with increased risk of AL or recurrence of the fistula in TEF/EA.

Design

This retrospective cohort study was reported using the STROBE guidelines.¹⁷

Study period

The study period included the years 2007–2014.

Ethics approval

The study was approved by hospital's quality improvement committee as having met the 'Australian National Health and Medical Research Council requirements for quality assurance and audit projects'.¹⁸

Participants and setting

Neonates with EA and/or TEF admitted to the Neonatal Intensive Care Unit of Princess Margaret Hospital for Children, Perth, Western Australia were included in the study.

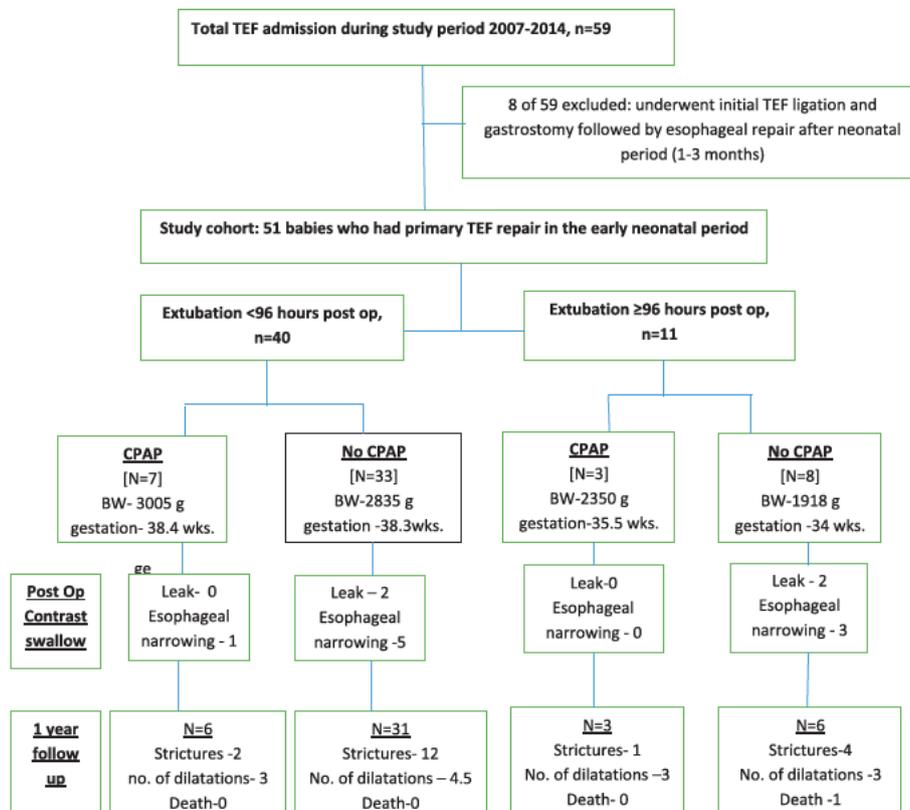


Fig. 1 Flow diagram of study selection and outcomes of neonates with tracheoesophageal fistula (TEF). BW, birthweight; CPAP, continuous positive airway pressure.

Method

Cases were identified by interrogating Neonatal Database of the department. Relevant clinical details were extracted from the medical records.

Statistical analysis

stata 13.0 (StataCorp, Lakeway Drive, Texas, USA) was used for the statistical analysis of the data. Continuous variables were described using median and interquartile range (IQR). Fisher's exact test was used for comparison of the outcomes between the CPAP and 'no CPAP' group. Univariate analyses were carried out where appropriate to derive effect size estimates. Multivariate regression analysis was not attempted in view of unequal number of patients in each group and the relatively small sample size. For all analyses, a *P* value of less than 0.05 was considered as statistically significant. Effect size estimates are presented as unadjusted relative risk and 95% confidence intervals.

Results

A total of 51 neonates who underwent primary repair of TEF in the immediate newborn period were included. Eight infants who underwent gastrostomy and ligation of the fistula in the newborn period, followed by subsequent repair of the EA at 1–3 months were excluded; the reason being such infants are known to have higher rates of recurrence of fistula, ALs, gastroesophageal reflux and strictures compared with those who undergo primary repair.

The median (IQR) gestational age and birthweight were 38.0 (36.4, 39.5) weeks and 2783 (2325, 3142) grams, respectively. Details of the study cohort are given in Table 1.

Forty neonates were extubated within 96 h of surgery, of which seven required CPAP in the immediate post extubation period (within 72 h of extubation). Of the 11 neonates who were extubated more than 96 h post surgery, three received CPAP (Fig. 1).

All 51 infants underwent contrast study of the oesophagus at a median post operative day 6 (IQR: 6, 7). Among the 10 infants who received CPAP, the median post operative day when CPAP was commenced was 2.5 days (IQR: 1, 6; range: 1, 14). Details of the peri operative characteristics and neonatal course are given in Table 2.

Zero out of 10 in the CPAP group and 4/41 neonates in the 'no CPAP' group developed AL on the routine post operative contrast

Table 2 Perioperative characteristics

Age at surgery (<i>n</i> = 51) (hours of life)	24 (12, 24)
Long gap lesion (<i>n</i> = 47, excluding four with H type TEF)	11
Surgery (<i>n</i> = 51)	
Ligation and primary anastomosis	47
Ligation of fistula (H type TEF)	4
Respiratory support before surgery	
Self ventilating	41
CPAP	8
Mechanical ventilation	2
Respiratory support immediate post op (<i>n</i> = 51)	2
Self ventilating	0
CPAP mechanical ventilation	49
Duration of mechanical ventilation, h (<i>n</i> = 51)	60 (40, 93)
Extubation within 96 h of surgery	40/51
CPAP (<i>n</i>)	7
Duration (h)	21 (10, 155)
Re intubation in 72 h of CPAP	1
No CPAP	33
First contrast study	
Median age post op (days)	6 (6, 7)
Anastomotic constriction on contrast (<i>n</i>)	9
Anastomotic leak on contrast (<i>n</i>)	4
Recurrence of fistula	1
Death	1
Duration of hospital stay (days)	16 (11.5, 25)

All discrete data presented as *n*, (%) and continuous data presented as median (IQR). CPAP, continuous positive airway pressure; TEF, tracheoesophageal fistula.

study (*P* = 0.57). All four ALs resolved easily with conservative management. Oesophageal narrowing was noted on the initial contrast in 1/10 in the CPAP group versus 8/41 in the no CPAP group (relative risk (RR) 0.51, 95% CI: 0.07, 3.64; *P* = 0.667). Details of clinically important outcomes are given in Table 3.

Zero out of 10 in the CPAP group and 1/41 in the 'no CPAP group' developed recurrence of the TEF necessitating re surgery (*P* = 1.00). The infant with recurrent TEF had multiple, recurrent pneumothorax, pleural effusion and prolonged feed intolerance. The routine post operative contrast study had not demonstrated AL. Subsequent contrast studies also had failed to diagnose the recurrence of the fistula. It was diagnosed on bronchoscopy examination under general anaesthesia. Surgery for ligation of the recurrent fistula was performed at 3 months of age. The infant also

Table 3 Comparison of outcomes between infants who received continuous positive airway pressure (CPAP) versus those who did not

	CPAP (<i>n</i> = 10)	No CPAP (<i>n</i> = 41)	Relative risk (95% CI)	<i>P</i> value
Anastomotic leak on contrast study	0	4	NC	0.573
Oesophageal narrowing on contrast study	1	8	0.51 (0.07, 3.64)	0.667
Recurrence of TEF	0	1	NC	1.000
Mortality at 1 year follow up	0	1	NC	1.000
Oesophageal stricture at 1 year follow up (<i>n</i> = 46)	3/9	16/37	0.77 (0.28, 2.08)	0.716

NC because there were no anastomotic leaks or deaths in the CPAP group. CI, confidence interval; NC, not calculable; TEF, tracheoesophageal fistula.

had coarctation of the aorta, which was successfully repaired in the first week of life. The main reason for prolonged hospitalisation was respiratory and gastrointestinal morbidity secondary to the recurrent fistula.

One infant in the CPAP group had an upper pouch fistula in addition to the classical distal fistula. The upper pouch fistula had been missed during the initial surgery. The infant had associated anomalies including transposition of great vessels, pulmonary stenosis and imperforate anus, which were repaired in the neonatal period. Surgical ligation of the upper pouch fistula was performed at 50 days of life, and discharge home was achieved on day 85 of life. The main reason for prolonged hospitalisation was respiratory and gastrointestinal morbidity secondary to the second fistula.

There was one death (preterm infant, 31 weeks). The infant also had vestibular anus needing sigmoid colostomy, Fallot's tetralogy and sacral anomalies.

Follow up data until 1 year of age was available on 46/50 (92.0%) surviving infants. By 1 year, endoscopic dilatation of the oesophagus was required in 19/46 (43.5%) infants. Of these, 3/9 were in the CPAP group versus 16/37 in the 'no CPAP' group (RR: 0.77, 95% CI: 0.28, 2.08; $P = 0.716$). The median number of dilatations was 3 (IQR: 2, 7; range: 1, 10). No recurrence of fistula was reported post discharge.

Discussion

The results of our retrospective study suggest that CPAP after extubation in the post operative care of the newborn with TEF/EA appears to be safe and may not be associated with AL, recurrence of fistula, oesophageal strictures or mortality. To our knowledge, this is the first study to document the use of CPAP in neonates with TEF/EA.

It was reassuring to know that none of the 10 neonates who received CPAP developed AL or recurrence of the fistula. The only case in the CPAP group with significant morbidity had an additional upper pouch fistula, which had been missed at the time of initial surgery. Type C EA has been reported to be associated with a proximal TEF in up to 1.4% of cases.¹⁹ Although rare, this missed proximal TEF may present later and is often mistaken as recurrent TEF. Bronchoscopy is usually essential for its diagnosis. The primary distal TEF is usually located at the carina level, whereas the missed proximal TEF is usually located above this level.¹⁹

While there are adequate numbers of literature on the contemporary outcomes of TEF/EA,^{1,20-22} there is no information regarding the use of CPAP in the post extubation period. Published review articles²³ and surveys^{24,25} have evaluated various aspects of post operative care of TEF/EA, but none to our knowledge have explored the use of CPAP in post extubation period.

Hence, it is difficult to know if clinicians are using CPAP in the post extubation phase after the repair of EA/TEF. Hence, we believe that documentation of our experience with the use of CPAP is a step in the right direction.

The main strength of our study is that all neonates born with TEF/EA in the state of Western Australia are managed in our unit. Hence, it represents a complete data from this state. The major limitation is the small sample size because only 10 neonates received CPAP. For the same reason, we were unable to do multivariate regression analysis.

Conclusion

The use of CPAP in the immediate post extubation period after corrective surgery for TEF/EA in the newborn period appears to be safe and may not be associated with increased risk of AL or recurrence of the fistula. Information from other centres, surveys and large databases is needed to define the benefits and risks of the use of CPAP in these infants.

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ORIGINAL ARTICLE

Role of upper gastrointestinal contrast studies for suspected malrotation in neonatal population

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Aim: Upper gastrointestinal (UGI) contrast study is the preferred radiological investigation to diagnose malrotation of intestine. We aimed to review the role of UGI contrast in neonates (term and preterm) who were clinically suspected to have malrotation.

Methods: The study included a retrospective review of medical charts and radiology reports.

Results: A total of 164 newborn infants underwent UGI contrast study to rule out malrotation during the study period (2006–2015). Median gestational age at the time of presentation was 38 weeks (interquartile range: 35.5–39.6 weeks). Median age for clinical presentation was day 2 of life (interquartile range: 2–5 days). Out of the 164 contrast studies, 112 were normal, whereas 52 were reported to have malrotation. Of those 52 infants, 47 were confirmed to have malrotation on surgery (positive predictive value: 90). Of the 112 infants with normal UGI contrasts, nine infants underwent laparotomy for ongoing clinical symptoms out of which four infants were diagnosed to have malrotation on laparotomy. There were 22 infants born at gestational age <32 weeks, who underwent UGI contrast studies to rule out malrotation. Their clinical symptoms were similar to necrotising enterocolitis. Of 22 preterm contrast studies, six were reported to have malrotation; of these, five had surgically confirmed malrotation. No complications related to the contrast study were noted in both term and preterm infants.

Conclusion: Current study reaffirms the role of UGI contrast study as the investigation of choice for diagnosis of malrotation, in both term and preterm infants. UGI contrast is safe and well tolerated even in preterm infants.

Key words: contrast studies; intestinal malrotation; neonatology.

What is already known on this topic

- 1 Bilious vomiting is an important symptom of malrotation of intestines.
- 2 Upper gastrointestinal (UGI) contrast study has been the investigation of choice for diagnosis of malrotation.
- 3 High index of clinical suspicion, early UGI contrast study and prompt surgery can reduce mortality and morbidity associated with malrotation.

What this paper adds

- 1 Our study reaffirms the role of UGI contrast study as the investigation of choice for the diagnosis of malrotation.
- 2 In preterm infants, malrotation can present with clinical symptoms similar to necrotising enterocolitis.
- 3 UGI contrast studies with isotonic contrast media are safe and need to be considered even in extremely preterm infants with clinical suspicion of malrotation.

Malrotation of the intestines is an important surgical condition, defined by abnormal fixation of bowel within the peritoneal cavity. Its true incidence is unknown considering its variable presenting features. The incidence of malrotation has been reported variably from 1 in 6000 live births to as high as 1 in 500 births to 1% of the population on autopsy.^{1–4} Up to 75% of all cases of malrotation present clinically in the neonatal period.^{5–7} Early diagnosis and surgical correction is of paramount importance to avoid mortality and morbidity such as bowel ischaemia and short bowel syndrome (SBS). Upper gastrointestinal (UGI) contrast study has

been considered the best available investigation to diagnose malrotation.⁸ There are few recent studies that advocate ultrasound as the preferred modality for diagnosing malrotation.^{9–11} However, there is not enough data yet to support ultrasound as a primary imaging investigation for diagnosing malrotation. Although it is essential to request for UGI contrast studies in suspected cases, it is also important to utilise the radiology resources judiciously. Hence, we conducted a retrospective analysis of neonates who presented with clinical features suspicious of malrotation and had UGI contrast study.

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Conflict of interest: None declared.

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Methods

Ethics approval

The study was approved by hospital's quality improvement committee as having met the 'Australian National Health and Medical

Research Council requirements for quality assurance and audit projects'.

This study was conducted at the Princess Margaret Hospital for Children and King Edward Memorial Hospital for Women, Western Australia (duration 2006–2015). Radiological and neonatal database from both hospitals were screened to identify neonates who underwent UGI contrast study for clinical suspicion of malrotation. The clinical symptoms and signs for which the study was requested included bilious vomiting or bilious aspirates with or without abdominal distension or tenderness. In preterm infants, persistent bilious aspirates or tender abdomen or distended abdomen in absence of radiological signs of necrotising enterocolitis (NEC) were the common indications for undergoing UGI contrast study. Infants who had UGI contrast studies performed for conditions such as oesophageal atresia, trachea oesophageal fistula and gastro oesophageal reflux (GOER) were excluded. Neonates with abdominal wall defects such as gastro schisis, exomphalous and congenital diaphragmatic hernia were also excluded because nonrotation is considered as a part of the developmental defect in these conditions.¹²

The demographic and clinical data were collected from the medical records of patients. Reports of all radiological studies (X ray of abdomen, UGI contrast study and abdominal ultrasound with colour Doppler) were recorded. For neonates who underwent laparotomy, the operative findings were noted.

Diluted low osmolality water soluble contrast agent Iohexol (Omnipaque) was used for UGI contrast in the current study. A small amount (5–10 mL) of contrast agent was administered preferably orally or via nasogastric tube in extreme preterm infants. After the conclusion of the study, excessive contrast agent was aspirated via nasogastric tube.

The standard practice of the unit is to ensure UGI contrast study is done immediately upon admission to the neonatal intensive care unit (NICU). In the majority of the cases, infants were retrieved from the referring hospitals by the neonatal emergency transport team and directly taken to the radiology department for UGI contrast studies.

The paediatric surgeon or paediatric surgical fellow is present in the radiology department while UGI contrast is undertaken. Babies with radiological diagnosis of malrotation are directly transferred to operation theatre from the radiology department.

Statistical analysis

Statistical analysis was performed using Stata 11.0 (STATA Corp., College Station, TX, USA). Median, interquartile range (IQR) and range were calculated for data with skewed distribution. Mean and SD were calculated for data with normal distribution. Percentages were calculated for categorical items.

Results

The above search strategy (Fig. 1) led to a total study population of 164 neonates during the study period (2006–2015).

Demographic details and clinical features of neonates undergoing UGI contrast studies are given in Tables 1 and 2, respectively.

The study population's gestational age ranged from 26.1 weeks at birth to 41.4 weeks, with mean gestational age being 36.9 weeks.

UGI contrast studies performed on neonates at NICU during years 2006–2015

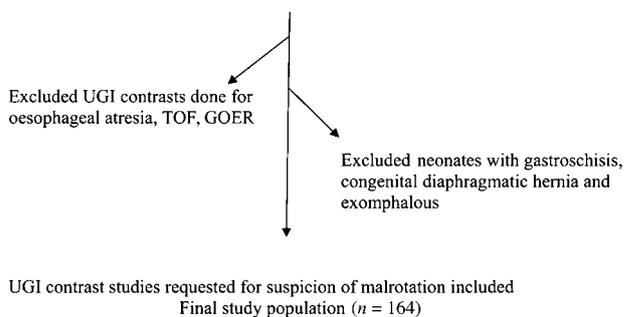


Fig. 1 Study population. GOER, gastroesophageal reflux; NICU, neonatal intensive care unit; TOF, tracheoesophageal fistula; UGI, upper gastrointestinal.

The mean birth weight was 2867 g (range: 860–4635 g). The median age at presentation with clinical symptoms suggestive of malrotation was 2 days (mean 5.6 days). After suspicion of malrotation, the majority of babies underwent UGI contrast study within 2 h (range 1–5 h). Laparotomy was performed in all cases immediately after radiological diagnosis.

Radiological investigations

A total of 164 infants underwent UGI contrast study for clinical suspicion of malrotation ($n = 164$; Fig. 2).

All but 15 contrast studies were preceded by plain X ray of abdomen, with anteroposterior and lateral decubitus views. Ultrasound was performed in selected cases where there was suspicion of volvulus or UGI contrast study was equivocal ($n = 48$ cases), more so in the later years of the study.

Lower gastrointestinal (GI) contrast was performed in 30 cases where UGI contrast was inconclusive or if there was suspicion of colonic pathology on plain abdominal film or UGI contrast.

UGI contrast study in 52 infants was suggestive of malrotation and hence they all underwent laparotomy, of whom 47 were confirmed to have intestinal malrotation. Hence, positive predictive value (PPV) of UGI contrast study in the diagnosis of malrotation was 90%.

Because the majority of infants with normal UGI contrast do not undergo laparotomy, true negative and false negative values for the test could not be obtained. Hence, it was not possible to calculate sensitivity, specificity, negative predictive value and likelihood ratios.

There were five infants whose UGI contrast study was suggestive of malrotation but at laparotomy did not have malrotation. Two of them had Hirschsprung disease and the other two had meconium ileus, of which one was later diagnosed with cystic fibrosis. In the remaining one, no pathology was found on laparotomy.

Of the 112 contrast studies which did not show malrotation, 103 did not require surgical intervention. In the remaining nine, further investigations such as plain X ray of abdomen, lower GI contrast study and ultrasound of abdomen were performed to find underlying cause for clinical concerns. All of these nine infants underwent laparotomy, four of whom were found to have malrotation on surgery. The remaining five had pathologies

Table 1 Demographic data of study population

Parameter	Median	25th centile	75th centile	Mean	Standard deviation
Gestational age (weeks)	38	35.5	39.6	36.9	3.7
Birth weight (g)	3030	2440	3390	2867	831
Age at presentation (days)	2	2	5	5.6	8.4
Age at upper gastrointestinal contrast study (days)	3	2	7	6	9

Table 2 Clinical features of neonates undergoing upper gastrointestinal contrast†

Clinical parameters	n (%), n	164
Gender		
Male	102 (62)	
Female	62 (38)	
Abdominal distension	56 (34)	
Tender abdomen	11 (7)	
Visible bowel loops	13 (8)	
Delayed by passage of meconium (>24 h)	32 (20)	
Antenatal suspicion	8 (5)	
Bilious vomits		
Yes	101 (61.5)	
No	63 (38.5)	
Number of bilious vomits (mean)	1.3	
Bilious aspirates	91 (55)	

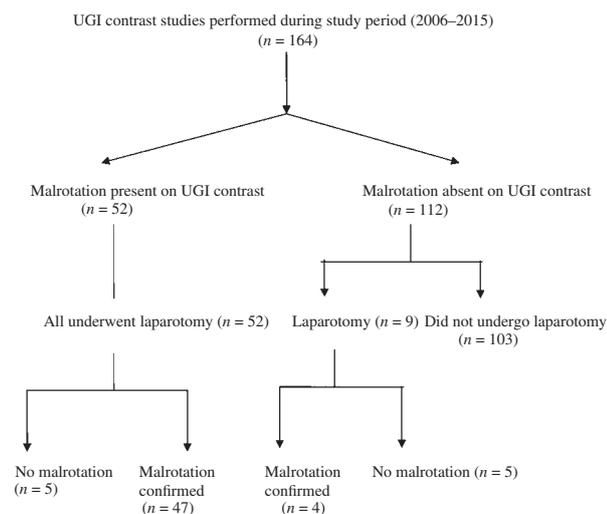
†One infant could have more than one symptom.

such as meconium ileus, NEC, jejunal stenosis, bowel atresia and Hirschsprung disease.

Three neonates with confirmed malrotation died. The first patient was 35 weeks gestation infant with multiple congenital anomalies that included malrotation, Hirschsprung disease and hydrocephalus. He died of pneumonia post ventriculo peritoneal

shunt surgery. The second infant was 37 weeks of gestation presented on day 1 with respiratory distress, abdominal distension and bilious aspirates. Her UGI contrast study revealed malrotation with volvulus. On exploratory laparotomy she had extensive bowel necrosis and died within 24 h. The third baby was 38 weeks of gestation at birth, was operated for malrotation on day 1 of life. He developed severe pulmonary hypertension post operatively, and died on day 4. Autopsy findings confirmed that he had the lethal condition of alveolar capillary dysplasia.

The standard protocol of our department is that all patients with suspected malrotation undergo UGI contrast, unless critically ill in which case they undergo exploratory laparotomy in the NICU or in the operation theatre, immediately upon admission. Ultrasound is not the first line investigation in our NICU or the hospital. It is done only on an *ad hoc* basis at the discretion of the clinicians/radiologists, to look for associated volvulus and in circumstances where UGI contrast is inconclusive. Of 48 neonates in the study cohort who had undergone ultrasound and colour Doppler examination, 20 had revealed either malrotation or malrotation with volvulus. In nine cases the examination was inconclusive as there was gaseous distension of abdomen, resulting in incomplete view of the superior mesenteric artery (SMA) and vein anatomy. In four cases, the ultrasound was reported to have other abnormal findings such as ischaemic bowel, pneumatosis or bowel atresia. Of remaining 15 cases where ultrasound examination was normal, three cases were confirmed to have malrotation on surgery.

**Fig. 2** Study flow diagram. UGI, upper gastrointestinal.

Upper gastrointestinal contrast studies in preterm babies less than 32 weeks

There were 22 babies born at gestational age less than 32 weeks who underwent UGI contrast studies. Preterm infants with persistent symptoms of bilious aspirates, tender abdomen or distended abdomen in the absence of clinical or radiological signs of NEC such as intramural gas on abdominal radiograph, underwent UGI contrast study. The median gestational age for this subgroup was 30 weeks (IQR: 28.1–31.1 weeks), median birth weight was 1222 g (IQR: 915–1700 g), median age at presentation was 4 days (IQR: 3–15 days) and median age at UGI contrast study was 6.5 days (IQR: 4–20 days). Six of these studies were suggestive of malrotation; all six underwent laparotomy. Five had malrotation, whereas one infant had normal intestinal rotation on laparotomy (PPV: 83%).

Of remaining 16, no further investigations were required in 12 as UGI studies were normal and clinical symptoms improved. The remaining four infants underwent laparotomy in view of

ongoing clinical concerns. Their operative findings included Hirschsprung disease, jejunal stenosis, meconium ileus and NEC.

Discussion

Clinical outcomes of malrotation and volvulus can be improved by having a high index of clinical suspicion, early radiological investigations and prompt surgery.¹³ This study reaffirms the role of UGI contrast study in malrotation. The PPV of 90% asserts its value in investigating a condition with low prevalence.¹⁴

The radiological diagnosis of malrotation requires an experienced radiologist to perform and analyse this dynamic study in real time. Based on positions of anatomical landmarks such as pylorus, duodenojejunal flexure and jejunum, radiological diagnostic criteria have been established.¹⁵ Localisation of duodenojejunal junction (DJJ) in frontal and oblique view is an important step in UGI contrast study to diagnose or exclude malrotation.¹⁶

Normally, DJJ should be to the left of the spinal pedicle on frontal view and should take a posterior course on oblique view (Figs 3,4). In malrotation, on frontal films, the DJJ is towards the right of midline (Fig. 5)¹⁷ and the distal duodenum takes an anterior course on oblique/lateral view rather than normal posterior location. However, there are few normal variations of its anatomical positions that can mimic as malrotation such as redundant duodenum and low DJJ in the presence of distended bowel.¹⁸ These anatomical variations in duodenal positions can lead to unnecessary laparotomy in newborn infants.

We had five infants where UGI contrast was suggestive of malrotation, but on laparotomy there was no malrotation. This gives a false positive rate of 9.6% that is comparable to (6–15%) reported rate in literature.^{18–20} Of these five false positive cases, four infants had other intestinal pathologies and only one had normal laparotomy findings.

The risk of malrotation associated volvulus in the newborn population is higher than any other age group (Fig. 6).^{21,22} Therefore it is necessary to investigate the neonatal population promptly. The mortality secondary to volvulus still remains significantly high at around 4%.⁶ Delay in identification of the volvulus can lead to bowel ischaemia and sometimes to SBS. In our study population, the mortality rate was 5.8% (3/51). Of these, two infants died because of non malrotation related conditions (alveolar capillary dysplasia, ventriculo peritoneal shunt complications). Only one died because of extensive bowel ischaemia (1/51). It was reassuring to know that there were no long term morbidities such as SBS. This may be due to enhanced awareness among the clinicians in the referring hospitals as well as tertiary units.

There is scant literature about UGI contrast studies for malrotation in preterm infants. There are few case reports or case series describing malrotation and/or volvulus in preterm and extremely preterm infants.^{23–27} The process of developmental and fixation of fetal gut is ongoing in extremely premature neonates (<28 weeks).²⁸ Bilious vomiting, a very important symptom of malrotation, could be absent in the extreme premature infants. On the other hand, symptoms such as abdominal distension, bilious aspirates from the nasogastric tube and abdominal tenderness are known to be associated with NEC and sepsis in preterm neonates and hence may result in a delayed diagnosis of malrotation. Persistent bilious aspirates could be a sign of gut immobility and anti peristalsis in these preterm infants. In our study

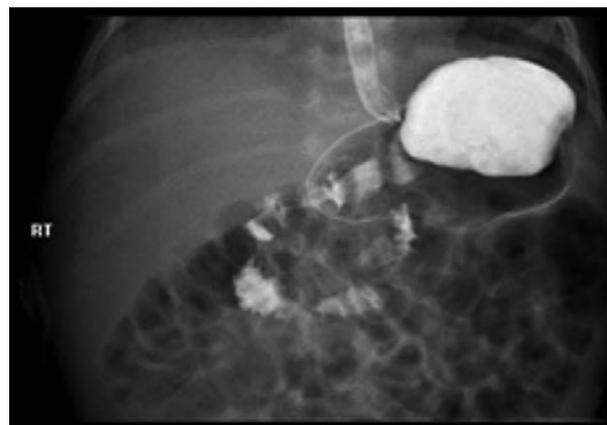


Fig. 3 Upper gastrointestinal contrast study frontal view: Normal position of duodenojejunal junction to left of spinal pedicle and at the level of pylorus.

population, there were 22 preterm infants who underwent UGI contrast for bilious vomiting or persistent bilious aspirates with additional signs of intestinal obstruction such as tender or distended abdomen. Of these 22, six had UGI contrast suggestive of malrotation. On laparotomy five babies (83%) were confirmed to have malrotation. This PPV of UGI contrast study to diagnose malrotation is close to term infant population (83 vs. 90%) (Fig. 6).

Iohexol, a safe contrast medium for UGI studies in paediatric population, was used in this study population.^{29,30} In extreme preterm infants, it is preferable to use an isotonic isosmolar preparation of contrast agents so as to reduce osmotic load as these infants are at risk of NEC. In our study population, there was no adverse event noted associated with use of contrast agent.

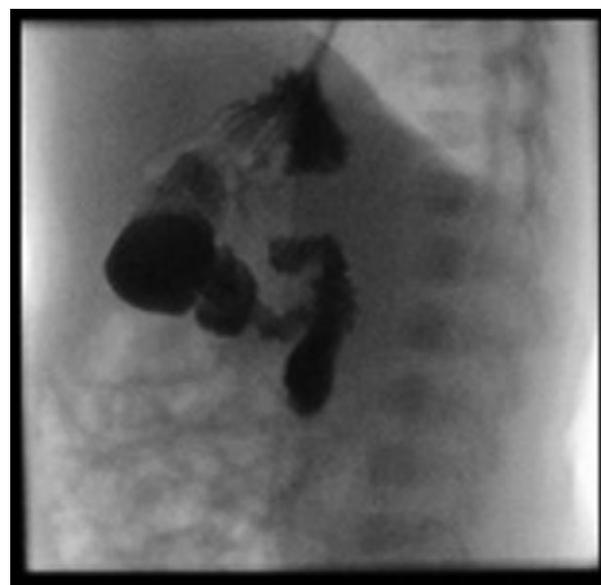


Fig. 4 Normal upper gastrointestinal contrast study oblique view: Posteriorly located duodenojejunal junction.

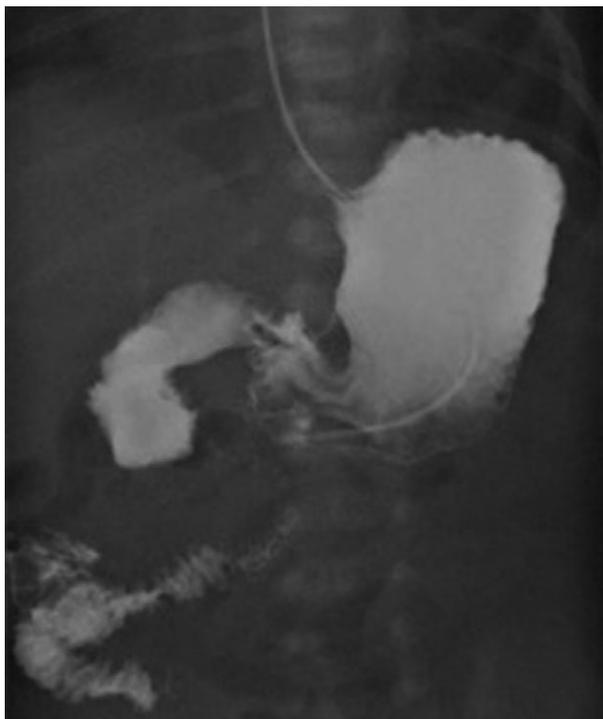


Fig. 5 Malrotation. On frontal film, duodenojejunal junction located to right of spinal pedicle and below the pylorus.

The radiological investigations in suspected case of malrotation are aimed at avoiding false negative result that is missing the diagnosis on contrast studies, in order to prevent fatal midgut



Fig. 6 Malrotation and volvulus. On lateral view, duodenojejunal junction located anteriorly with 'corkscrew' downward path of distal duodenum.

volvulus.^{18,31} In our study, we had four cases of malrotation that were not identified on the UGI contrast. However, additional radiological investigations such as lower GI enema and highly suspicious abdominal plain X ray prompted surgeons to carry out laparotomy that revealed malrotation of intestines. Due to the retrospective nature of the study and lack of complete data, the false negative rate in our study could not be calculated. However, it has been reported to be around 6.14% in the paediatric population.¹⁸

Ultrasound of the abdomen with colour Doppler has been reported as a preferred modality for diagnosis of malrotation in a few recent studies.^{9–11} In our study cohort, ultrasound was performed in conditions only when UGI contrast was inconclusive or if there was suspicion of volvulus. In many instances, for the preterm infants, the investigation was requested at the clinicians'/radiologists' discretion rather than as a standard protocol. Therefore, the role of ultrasound in this condition could not be evaluated in detail. In addition, due to the presence of a skewed number of infants undergoing UGI contrast ($n = 164$) and ultrasound ($n = 48$), we were unable to compare these two modalities.

The choice of ultrasound over UGI contrast study necessitates having a skilful and available team of sonographers and radiologists. This might not be the case in all jurisdictions. Paediatric radiologists are familiar with UGI contrast studies and the images provide a permanent record that can be interpreted retrospectively. Reversal of mesenteric vessel positioning and whirlpool sign, the typical pattern of SMA wrapped by coils of superior mesenteric vein and bowel, are considered as useful diagnostic features for determining midgut volvulus.^{32–34} Although reversed SMA/superior mesenteric vein position is frequently recognised in patients with malrotation and volvulus at ultrasound examination, this sign is not considered specific. Approximately one third of the affected patients have been reported to have normal vessel anatomic position in cases of malrotation.^{32,35–37} In addition, these studies were performed in older paediatric age groups and the number of participants was small. UGI radiographic examination still remains the criterion standard in isolated malrotation diagnosis when it is suspected, despite a negative ultrasound examination.⁹

Any newborn presenting with bilious vomiting needs to be investigated for intestinal pathology. The common causes include Hirschsprung disease, malrotation, small bowel atresia and meconium ileus. In our study, 30% of infants presenting with bilious vomiting turned out to have surgically confirmed malrotation. In a prospective study for evaluation of bilious vomiting in first 72 h by Lilien *et al.*, it was noted that 31% neonates had bowel obstruction of which malrotation was the most common pathology (11%).³⁸ In another prospective study by Godbole and Stringer, 38% of neonates with bilious vomiting were prospectively diagnosed with surgical pathology.³⁹ In this study, the commonest surgical condition presenting as bilious vomiting was Hirschsprung disease (14%), followed by small bowel atresia and malrotation. In our study, those babies who had abnormal UGI contrast and no malrotation on laparotomy, the second commonest surgical pathology was Hirschsprung disease. In all those instances where a baby had suspicion of malrotation and abnormal radiological investigation but laparotomy not revealing malrotation, then other surgical conditions such as meconium ileus,

small bowel atresia and jejunal stenosis were diagnosed. This reiterates the importance of radiological investigations to rule out surgical cause of obstruction in the neonatal population presenting with bilious vomiting. However, it needs to be noted that in around 62–69% of cases of neonatal population, there is no definite cause found.^{38,39}

Conclusion

Malrotation of the intestine is an important surgical condition in the neonatal population. Delayed diagnosis could lead to potentially fatal complication of volvulus and bowel ischaemia. UGI contrast study is a cornerstone investigation for diagnosis. Additional investigational modalities such as abdominal Doppler studies and lower GI enema can be useful when the UGI contrast studies are inconclusive. Although sparsely reported, malrotation and volvulus can occur in premature infants with clinical features overlapping with NEC. In suspicious cases, it is prudent to perform UGI contrast study even in extreme preterm infants.

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Neurodevelopmental outcomes of neonates undergoing surgery under general anesthesia for malrotation of intestines



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ABSTRACT

Background: It is difficult to differentiate between the potential adverse effects of general anesthesia (GA) on the developing brain and the role of associated co-morbidities and syndromes that can adversely affect neurodevelopmental outcomes in neonates undergoing GA. Neonates with malrotation of the intestines without volvulus usually do not have co-morbidities or syndromes. In addition, majority of them recover very well after surgery and are discharged home within a few days. Neonates with malrotation are a clean cohort of babies to study the role of a single episode of GA on the developing brain.

Aims: The study aimed to evaluate the neurodevelopmental outcomes of neonates undergoing GA for malrotation surgery.

Study design: Retrospective review of neonates born at gestational age of ≥ 32 weeks undergoing laparotomy for malrotation.

Outcome measures: Neurodevelopment in the study cohort at the age of one year.

Results: 33 eligible infants were identified from the departmental database. All 33 survived and were assessed using the Griffiths Mental Development Scales (GMDS) at one year. Mean general quotient (GQ) of the study population was 98 (SD 7.33) which was similar to the population norms (100.2, SD 12.8); p value 0.10. None of the infants developed cerebral palsy, tone abnormality, sensorineural deafness or blindness. There was no significant difference in the centiles at birth versus one year for weight and length (p values 0.454 and 0.178 respectively). Reassuringly, the head circumference centiles at one year showed a trend towards higher values (p value: 0.0735).

Conclusion: One year developmental outcomes of neonates undergoing surgery under GA for malrotation were similar to population norms.

1. Introduction

In recent years, animal studies have suggested that the commonly used general anesthetic agents are neurotoxic to the developing brain and can cause adverse effects on cognition and behaviour [1,2]. Observational studies in human children have also suggested that exposure to general anesthesia in children younger than 4 years may be associated with developmental and behavioural disorders such as language and mathematical learning disabilities or abstract reasoning deficits [3–7]. However, the study population included serious conditions such as congenital heart disease, esophageal atresia, necrotizing enterocolitis, gastroschisis etc. [7,8]. These infants are at high risk of

adverse events such as infection, hypotension, hypoxia, acidosis, hypoglycemia, hypocarbia and electrolyte disturbances in the perioperative period, all of which are known to result in adverse neurological problems [9]. In addition, the presence of associated anomalies and syndromes can also influence the neurodevelopmental outcomes.

To test the effects of general anesthesia on neurodevelopmental outcomes, it is important to avoid such confounding factors. Malrotation of intestines is one such condition that lends itself to study the long term effects of a single episode of general anesthesia in the neonatal period. This is because malrotation usually presents in the neonatal period and is not associated with other comorbidities in majority of the instances. Early identification and prompt surgical

Abbreviations: UGI, upper gastrointestinal; GA, general anesthesia; GMDS, Griffiths mental development scales; GQ, general quotient; ND, neurodevelopment; SD, standard deviation; MASK, Mayo Anesthesia Safety in Kids; PANDA, pediatric anesthesia neurodevelopment assessment; GAS, general anaesthesia and awake-regional anaesthesia in infancy

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intervention with Ladd procedure results in correction of this anomaly, with recurrences being unusual [10]. In our experience, post operative complications are rare and the length of stay in hospital is usually minimal if malrotation is diagnosed and treated in a timely fashion before the onset of volvulus. Hence, we aimed to evaluate the neurodevelopmental outcomes of neonates with malrotation.

2. Ethics approval

The conduct of this retrospective study was approved by the Institutional Clinical Audit Committee.

3. Methods

All full term and moderate to late preterm infants (≥ 32 weeks at birth) undergoing laparotomy for malrotation under general anesthesia (years 2005–2014) were included in this study. The diagnosis of malrotation was based on upper GI contrast study in all patients. It is the standard modality of investigation in our unit. The final confirmation of diagnosis was done at laparotomy. Infants with dysmorphic features and genetic anomalies were excluded.

General anesthesia was defined as the use of intravenous or inhalational agents to achieve amnesia, analgesia, muscle paralysis, and sedation. Intra operatively vital parameters such as temperature, heart rate, respiratory rate, pulse oximeter saturation and end tidal carbon dioxide (etCO_2) were continuously monitored. Regular blood gas analyses were performed as per the needs of the infant during the intra operative period. If arterial access was present, blood pressure was monitored continuously; if arterial access was not present, non invasive blood pressure (BP) was recorded intra operatively every 10 min during surgery. The infants were closely monitored in the postoperative period with regular blood gas analyses (arterial or capillary) and all the other vital parameters.

Hypertension was defined as mean BP below the 10th percentile for age [11]. Mild hypocapnia was defined as $\text{CO}_2 = 34$ – 25 mm Hg, moderate hypocapnia was defined as $\text{CO}_2 = 25$ – 20 mm Hg and severe as $\text{CO}_2 < 20$ mm Hg [12]. Since the majority of the blood gas analyses were from capillary sample, normal lactate level was defined as 2.6 (SD 0.7) mmol/L [13].

3.1. Neurodevelopmental assessment

All neonates in Western Australia who undergo general anesthesia for surgical procedures are routinely enrolled in a formal developmental follow up program and are seen at 4, 8, and 12 months' corrected age. At the 12 month visit, development is formally assessed using the Griffiths Mental Development Scales [14,15]. The Griffiths Mental Development Scales assess development in 5 separate areas: locomotor, personal and social, hearing and speech, eye and hand coordination, and performance. The locomotor sub scale measures the earliest motor milestones as the child moves from horizontal to vertical and becomes mobile. The personal social sub scale assesses early adaptive behaviour using interaction with the environment and skill in dressing and feeding as well as pointing out body parts as the child approaches 2 years of age. This sub scale uses caregiver reports. The hearing and language sub scale measures the earliest forms of expressive language such as babbling, the development of words with meaning, and receptive speech through the ability to follow commands and identify objects. The eye hand co ordination sub scale measures the development of hand grasp, fine motor and visual abilities. The performance sub scale measures fine motor manipulative skill as well as visual spatial orientation [16]. The 5 subscales are assessed and scored separately and then combined to provide an overall general quotient (GQ) reflecting the child's developmental performance level relative to the general population. The normal population mean score is 100.2 with a SD of 12.8 [16,17]. 80% of the Griffiths assessments were

conducted by a single developmental pediatrician (J.M.). For our study, the main outcomes of interest were Griffiths scores, sub optimal developmental outcome ($\text{GQ} < 75$), cerebral palsy, blindness (visual acuity of $< 6/60$ in the better eye), or sensorineural deafness requiring hearing aids. Cerebral Palsy was defined as abnormal muscle tone and a Gross Motor Function Classification System (GMFCS) level ≥ 1 [18]. Other outcomes of interest were mild developmental delay ($\text{GQ} 76$ – 88), and physical growth at 1 year of age.

3.2. Statistical analysis

Statistical analysis was performed using Stata 12.0 (StataCorp LP 4905 Lakeway Drive, College Station, Texas 77845 4512, USA). Mean and SD values were calculated for normally distributed data. Median, IQR, and range values were calculated for continuous data with non normal distribution. The mean GQ of the study sample was compared with the published healthy population mean (100.2, SD 12.8) using the *t* test, and the magnitude of this difference was evaluated using Cohen *d*, where *d* is the difference between the study and population means divided by the population SD. A *d* of 0.2 is considered a small effect; 0.5, a medium effect; and 0.8, a large effect size [19]. The physical growth parameters (centiles) at birth versus one year were compared using the Wilcoxon matched pairs rank sum test.

4. Results

After excluding three neonates with dysmorphic features and genetic anomalies (22q13 deletion, Mowat Wilson syndrome), the final study population comprised of 33 patients (Fig. 1). There were 21 males (67%) and 12 females (33%); the difference was not statistically significant ($p = 0.059$). The study population was uniform with respect to their birth weight, gestational age and birth length. There were two growth restricted infants (6%) and the remaining 31 infants (94%) were not growth restricted. The difference was statistically significant ($p = 0.0001$). Clinical details of the study population are given in Table 1.

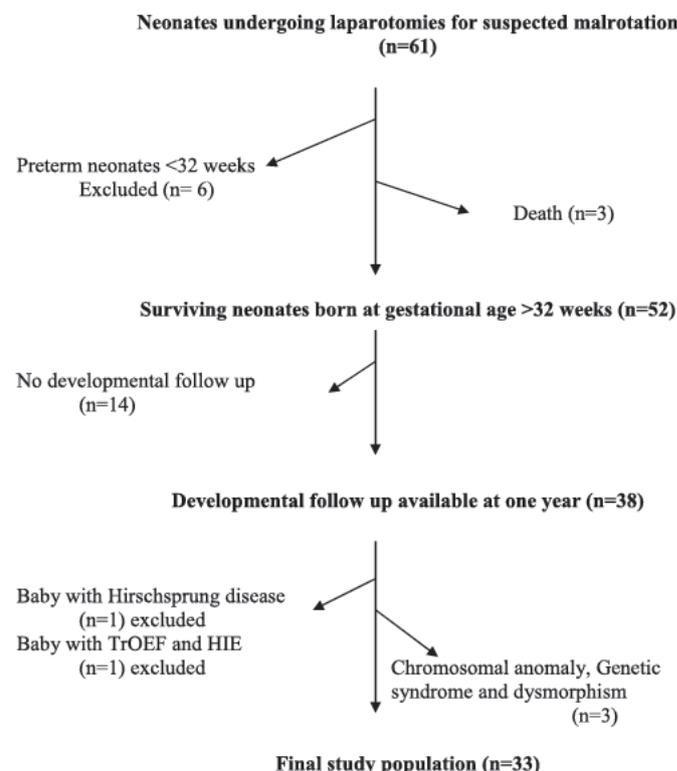


Fig. 1. Study flow diagram.

Table 1
Clinical details of neonates undergoing surgery for malrotation.

Clinical characteristics	Median	IQR	Range	Mean	Standard deviation
Gestational age, weeks	37.4	35–39	33.1–41	37.2	2.29
Birth weight, grams	3080	2600–3290	2020–4335	3018	591
Birth weight, centile	55	37–77	2–97	54.8	27
Birth head circumference, cm	34	32.5–35	31–47	34.1	2.89
Birth head circumference, centile	68	51–82	7–100	63	24.74
Birth length, cm	49	47–51	44.5–56	49.12	2.68
Birth length, centile	65	45–91	8–97	61	28.9
Apgar score at 5 min	9	9–9	6–10	8.68	0.93
Umbilical cord pH (n = 18)	7.31	7.28–7.36	7.1–7.46	7.31	0.084
Age at presentation, d	2	1–5	1–22	4.27	4.98
Age at surgery, d	2	1–5	1–22	4.27	4.98
Weight at surgery, gram	3000	2450–3250	2000–4300	2940	582
Ventilation duration, hours	35	14–58	0–207	40.7	42.7
Lowest pH, perioperative	7.3	7.24–7.33	7.17–7.55	7.29	0.08
Highest lactate	3.1	2.5–4.2	1.7–6.1	3.31	1.1
Highest fiO ₂	0.5	0.4–0.6	0.27–0.9		
Lowest CO ₂	32.5	29–36.5	27–41	31.7	5.4
SpO ₂	98	94–99	91–100	98	1.9

IQR = inter quartile range.

None of the infants undergoing malrotation correction surgery suffered from hypoxia, hypotension or significant lactic acidosis during intraoperative period or subsequently during their stay in neonatal intensive care unit. The mean of the lowest CO₂ in the study cohort was 31.7 mm Hg (range 27–41), which is mild hypocarbia (for normal values 35–45).

4.1. Anesthesia details

Sevoflurane was the most commonly used inhalational anesthetic agent; isoflurane was used in only one case. Propofol was the sole intravenous anesthetic agent and was used in 17 cases (dose 3–4 mg/kg). Opioid analgesics used intraoperatively were fentanyl (n = 28), alfentanil (n = 2) and morphine (n = 3). The dose of fentanyl ranged from 3 to 10 µg/kg. All patients received morphine infusion postoperatively at the rate of 10 to 40 micrograms per kg per hour. Intravenous paracetamol was used as needed to facilitate pain management while weaning from morphine. Majority of the neonates (n = 23) were extubated within 48 h after surgery. The details of the anesthetic management are given in Table 2.

Table 2
Details of anesthesia.

Clinical parameter	Frequency	Percentage
General anesthesia induction route		
Inhalation only	16	49
Intravenous only	3	9
Combination (Inhalation + IV)	14	42
Analgesic agents		
Fentanyl	28	85
Alfentanil	2	6
Morphine	3	9
Ventilation post-operative		
Yes	28	85
No	5	15

4.2. Neurodevelopmental outcomes

All 33 infants survived and were followed up until one year of age. None of them had readmission to the hospital or underwent procedure under general anesthesia. The mean GQ of the study population on Griffiths scale was 98 (SD 7.33), which compared favorably with the published population norms (mean 100.2, SD 12.8) [15]. The magnitude of the difference between the mean GQ of the study population and the general population mean using Cohen d was 0.125, representing a small effect size. The p value for this difference was 0.10, suggesting that it was not statistically significant. None of the infants developed cerebral palsy, tone abnormality, sensorineural deafness or blindness. The details of developmental outcomes are given in Table 3. The outcomes of the 12 moderate to late preterm infants versus 21 term infants were compared. Within the constraints of the small comparison sizes, there was no statistically significant difference between the mean GQ in preterm infants (< 37 weeks) versus full term [98.0 (SD 7.0) versus 98.1 (SD 7.7); p = 0.972].

Information on the weight, length and head circumference at one year was available in all 33 infants. There was no significant difference in the weight centiles at birth and at one year of age (p = 0.454) (Fig. 2). There was no significant difference in the length centiles at birth and at one year of age (p = 0.178) (Fig. 3). There was no significant difference in the head circumference centiles at birth and at one year of age (p = 0.073) (Fig. 4). The details are given in Figs. 2, 3 and 4.

5. Discussion

The results of our study (n = 33) showed that the developmental outcomes of children who had undergone surgery under general anesthesia for malrotation in the neonatal period were within the normal range. In fact, the lowest GQ observed was 86 (normal population range: 88–113). None of the study infants developed cerebral palsy, sensorineural deafness or blindness. The physical growth of these infants at one year was also normal, with the head circumference centiles at one year being better than at birth. These findings provide some reassurance, more so because our study cohort also included 12 moderately preterm infants; evidence is emerging that late and moderate preterm infants have a higher of adverse developmental outcomes compared to healthy term infants [20].

Malrotation of the intestine presents in neonatal period in 75% of cases [21]. Recurrence is rare once the condition is corrected surgically [22]. Unless there are associated comorbidities or complications such as ischemia of intestines or short bowel, infants with this condition don't undergo prolonged or repeated exposure to anesthesia. We excluded three neonates from available cohort as they had dysmorphic features or genetic syndromes, which are known to adversely affect the neurodevelopment. Each of these three infants had Griffiths GMDS score of < 50 each at 12 months. This finding reiterates the importance of associated anomalies and co morbidities that can adversely influence the developmental outcome rather than the anesthetic agent per se.

Even though vast amount of information is available from animal studies [23–25], evidence regarding the adverse effects of general anesthesia on the developing brain remains inconclusive in humans. Many factors that can adversely affect neurocognitive function commonly occur during surgery under general anesthesia as well as in the perioperative period. They include hypotension, hypoglycemia, hyperglycemia, hypocarbia, hypercarbia, abnormalities of the acid base homeostasis, sepsis and many more [9]. Risk of anesthetic complications is also higher in neonates and infants compared to adults due to physiological differences.

Recently there have been two studies that investigated neurodevelopmental outcomes after general anesthesia; GAS study and PANDA study [26,27]. GAS study was conducted to assess neurodevelopmental outcome at 2 years of age after general and awake regional anesthesia

Table 3
One year outcomes of neonates undergoing surgery for malrotation.

Clinical characteristics	Median	IQR	Range	Mean	Standard deviation
Weight at 12 months, kg	9.95	9.04–10.94	7.8–12.65	10.03	1.27
Weight at 12 month, centile	66.5	31.5–87	4–99	60.5	30
Length at 12 months, cm	76	73.35–78.35	71.5–82	75.8	2.8
Length at 12 months, centile	54	16.5–83.5	3–97	51	32.8
Head circumference at 12 months, cm	47.15	46–47.8	44–50	46.8	1.4
Head circumference at 12 months, centile	79	63–91	11–99	72.8	23.6
Chronological age at Griffiths assessment (months)	12	11.25–12.5	10–15.25	12	1.288
GQ on Griffiths assessment at one year (n = 33)	98.6	94–103	86–112	98	7.33
Performance score, months	12	11.25–12.5	10–15.25	12	1.28

GQ = general quotient, IQR = inter quartile range.

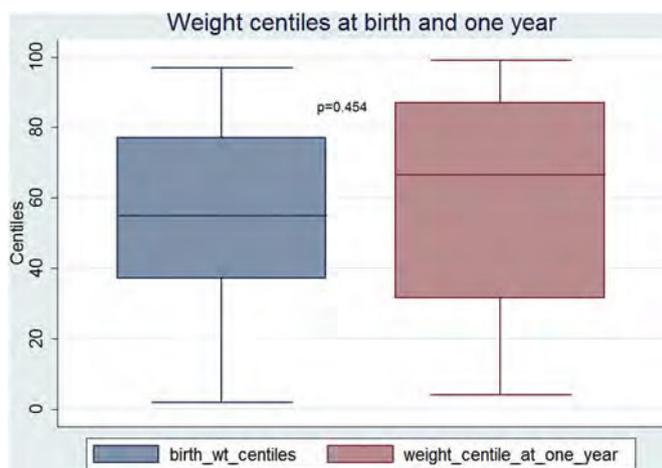


Fig. 2. Weight centiles in the study population at birth and at one year of age.

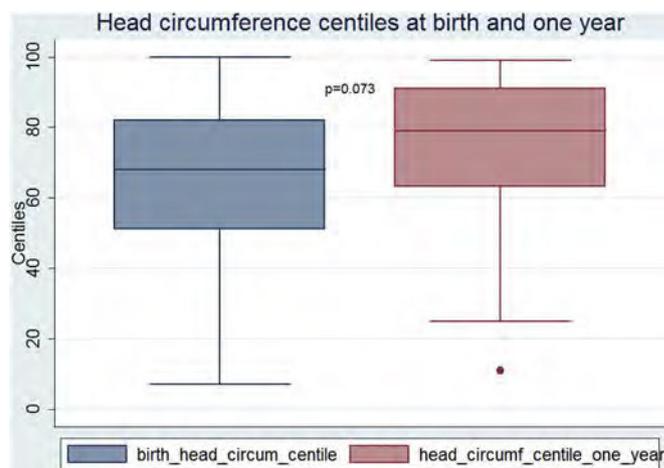


Fig. 4. Head circumference centiles in the study population at birth and at one year of age.

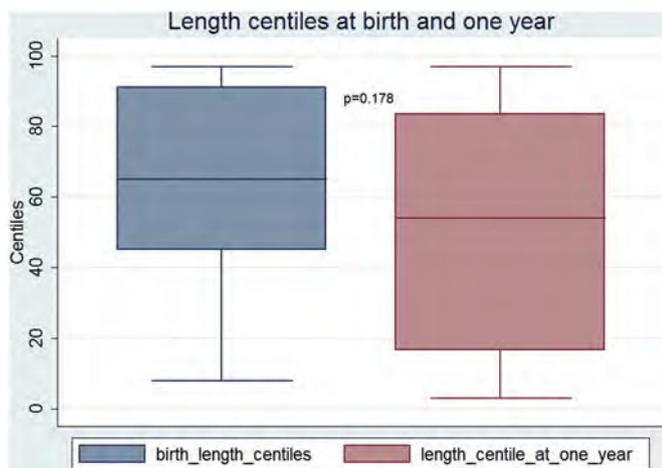


Fig. 3. Length centiles in the study population at birth and at one year of age.

in infants. In this multicenter RCT infants younger than 60 weeks' postmenstrual age, born at > 26 weeks' gestation and undergoing inguinal herniorrhaphy were randomized either to sevoflurane general anesthesia or awake spinal or caudal anesthesia. There was no evidence for increased risk of adverse neurodevelopmental outcome at 2 years of age in general anesthesia group compared with awake regional anesthesia [26,28].

The PANDA study was a sibling matched cohort study that included sibling pairs with cases having single exposure to general anesthesia before 36 months of age and controls were their siblings with no anesthesia exposure. A detailed neuropsychological assessment in this retrospective cohort was performed at the current age of 8 to 15 years. The study revealed no significant differences in IQ scores of anesthesia

exposed and non exposed groups [27]. Our study results are congruent with both of above studies with regards to single exposure to general anesthesia in early life not resulting in adverse neurodevelopmental outcomes compared to non exposed population.

The main strength of our study is that none of the study infants had associated malformations/syndromes that could affect the ND outcomes. In addition, the study cohort did not have perinatal asphyxia, peri operative acidosis, significant hypocarbia, hypoglycemia, hypoxia or hypotension, all of which are known to adversely affect the ND outcomes. The main limitation of our study is the lack of healthy controls, because we do not perform ND assessments on healthy infants. We have tried overcoming this limitation by comparing the results to the population norms. An additional limitation of the study is the retrospective nature of study design and the fact that the follow up was only up to one year of age. Another important limitation is the lack of data regarding socio economic status of the family, a factor known to influence the neurodevelopmental outcomes [29].

In summary, the one year neurodevelopmental outcomes of neonates undergoing general anesthesia for malrotation surgery were similar to the population norms.

Disclosures

None of patients' identity disclosed. This research was carried out without funding. Conflicts of interest: No conflicts of interest declared. As per institutional protocols no ethics committee approval required for retrospective study.

Conflict of interest

None declared.

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Probiotics for Preterm Infants in India – Systematic Review and Meta-Analysis of Randomized Controlled Trials

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Abstract

The objective of the present study is to review current evidence from randomized controlled trials (RCTs) of probiotics for preterm infants in India. A systematic review of RCTs of probiotics for preterm infants in India was conducted using Cochrane methodology and PRISMA guidelines. Fixed effects model was used for meta-analysis. Nine RCTs ($n = 1514$) were included. Meta-analysis showed reduced risk of necrotizing enterocolitis (NEC) \geq Stage II {Risk ratio (RR): 0.36 [95% confidence interval (CI): 0.20, 0.66], $p = 0.0009$, (9 RCTs)}, late onset sepsis [RR: 0.56 (95% CI: 0.45, 0.71), $p < 0.00001$, (7 RCTs)] and mortality [RR: 0.62 (95% CI: 0.41, 0.95), $p = 0.03$ (8 RCTs)] in the probiotic group. Probiotics also reduced the time to full feeds [Mean difference (MD): -4.09 d (95% CI: -4.52 , -3.65), $p < 0.00001$, 5 RCTs] and duration of hospital stay [Fixed effects model (FEM): MD: -2.00 d (95% CI: -2.46 , -1.53), $p < 0.00001$, 6 RCTs]. Current evidence from RCTs supports probiotic supplementation for optimizing outcomes of preterm infants in India.

Keywords India · Necrotizing enterocolitis · Preterm · Probiotic · Sepsis

Introduction

India has made rapid progress in neonatology in last few decades [1, 2]. With increasing survival of preterm infants, the burden of morbidities such as necrotizing enterocolitis (NEC), late onset sepsis (LOS), and postnatal growth failure due to suboptimal nutrition in early postnatal period, especially in extremely preterm infants, is expected to rise, stretching the

limited resources further. Furthermore, these morbidities have significant adverse impact on long-term neurodevelopment. Strategies for preventing all-cause mortality, NEC, LOS, and optimizing enteral nutrition are thus urgently needed to optimize long term outcomes of preterm infants in India.

A previous systematic review (2017) by authors has shown that probiotics reduce all-cause mortality, NEC, LOS, and feed intolerance in preterm very low birth weight (VLBW) infants in high as well as low middle income countries (LMIC) [3]. However, it contained only 4 RCTs from India. The number of probiotic RCTs reported from India has increased significantly in the last two years [4–7]. Considering the rapidly rising burden of prematurity in the country, authors aimed to review current evidence from probiotic RCTs conducted in India to guide research and clinical practice.

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Material and Methods

The authors systematically reviewed randomized controlled trials (RCTs) of probiotics for preterm infants (gestation <37 wk) conducted in India using Cochrane methodology and PRISMA guidelines as reported in their previous systematic review [3]. Fixed effects model (FEM) was used for meta-analysis.

Only RCTs were included in the review. Preterm infants born at a gestation <37 wk in India were included. Enteral administration of probiotic (in any dose, commenced within the first 10 d of life and continued for at least 7 d) vs. control (placebo/no probiotic) was compared. The study outcomes were: mortality prior to discharge, definite NEC (\geq Stage II) [8], LOS: positive blood/cerebrospinal fluid culture after 48 to 72 h of birth, time to full feeding (TFF: \geq 120 ml/kg/d), duration of hospital stay.

The databases, Medline searched via PubMed (<https://www.ncbi.nlm.nih.gov> 1946–2019), Embase (ExcerptaMedica database) via Ovid (<http://ovidsp.tx.ovid.com>, 1947–2019), Cochrane Central Register of Controlled Trials (<http://www.TheCochranelibrary.com>) and, Cumulative Index of Nursing and Allied Health Literature via OVID (<http://ovidsp.tx.ovid.com>, 1982–2019) were searched in July 2019. Abstracts of other conference proceedings such as European Academy of Paediatric Societies, and the British Maternal and Fetal Medicine Society were searched in Embase. ‘Google Scholar’ was searched for articles that might not have been cited in the standard medical databases. The authors searched Clinical Trials.gov (<https://clinicaltrials.gov>) for ongoing RCTs. The reference lists of eligible studies, and review articles were searched to identify additional studies. Reviewers HB, AA and SP conducted the literature search independently. Authors of the included studies were contacted through emails to obtain additional data and clarification of methods if required.

Medline was searched using the following terminology: a) Population: ‘Infant, Newborn’ [MeSH] OR ‘Infant, Premature’ [MeSH] OR ‘Infant, Low Birth Weight’ [MeSH] OR ‘Infant, Extremely Low Birth Weight’ [MeSH] OR ‘Infant, Very Low Birth Weight’ [MeSH] OR ‘Infant, Small for Gestational Age’ AND b) Intervention: ‘Probiotics’ [MeSH] OR ‘Probiotic agent’ [MeSH] OR ‘Bifidobacterium’ [MeSH] OR ‘Lactobacillus’ [MeSH] OR ‘Saccharomyces’ [MeSH] OR probiotic (tw) AND c) Randomized Controlled Trial (publication type). The other databases were searched using similar terminologies. Animal studies were excluded.

The abstracts of citations obtained from the initial broad search were read independently by reviewers HB, AA and SP to identify potentially eligible studies. Full-text articles of these studies were obtained and assessed for eligibility by reviewers HB, AA and SP independently, using the predefined eligibility criteria. Differences in opinion were resolved by group discussion to reach consensus. Multiple publications of the same study were excluded to avoid data duplication.

Reviewers HB, AA and SP extracted the data independently using a data collection form designed for this review. Information about the study design and outcomes was verified by all reviewers. Discrepancies during the data extraction process were resolved by group

discussion. If required, they contacted authors for additional information/clarifications.

Risk of bias (ROB) was assessed using the Cochrane ‘Risk of Bias Assessment Tool’. Authors HB, AA and SP independently assessed the ROB in all domains including random number generation, allocation concealment, blinding of intervention, and outcome assessors, completeness of follow-up, selectivity of reporting, and other potential sources of bias. For each domain, the ROB was assessed as low, high or unclear risk based on the Cochrane Collaboration guidelines [9].

Meta-analysis was conducted using Review Manager 5.3 (Cochrane Collaboration, Nordic Cochrane Centre). Fixed effects model (FEM; Mantel-Haenszel) was used as it is the preferred meta-analytic method in CNRG reviews (<http://neonatal.cochrane.org/resources-review-authors> accessed on 12 July 2019). Meta-analysis using Random effects model (REM; derSimonian and Laird) was used to check consistency of the results, when there was significant heterogeneity between the individual studies.

Effect size was expressed as risk ratio (RR), and 95% confidence intervals (CI). Statistical heterogeneity was assessed by the χ^2 test, I^2 statistic, and visual inspection of the forest plot (overlap of CIs). I^2 statistic values were interpreted as per the Cochrane Handbook guidelines as follows: 0% to 40%: might not be important; 30% to 60%: may represent moderate heterogeneity; 50% to 90%: may represent substantial heterogeneity; 75% to 100%: considerable heterogeneity [10]. The risk of publication bias was assessed by visual inspection of the funnel plot (Supplementary Fig. 1) [9].

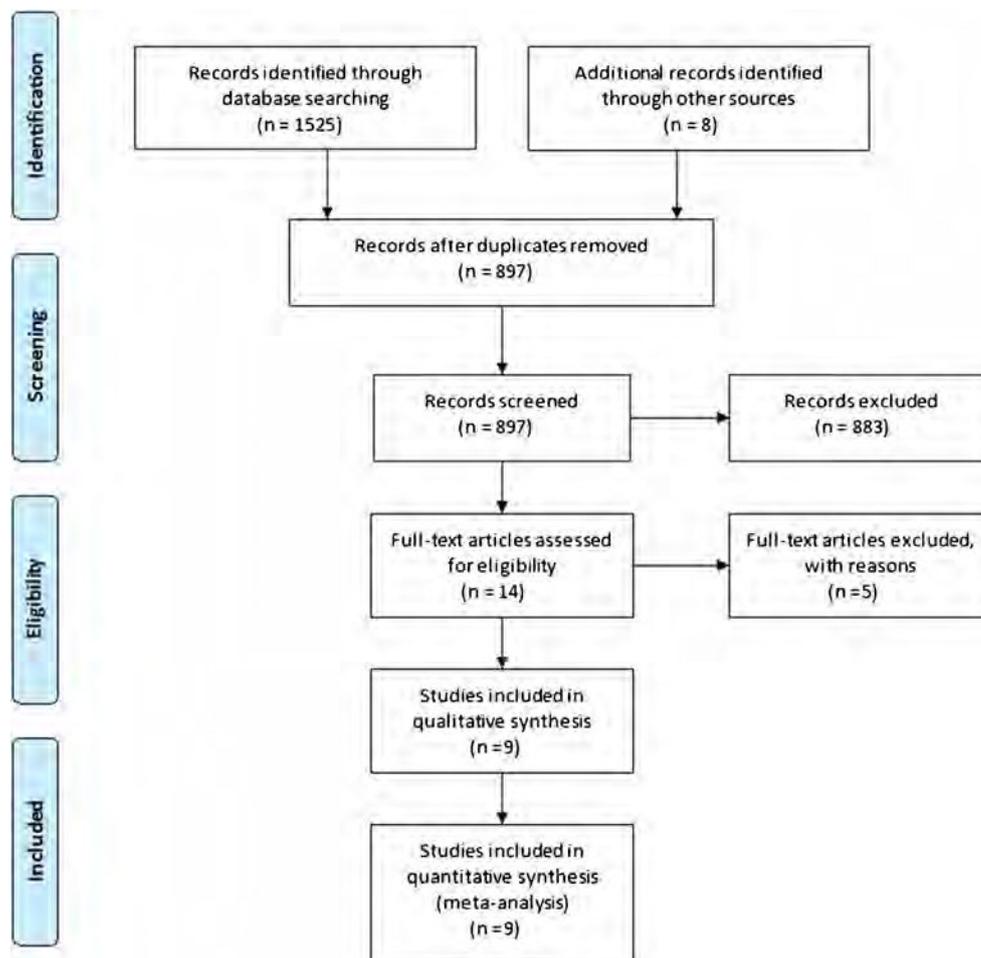
Sensitivity analysis was performed after excluding RCTs with high ROB in the domains of random sequence generation, allocation concealment, and blinding of intervention.

The key information concerning the quality of evidence, based on the (a) sample size for clinically important outcomes, (b) magnitude and precision of effect of the intervention, (c) ROB (d) directness of evidence (e) consistency of results given the heterogeneity in patient population, type, dose and duration of probiotic supplementation was presented as per the Grades of Recommendation, Assessment, Development and Evaluation (GRADE) guidelines [11].

Results

The literature search identified 1533 potentially relevant citations. After carefully reviewing the abstracts, 14 studies were identified and finally nine RCTs ($n = 1514$) were included in the meta-analysis (Fig. 1). The characteristics of the included studies are given in Table 1 [4–7, 12–15] and [Rajapriya K SM RS. Role of probiotics in prevention of necrotising enterocolitis in preterm babies (Randomized controlled trial). Thanjavur: The Tamil Nadu Dr. M.G.R. Medical University 2016].

Fig. 1 Flow diagram showing study selection process



Seven out of the nine included RCTs used multi strain probiotics [5–7, 12–14], [Rajapriya 2016] and two used single strain probiotics [4, 15]. Six studies used exclusive expressed breast milk [5, 7, 13–15], [Rajapriya 2016]. Probiotic supplementation was continued till discharge [6, 13–15], till reaching full feeds [7], [Rajapriya 2016] or for a pre-specified duration between 7 and 21 d [4, 5, 12]. Six studies focused on preterm infants <34 wk [5, 7, 12, 14, 15], [Rajapriya 2016] while three studies also included infants between 34 and 37 wk of gestation [4, 6, 13]. Outcomes of extremely low birth weight (ELBW) infants and extremely preterm infants were reported separately in one study [15].

Four of the 9 included RCTs had low ROB for the domain of ‘random sequence generation’ [6, 12–14], four had low ROB for allocation concealment and blinding of intervention [6, 12, 13, 15] (Supplementary Table 1).

Meta-analysis of data from 9 included trials ($n = 1514$) showed significantly lower risk of NEC \geq Stage II (Bell staging) [16] [RR: 0.36 (95% CI: 0.20, 0.66), $p < 0.0001$, Fig. 2, $I^2 = 0\%$] in the probiotic group [4–7, 12–15], [Rajapriya 2016].

Meta-analysis of data from 7 trials ($n = 1145$) showed significantly lower risk of blood culture positive LOS after 48 h of birth [RR: 0.56 (95% CI: 0.45, 0.71), $p < 0.00001$, Fig. 3, $I^2 = 48\%$] in the probiotic group [5, 6, 12–15], [Rajapriya 2016].

Meta-analysis of data from 8 trials ($n = 1314$) showed decreased risk of mortality prior to discharge in the probiotic group [RR: 0.62 (95% CI: 0.41, 0.95), $p = 0.03$, Fig. 4, $I^2 = 0\%$] [4–7, 12–15].

Meta-analysis of data from 5 trials ($n = 796$) showed significant reduction in TFF in the probiotic vs control group [Mean difference (MD) = -4.09 d (95% CI: -4.52 , -3.65), $p < 0.00001$] [5, 6, 13–15]. However, there was significant heterogeneity ($I^2 = 90\%$, $p < 0.00001$) among the trials. These results were hence checked by using random effects model (REM), and remained significant [MD = -3.48 d (95% CI: -5.14 , -1.83), $p < 0.0001$; Fig. 5].

Meta-analysis of data from 6 trials ($n = 865$) showed significant reduction in hospital stay in the probiotics vs. control group [MD: -2.00 d (95% CI: -2.46 , -1.53), $P < 0.00001$] [4–6, 13–15]. However, there was significant heterogeneity ($I^2 = 96\%$, $P < 0.00001$) among the trials. These results were

Table 1 Characteristics of included studies

First author, year of publication, place (reference)	Characteristics of included RCTs
Ambrin et al. 2015 (Mahaboob Nagar, Telangana) [4]	<p>Participants: Preterm infants <37 wk gestation and birth weight < 2 kg</p> <p>Mean gestational age and birth weight: 33.37 vs. 33.21 wk, 1509 g vs. 1564 g</p> <p>Intervention: <i>Saccharomyces Boulardi</i> 250 mg once a day vs. control (No probiotic)</p> <p>Duration of supplementation: 7 d from initiation of probiotic</p> <p>$n = 69$ (Probiotic: 35) vs. (Control: 34)</p> <p>Type of milk: EBM/Formula</p> <p>Type of delivery: CS 48.6% vs. 38.2%</p> <p>Primary outcome: Clinical sepsis: 3/35 (8.6%) vs. 3/34 (8.8%); $p = 0.7$</p> <p>Other outcomes: Mortality: 3/35 vs. 3/34, $p = 0.7$; Hospital stay (days): 6.71 ± 1.07 vs. 7.12 ± 1.27, $p = 0.16$</p>
Arora et al. 2017 (Amritsar) [5]	<p>Participants: Preterm infants ≤ 34 wk</p> <p>Mean gestational age and birth weight: Not reported</p> <p>Intervention: Probiotic supplement (1 g containing 1.25 billion cells of: <i>L. acidophilus</i>, <i>L. rhamnosus</i>, <i>B. longum</i>, <i>S. boulardii</i>) vs. control (No probiotic)</p> <p>Duration of supplementation: 2 wk from onset of enteral feeds</p> <p>$n = 150$ (Probiotic: 75) vs. (Control: 75)</p> <p>Type of milk: EBM</p> <p>Type of delivery: CS: 81.33% vs. 82.67%</p> <p>Primary outcome: Incidence of NEC (any stage): 1/75 (1.33%) vs. 12/75 (16%); $p = 0.016$; NEC (Stage ≥ 2): 0/75 vs. 4/75</p> <p>Other outcomes: Time to reach full feeds: 8.53 ± 2.14 vs. 10.70 ± 3.25; $p = 0.245$; Duration of hospital stay: 16.06 ± 0.49 vs. 20.04 ± 7.85; $p = 0.001$; Daily weight gain of 15–20 g: 9/75 vs. 1/75; $p = 0.001$; Nosocomial sepsis: 2/75 vs. 21/75; $p = 0.001$</p>
Shashidhar et al. 2017 (Bangalore) [6]	<p>Participants: Preterm newborns (< 37 wk) with birth weight 750–1499 g</p> <p>Mean gestational age and birth weight: 31.2 vs. 31 wk, 1256 g vs. 1190 g</p> <p>Intervention: Multicomponent probiotic supplement (1.25×10^9 CFU of <i>L. acidophilus</i>, <i>L. rhamnosus</i>, <i>B. longum</i> and <i>S. boulardii</i>) vs. control (No probiotic)</p> <p>Duration of supplementation: Time of initiation of feeds (15–17 h) till discharge</p> <p>$n = 104$ (Probiotic: 52) vs. (Control: 52)</p> <p>ELBW neonates: 15 (5 vs. 10), ELGAN: 5 (1 vs. 4)</p> <p>Type of milk: EBM/ 5% dextrose</p> <p>Type of delivery: LSCS: 52% vs. 73%</p> <p>Primary outcome: Time to reach full enteral feeds of 150 ml/kg/d (days): 11.2 ± 8.3 vs. 12.7 ± 8.9; $p = 0.4$</p> <p>Other outcomes: Duration of hospital stay (days): 27.6 ± 18.5 vs. 31.2 ± 22.9; $p = 0.4$; NEC \geq Stage 2: 2/48 vs. 6/48; $p = 0.3$; Mortality: 1/48 vs. 3/48; $p = 0.6$</p>
Meha et al. 2016 (Patna) [7]	<p>Participants: Preterm infants <34 wk of gestation</p> <p>Mean gestational age and birth weight: Not reported</p> <p>Intervention: Probiotic supplement (125 mg/kg/dose twice daily of mixture containing <i>S. boulardii</i> 250 mg yeast, <i>L. rhamnosus</i> 0.24 billion, <i>L. acidophilus</i> 0.24 billion, <i>B. longum</i> 0.24 billion, <i>S. thermophilus</i> 0.24 billion in 1 g sachet) vs. control (No probiotic)</p> <p>Duration of supplementation: From onset of enteral feeds till full feeds</p> <p>$n = 300$ (Probiotic: 150) vs. (Control: 150)</p> <p>Type of milk: EBM</p> <p>Type of delivery: Not reported</p> <p>Primary outcome: NEC \geq Stage 2: 1/50 vs. 9/150</p> <p>Other outcomes: Mortality due to NEC: 0/4 vs. 3/14</p>

Table 1 (continued)

First author, year of publication, place (reference)	Characteristics of included RCTs
Dutta et al. 2015 (Chandigarh) [12]	<p>Participants: Preterm infants 27–33 wk gestation</p> <p>Intervention: High dose (10 billion CFU: <i>L. acidophilus</i>, <i>L. rhamnosus</i>, <i>B. longum</i>, <i>S. boulardii</i>) vs. low dose (1 billion CFU: <i>L. acidophilus</i>, <i>L. rhamnosus</i>, <i>B. longum</i>, <i>S. boulardii</i>) vs. placebo (potato starch, maltodextrin)</p> <p>Duration of supplementation: Probiotic groups: (A): high dose (10^{10} cells 12 hourly) for 21 d, (C): low dose (10^9 cells 12 hourly) for 21 d, (B): high dose short course (high dose from D1–D14 and placebo from D15–D21)</p> <p>N: probiotic (114) vs. placebo (35)</p> <p>Type of milk: EBM/formula</p> <p>Type of delivery: Probiotic group vs. placebo: SVD (69% vs. 60%), CS: Data NA</p> <p>Primary outcome: Stool colonization rates on D14, D21, D28 with three different probiotic regimens (Lactobacillus and Bifidobacterium colonisation was significantly higher in groups A, B and C vs placebo, respectively). Groups A, B and C did not differ from each other. There were trends towards more CFU of Lactobacillus and Bifidobacterium per millilitre of stool in group A vs. B, and B vs. C.</p> <p>Clinical outcomes: LOS: 10/114 (8.8%) vs. 6/35 (17.1%), $P = 0.14$; Mortality: 8/114 (7%) vs. 2/35 (12.7%), $p = 0.85$; NEC (\geq Stage 2): 6/114 (5.3%) vs. 0/35 (0%), $p = 0.35$</p>
Roy et al. 2014 (Kolkata) [13]	<p>Participants: Preterm infants <37 wk and birth weight <2500 g</p> <p>Mean gestational age and birth weight: 32 vs. 32.2 wk, 1192 g vs. 1069 g</p> <p>ELBW neonates = 22 (11 in each group)</p> <p>Intervention and dosage: Half of the 1 g sachet that contained <i>L. acidophilus</i> 1.25×10^9 + <i>B. longum</i> 0.125×10^9 + <i>B. bifidum</i> 0.125×10^9 + <i>B. lactis</i> 1×10^9 vs. sterile water (placebo)</p> <p>Duration of supplementation: Commenced within 72 h of birth for 6 wk or until discharge</p> <p>$n = 112$ (Probiotics: 56; Placebo: 56)</p> <p>Type of milk: EBM</p> <p>Type of delivery: CS 83.9% vs. 76.8%</p> <p>Primary outcome: Enteric fungal colonization: $3.03 \pm 2.33 \times 10^5$ CFU vs. $3 \pm 1.5 \times 10^5$, $p = 0.03$ and LOS (Bacterial and Fungal): 31/56 (55.4%) vs. 42/56 (75%); $p = 0.02$</p> <p>Other outcomes: TFEF: 11.22 ± 5.04 vs. 15.41 ± 8.07 d; $p = 0.016$; NEC: 2/56 vs. 2/56; $p = \text{NS}$</p>
Samanta et al. 2009 (Kolkata) [14]	<p>Participants: Preterm (<32 wk) and VLBW (<1500 g) infants</p> <p>Mean gestational age and birth weight: 30.12 wk vs. 30.14 wk, 1172 g vs. 1210 g</p> <p>Intervention and dosage: probiotic mixture (<i>B. infantis</i> + <i>B. bifidum</i> + <i>B. longum</i> + <i>L. acidophilus</i>, each 2.5×10^9 CFU), administered two times per day vs. no probiotic</p> <p>Duration of supplementation: From D5–6 of postnatal age until discharge</p> <p>$n = 186$ (Probiotics: 91; Controls: 95)</p> <p>Type of milk: EBM</p> <p>Type of delivery: CS 46.15% vs. 49.47%</p> <p>Primary outcomes: Incidence of NEC (\geq Stage 2): 5/91 (1.1%) vs. 15/95 (15.8%); $p = 0.042$; Mortality: 4/91 (4.4%) vs. 14/95 (14.7%); $p = 0.032$; Time to full feeds: 13.76 ± 2.28 vs. 19.2 ± 2.02 d; $p < 0.001$</p> <p>Other outcomes: LOS: 13/91 (14.3%) vs. 28/95 (29.5%); $p = 0.02$; Hospital stay: 17.17 ± 3.23 vs. 24.07 ± 4 d; $p < 0.001$</p>
Tewari et al. 2015 (New Delhi) [15]	<p>Participants: Preterm infants <34 wk (Two groups: EPT: 27–30⁺⁶ wk and VPT: 31–33⁺⁶ wk)</p>

Table 1 (continued)

First author, year of publication, place (reference)	Characteristics of included RCTs
	Intervention: <i>Bacillus clausii</i> (2.4×10^9 spores per day) vs. placebo Duration of supplementation: Commenced D5 in asymptomatic and D10 in symptomatic neonates, and continued for 6 wk/discharge/death/occurrence of LOS whichever was earlier. $n = 244$ (Study: EPT: 61 and VPT: 62) vs. (Placebo: 121) ELBW neonates: 45 (probiotic 23, placebo 22) Type of milk: EBM/PDHM Type of delivery: CS: EPT: 66% vs. 59% and VPT: 58% vs. 60% Primary outcome: Incidence of definite and probable LOS: definite LOS: EPT: 6/61 (10%) vs. 8/59 (14%); $p = 0.26$; VPT: 2/62 (3%) vs. 3/62 (5%); $p = 0.39$; probable LOS: EPT: 8/61 (12%) vs. 9/59 (15%); VPT: 4/62 (6%) vs. 5/62 (7%) Other outcomes: Death: EPT: 8/61 (13%) vs. 9/59 (15%); $p = 0.84$; VPT: 4/62 (7%) vs. 5/62 (8%); $p = 0.79$; NEC (\geq Stage 2): EPT: 0/61 vs. 0/59; VPT: 0/62 vs. 0/62
Rajapriya K SMRS. Role of probiotics in prevention of necrotising enterocolitis in preterm babies (Randomized controlled trial). Thanjavur: The Tamil Nadu Dr. M.G.R. Medical University 2016	Participants: Preterm infants <34 wk of gestation Mean gestational age and birth weight: 31.9 vs. 32.1 wk, 1460 g vs. 1470 g Intervention: Probiotic supplement Darolac 1.25 billion cells in two divided doses vs. control (No probiotic) Duration of supplementation: From onset of enteral feeds till full feeds $n = 200$ (Probiotic: 100) vs. (Control: 100) Type of milk: EBM Type of delivery: CS: 27% vs. 17% Primary outcome: NEC \geq Stage 2: 0/100 vs. 4/100; $p = 0.46$ Other outcomes: Sepsis: 15/100 vs. 23/100; $p = NS$

B. bifidum *Bifidobacterium bifidum*; *B. infantis* *Bifidobacterium infantis*; *B. longum* *Bifidobacterium longum*; CFU Colony forming units; CS Cesarean section; EBM Expressed breast milk; ELBW Extremely low birth weight; ELGAN Extremely low gestational age newborns; EPT Extreme preterm; *L. acidophilus* *Lactobacillus acidophilus*; *L. rhamnosus* *Lactobacillus rhamnosus*; LSCS Lower segment cesarean section; LOS Late onset sepsis; NA Not applicable; NEC Necrotizing enterocolitis; NS Not significant; PDHM Pasteurized donor human milk; *S. boulardii* *Sacharomyces boulardii*; SVD Spontaneous vaginal delivery; TFEF Time to reach full enteral feeds; VLBW Very low birth weight; VPT Very preterm

hence checked by using REM and were not significant [MD: -3.48 d (95% CI: -5.14, 1.83), $p = 0.06$; Supplementary Fig. 2].

The effect of probiotic supplementation was analysed in studies with low ROB for random sequence generation, allocation concealment and blinding, and in studies where

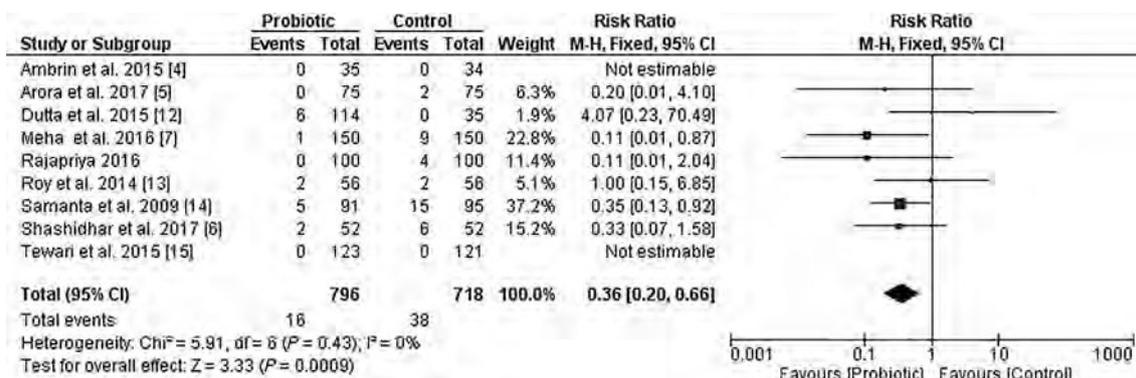


Fig. 2 Definite necrotizing enterocolitis (NEC)

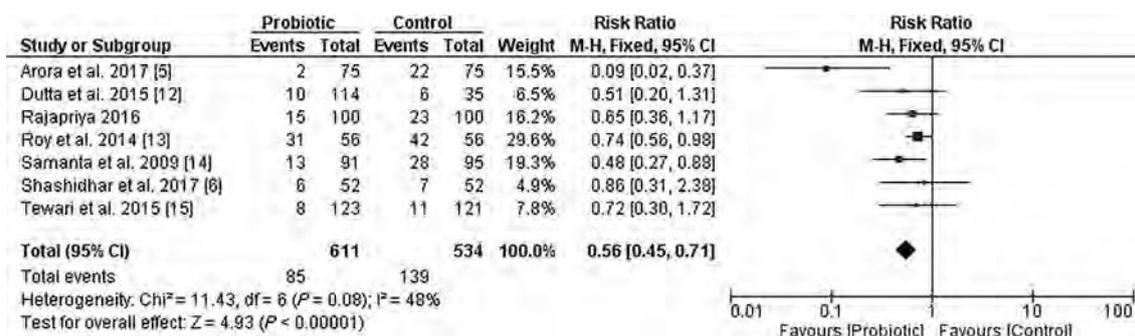


Fig. 3 Late onset sepsis

probiotic was supplemented till discharge (Supplementary Table 2). There was no significant effect on definite NEC in studies with low ROB. However, the beneficial effect on definite NEC was observed in studies where probiotic was supplemented till discharge. Probiotic supplementation significantly reduced the incidence of culture positive sepsis in studies with low ROB and prolonged probiotic supplementation. There was no significant effect on mortality prior to discharge on sensitivity analysis.

The overall evidence as per GRADE guidelines is provided in Supplementary Table 3.

Publication bias was less likely considering the symmetrical distribution of the funnel plot (Supplementary Fig. 1).

Discussion

The present systematic review which focused on RCTs of probiotics for preterm infants in India suggests a significant reduction in the risk of mortality, NEC \geq Stage II, and culture positive LOS. The findings are in agreement with the previous systematic review (2017) of probiotics for preterm infants in LMICs in general [3]. The strengths of present review include its robust methodology and relevance to India considering the

nation's rapidly increasing burden of prematurity and associated morbidities. Unlike the previous systematic review by authors which included only 4 RCTs from India, this focused systematic review provides data from 5 additional RCTs ($n = 823$) from the country. The large effect size for major neonatal outcomes, and the lack of statistical heterogeneity for outcomes, NEC and LOS add to the validity of present results. These results are important, especially in the context of the burden of LOS in preterm neonates in India.

Nearly one-third of the world's one million annual preterm deaths are estimated to occur in India [17, 18]. The 'Born Too Soon' preterm birth action group has reported that India can reduce its neonatal deaths by 50% by improving management of infections and feeding support of preterm neonates besides optimizing thermal care, and scaling up Kangaroo Mother Care [19]. The benefits of an additional affordable strategy such as probiotics for preterm infants cannot be overemphasized in this context.

Given the predominance of mature and breast milk fed neonates in the present systematic review cohort, the baseline incidence of NEC of 6% would be considered as high, reflecting the burden of NEC in India. The lack of significant effect on definite NEC or mortality in the sensitivity analysis of studies with lower risk of bias, may be because of the

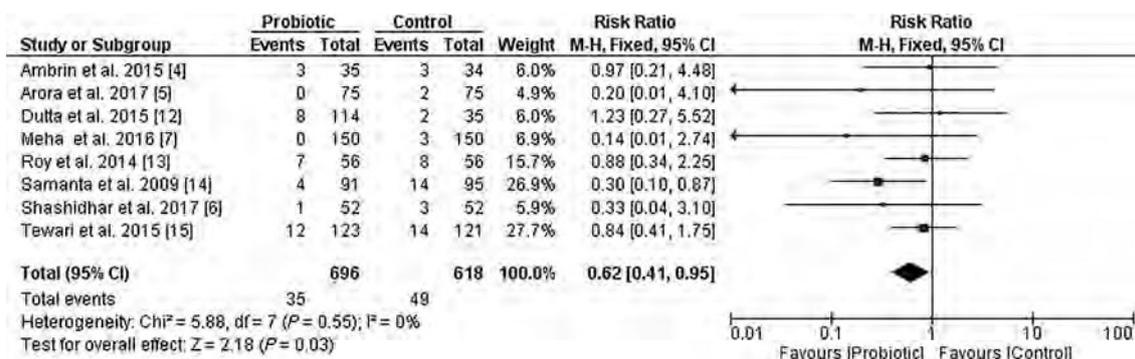


Fig. 4 Mortality prior to discharge

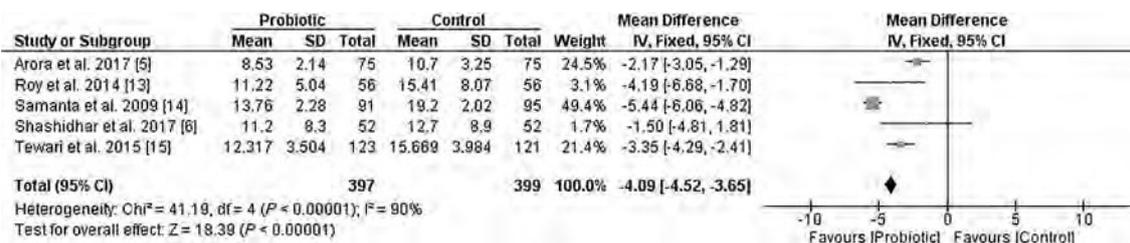


Fig. 5 Time to full feeds

smaller cumulative sample size in the subset of studies. A trend towards benefit with the use of prophylactic probiotic was still observed.

The lack of benefit in NEC on sensitivity analysis, and the wide variation in baseline incidence (1–16%) in present systematic review suggests the need for standardized reporting of NEC, and RCTs with robust methodology. The magnitude of benefit for culture positive LOS in the present meta-analysis (10%) was higher than the 2 to 3% absolute reduction reported in previous meta-analyses [3, 20]. This may relate to the high baseline incidence of LOS. Considering that death competes with NEC and LOS, primary composite outcomes such as ‘Death or LOS’ and ‘Death or NEC ≥ Stage II’ deserve attention for future trials in this field.

The sensitivity analysis also revealed that probiotic supplementation till discharge reduced the incidence of NEC and sepsis, while the benefit was not evident when probiotic was supplemented for shorter duration. However, the subgroup with shorter probiotic duration included more mature neonates with shorter hospital stay. It was also unclear if the duration of supplementation was linked to the median time to development of the first episode of NEC in the study groups, as the latter outcome was reported only in one study [5]. Hence, head to head comparison of studies of differing probiotic duration will be required to ascertain the effect of the probiotic duration on important neonatal outcomes.

The limitations of present review include variation in the patient population, and intervention (*i.e.*, probiotic strain/s, dose, duration) among the individual RCTs. Half of the trials had high ROB, especially for the domains of random sequence generation, and allocation concealment which are the critical aspects of an RCT. None of the included trials were adequately powered for clinically important outcomes such as mortality, sepsis, and NEC. The data was inadequate to assess effects of probiotics in extremely preterm or ELBW infants. However, it is important to note that the cohort included in present systematic review is representative of India’s preterm population structure, 85% of which comprises of those born >32 wks’ gestation [21].

The significance of present results in the context of research and clinical practice for preterm infants in India

needs to be discussed. The options include adopting probiotic supplementation as a standard practice for preterm infants or do so only after reproducing the results in a well-designed trial powered adequately for important outcomes. The first approach may be justifiable given present results, the evidence in totality for benefits of probiotics for preterm infants [22–24]. However, the recent example of ‘increased’ mortality following antenatal glucocorticoids points to the importance of reproducing results by a definitive large RCT in India [25]. Disparities in resources and standard of care in public vs. private neonatal intensive care units are the difficulties in designing and conducting large RCTs in this field in India. On site microbiology facility is important to optimize safety during research and/or routine use of probiotics.

Irrespective of the selected approach, optimizing access to high quality, safe and clinically proven probiotic strains/products and development of high quality indigenous probiotics is important. A survey of 21 probiotic products in Delhi revealed that half of them did not specify the number of viable organisms in the product [26]. Yadav et al. reported that none of the 12 commercially available probiotics had the colony count claimed on the product label [27]. Strict adherence to the Food Safety and Standards Authority of India guidelines is thus crucial to avoid unscrupulous use of probiotics for research or routine use [28–30].

In summary, the present results indicate the urgent need for considering probiotics as a strategy to optimize outcomes of preterm infants in India. They will be helpful in guiding research, and clinical practice in this field.

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Compliance with Ethical Standards

Conflict of Interest None.

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A modern era comparison of right versus left sided congenital diaphragmatic hernia outcomes



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ABSTRACT

Background/purpose: This study aims to retrospectively review outcomes, including neurodevelopmental outcomes, of neonatal right sided congenital diaphragmatic hernias (RCDH) compared with left sided congenital diaphragmatic hernias (L CDH) treated surgically at our institute.

Methods: A retrospective review was undertaken of all cases of congenital diaphragmatic hernia (CDH) treated at Princess Margaret Hospital for Children (PMH), Perth, born between 1st January 2002 and 1st August 2012. The outcomes of R CDH cases were compared with L CDH cases. We examined duration of ventilatory support, use of patch versus primary closure, the CDH recurrence rates, the number of reoperations and neurodevelopmental follow up at one year of age.

Results: Forty nine cases of CDH were operated on at PMH during the 10 year period. Of these, ten cases were R CDH with 39 L CDH cases. Of 49 cases, 34 were diagnosed antenatally, 5 R CDH versus 29 L CDH. Only 8/39 cases of L CDH required patch repair for larger defects, while 5/10 R CDH required patch repair. Postoperative mortality was 6/49 (1/10 right sided versus 5/39 left sided). Recurrence was observed in 5/10 R CDH versus 6/39 L CDH with $p = 0.03$. Thirty three of 43 surviving patients received one year follow up with Griffiths general quotient (GQ) assessment demonstrating a median score of 98 for L CDH (IQR 86 to 104.25) and 91 for R CDH (IQR 76.5 to 93).

Conclusions: R CDH required patch repair more commonly than L CDH because of larger defect size or complete agenesis. The rate of recurrent herniation was the only morbidity significantly higher in the R CDH group. Survivors of R CDH did not have a significant difference in neurodevelopmental outcome compared to L CDH cases, with both groups exhibiting normal median GQ scores at one year of age.

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Congenital diaphragmatic hernia (CDH) is a birth defect with abnormal diaphragmatic development that allows herniation of abdominal viscera through a diaphragmatic defect into the thoracic cavity. CDH occurs in approximately 1 in 2500 live births [1,2]. The severity of disease is highly variable and is chiefly dependent upon comorbidities and the degree of concomitant pulmonary hypoplasia and pulmonary hypertension [3,4]. Left sided CDH (L CDH) comprises the majority of cases, with right sided CDH (R CDH) accounting for approximately 15–20% of all cases [5,6]. Rarely, CDH will present bilaterally [5]. There is some literature comparing the outcomes of L CDH versus R CDH, however results have been mixed, with multiple treatment strategies utilized, confounding the data. Recent developments in treatment methods have increased the overall rate of survival in CDH, which is now as high as 80% postoperatively [7]. Certainly with the increasing survival of more

severe cases of CDH, long term complications are also becoming more evident. These include respiratory, gastroesophageal, nutritional complications and recurrence, not to mention morbidity attributed to associated congenital syndromes [3]. Studies have also looked at the neurodevelopmental outcomes of CDH survivors and mechanisms for impairment, with many reporting significant impairments in multiple fields of development [8–11]. Our study aims to retrospectively examine several outcomes including duration of ventilation, surgical repair options and recurrence. Furthermore, ours is one of the very few published studies to compare validated neurodevelopmental outcomes of R CDH and L CDH at one year of life.

1. Materials and methods

We performed a retrospective audit of all cases of CDH managed by seven surgeons at Princess Margaret Hospital (PMH) in the 10 and a half years between 1st January 2002 and 1st August 2012. Institutional ethics approval was obtained (GEKO 6683). Cases were identified

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from the neonatal and theater databases at our institute, as well as from the accurate and well maintained Western Australian Birth Defect Registry. Owing to the closed population base, all cases of CDH diagnosed antenatally or postnatally in the neonatal period (less than 28 days of age) in Western Australia are accounted for in this cohort, including those terminated and those born at peripheral centers. The medical records were reviewed to obtain the data, including sidedness, associated comorbidities, duration of ventilatory support, use of patch versus primary closure of diaphragm and abdomen, CDH recurrence rates, number of reoperations and neurodevelopmental follow up at one year of age (performed using a Griffiths developmental assessment to obtain general quotient (GQ) score). Antenatal ultrasound scans, where available, were used to determine whether the liver was up in the thorax; and the Lung Head Ratio (LHR) based on the Longest Axis Method. Observed/Expected LHR (O/E LHR) was calculated using the TOTAL Trial online calculator [12]. O/E LHR was calculated for cases with scans before 32.6 weeks of gestation, when the formula is considered valid, with well defined normal ranges [13].

Data analysis was performed using VassarStats, with a Fisher exact test for binomial data and Mann Whitney test for ordinal data. Owing to the small sample size, median and interquartile ranges (IQR) are reported for our continuous data. Two tailed p values are reported. Statistical significance was considered to be achieved with a p value of less than 0.05.

2. Results

2.1. Incidence of CDH

The calculated incidence of CDH in Western Australia during the study period was 0.4 cases per 1000 births. During this time there were a total of 113 cases of CDH recorded in Western Australia. In 35 cases of CDH the pregnancy was terminated and in a further 11 cases the fetus was stillborn. Almost all stillbirths and terminations of pregnancies had multiple congenital anomalies. During the study period there were a further 19 liveborns with CDH in the state of Western Australia who did not survive transfer from place of birth to PMH, dying either at or near delivery. Of these 19 very early neonatal deaths, six were R CDH, ten were L CDH and two bilateral, with one side unspecified. A total of 49 CDH cases were transferred to PMH and underwent surgical management during the 10 year period of the study. PMH, the sole pediatric tertiary center in Western Australia with surgical services, is not attached to an obstetric center. As such, all cases of CDH require transfer for surgery via the Newborn Emergency Transport Service (NETS) team.

2.2. Demographic and antenatal data

We managed ten cases of R CDH and 39 cases of L CDH. The demographic data for these cases is reported in Table 1. There was no statistically significant difference between the left and right groups in terms of demographics. Of cases where antenatal scans were available (two R CDH and 23 L CDH), liver was noted to be up in the thorax in both R CDH cases and four L CDH cases. O/E LHR calculations showed

severe hypoplasia (O/E LHR <25% before 29.5 weeks gestation) in five cases of L CDH (median 20.1%, range 19.1–23.8%). Moderate hypoplasia (O/E LHR 25–34.9% irrespective of liver position) was noted in both R CDH cases and six L CDH cases (median 32.25%, range 25.1–34.6%). Seven L CDH cases had mild hypoplasia (O/E LHR median 40.5%, range 35.8–66.3%), and in five L CDH cases antenatal data was beyond 32.6 weeks of gestation, too late to calculate validated O/E LHR.

2.3. Ventilation

Our neonatal intensive care units use advanced lung sparing and pulmonary hypertension managing strategies including permissive hypercapnea, high frequency oscillation and jet ventilation, nitric oxide and lower pressure conventional ventilation. As Table 2 demonstrates, there was no significant difference in the preoperative and postoperative invasive ventilatory duration and maximal requirements of the L CDH and R CDH group.

2.4. Operative findings

The operative findings demonstrated a greater proportion of R CDH with larger defects or total agenesis requiring patch closure when compared with L CDH (60% vs. 20.5% $p = 0.02$) (see Table 3). Operative repair was performed later for R CDH compared with L CDH (median 7 vs. 2 days, $p = 0.01$).

2.5. Total parenteral nutrition

Both left and right sided groups had an expected high requirement for perioperative total parenteral nutrition (TPN) with median duration of 10 days for L CDH and 17 for R CDH ($p = 0.25$) (Table 4).

2.6. Morbidity, mortality and neurodevelopment

The postoperative mortality was 10% for R CDH, compared with 13% for L CDH ($p = 1.00$). When liveborn babies who did not survive long enough to undergo surgical intervention are included in the data set, the mortality rates are 43% for right sided compared with 31% for left sided CDH ($p = 0.41$).

Our data demonstrates substantial short term postoperative morbidity for CDH babies. These morbidities include prolonged hospital admission, multiple reoperations including a large number for recurrence (with 50% of the right sided CDH group requiring rerepair, compared with 15% for the left sided group, $p = 0.03$) and several readmissions for respiratory problems or bowel obstruction.

Despite the high levels of ventilatory support required, the recurrence and readmission rates for the R CDH and L CDH babies, our data demonstrated pleasing Griffiths assessment scores obtained at one year of age. Thirty three of 43 CDH cases (76.7%) received a Griffiths assessment score at one year follow up. Both the left and right sided group medians GQ scores (L CDH 97, R CDH 91) fell within the range considered normal. Only two of the 33 cases with GQ scores performed fell in the abnormal range of below 70, one from each group. These scores

Table 1
Demographic Data of CDH Patients.

(Median and IQRs presented for continuous data)	Right CDH (n = 10)	Left CDH (n = 39)	p Value
Birth weight (kg)	2.66 (2.44–2.94)	2.95 (2.50–3.42) (n = 38)	0.27
Gestational age (weeks + days)	37 + 1.5 (34 + 6–38 + 1.5)	38 + 3 (37 + 0.5–39 + 2)	0.06
Sex M:F	8:2 (80%:20%)	21:18 (54%:46%)	0.17
Antenatal: postnatal diagnosis	5 Ante:5 Post (50%:50%)	29 Ante:10 Post (74%:26%)	0.25
Apgars	1 m: 6.5 (5.25–7.75)	1 m: 6 (5–7.5)	0.83
	5 m: 8 (7.25–9)	5 m: 9 (8–9)	0.75
Age at diagnosis (day of life)	0 (0–0.75)	0 (0–0)	0.28

*One patient excluded from left weight data because of estimate only.

Table 2
Maximum Preoperative and Postoperative Ventilation Required.

Outcome	Right CDH (n = 10)	Left CDH (n = 39)	p Value
No preoperative invasive ventilatory support	2 (20%)	2 (5%)	0.18
Preoperative conventional ventilation only	2 (20%)	19 (49%)	0.16
Preoperative CV with NO	1 (10%)	4 (10%)	1.00
Preoperative HFOV only	1 (10%)	9 (23%)	0.44
Preoperative HFOV with NO	4 (40%)	5 (13%)	0.07
Postoperative conventional ventilation only	6 (60%)	29 (74%)	0.44
Postoperative CV with NO	1 (10%)	2 (5%)	1.00
Postoperative HFOV required	1 (10%)	3 (8%)	1.00
Postoperative HFOV with NO	2 (20%)	5 (13%)	0.62
Time to first extubation (days postoperative)	6 (3.25–44.25)	4 (2–10) n = 38	0.16
Time to spontaneous ventilation RA (days postoperative)	13 (4.25–45) with one outlier	6 (3–12.5) n = 35 with four deaths (excluded) and one severe outlier	0.25

CV, conventional ventilation.

NO, nitric oxide.

HFOV, high frequency oscillation ventilation.

RA, room air.

*Two cases not requiring invasive ventilatory support were diagnosed in the perinatal period, presenting with respiratory distress while the other two cases were diagnosed postnatally after initially being discharged home as well neonates and representing within the first few weeks of life with various symptoms of respiratory distress.

*Severe outlier went home on supplemental oxygen.

show that most CDH babies in our cohort surviving to operation did not have abnormal neurodevelopmental outcomes at one year.

3. Discussion

In 2005 Colvin et al. reported a 33% rate of pregnancy termination in cases of CDH [14]. Termination rates remained high within the current study period, with 35 cases (31%) of prenatally identified CDH undergoing an abortion. Based on data from the Western Australian Birth Defects Registry, almost all stillbirths and pregnancy terminations with CDH had other congenital anomalies.

We found that a higher proportion of L CDH was diagnosed antenatally compared with R CDH, which tended to be diagnosed postnatally. The unexpected postnatal diagnosis of CDH required stabilization and organization of transfer and hence a comparative delay to referral. This lower prenatal recognition may explain the reason R CDH cases had surgery later than L CDH cases at PMH. As shown in the ventilator data, these neonates with R CDH did not have a higher ventilatory requirement than L CDH babies.

Our institutions practice is in line with modern techniques to manage CDH pathophysiology such as pulmonary hypertension and hypoxia secondary to lung hypoplasia. Our abilities include high frequency oscillation and jet ventilation depending on the clinical requirements. Our center offers surgery to repair diaphragmatic hernia through open surgical techniques at a point where the multidisciplinary team, including the neonatologists and surgeons, feel that the baby is at a period of medical stability. Achieving satisfactory medical stability prior to proceeding to surgery is an endpoint that is difficult to define with a heterogeneous spectrum. Our center takes a case by case approach in determining the optimum window for operation, balancing the cardiorespiratory stability of the patient and need for operation. As a result some infants were still weaning off nitric oxide at the time of surgery, whereas others had improved rapidly and were on conventional ventilation only. Ultimately, the key consideration is allowing sufficient time to permit the severity of the neonate's cardiopulmonary pathophysiology to declare itself.

Table 3
Operative Findings and Treatment.

(Median and IQRs presented for continuous data)	Right CDH (n = 10)	Left CDH (n = 39)	p Value
Total agenesis requiring patch	2 (20%)	1 (3%)	0.10
Large defect requiring patch	4 (40%)	7 (18%)	0.20
TOTAL PATCH	6 (60%)	8 (20.5%)	0.02
Primary defect closure	4 (40%)	32 (82%)	0.02
Abdominal patch at closure	1 (10%)	7 (18%)	0.67
Age at repair (days)	6.5 (4.25–10.25)	2.0 (1.5–4.0)	0.01

This in turn determines their capability of tolerating surgery, and allows the treating team to plan and maintain an ability to escalate cardiopulmonary supports if required in the perioperative period.

Traditionally, surgical management of CDH aims to achieve primary closure of the diaphragmatic defect [15]. Where primary closure cannot be performed, a “patch” of synthetic or biosynthetic material is used to close the defect [15]. The need for patch repair is indicative of the size of the diaphragmatic defect and is thought to correlate with worse outcomes [1,4]. However, recent evidence shows that patch repairs, which were previously thought to contribute to higher morbidity and mortality, can be performed with low recurrence rates [16–18]. In our series, R CDH cases were more likely to require patch repair, and were also more likely than L CDH to have recurrent herniation. We postulate that this relates to the larger defects that R CDH cases tend to have, with greater requirement for patch repair.

Colvin et al. [14] had previously presented mortality rates of 20% for neonates that reach the tertiary treating center in Western Australia. Our data demonstrates an improvement in this rate to 12% during this modern era. Furthermore, when all liveborns with CDH are included, the overall mortality rate has decreased from 50% to 36%. We speculate that this improvement relates to advancements in neonatal intensive care with advanced lung sparing and pulmonary hypertension management strategies.

Studies throughout the literature have not conclusively answered whether sidedness influences mortality rates. For example, a metaanalysis [19] of all English language literature on CDH from 1975 to 1998 was performed to assess mortality factors influencing outcome. A significant finding of the study was that the presence of major malformations associated with CDH conferred higher mortality. On examination of multiple studies, the metaanalysis also found that R CDH has higher mortality rates than in L CDH. However, these studies were small and did not account for associated major malformations, resulting in limited sample size and loss of statistical power. As such, the metaanalysis was unable to determine the effect of sidedness on mortality.

Other studies also have also been inconclusive. A study performed by Migliazza et al. in Bergamo, Italy consecutively followed 111 cases of CDH admitted to the Neonatal Intensive Care Unit [20]. There was no statistical difference in mortality between left sided and right sided CDH with 66 of 93 left sided CDH surviving compared to 11 of 18 right sided CDH ($p = 0.40$).

A retrospective multicenter cohort study conducted in Scandinavia comprised 195 cases across the years 1995 to 1998 [21]. A significant difference in mortality was demonstrated in right versus left sided hernias ($p = 0.042$) with a hazard ratio of 2.1 on multivariate Cox regression model analysis.

Table 4

Postoperative TPN, feeding, mortality and developmental outcomes, length of admission, readmissions, reoperation and recurrence rates.

(Median and IQRs presented for continuous data)	Right CDH (n = 10)	Left CDH (n = 39)	p Value
Number needing perioperative TPN	9 (90%)	38 (97%)	0.37
Total days of TPN	17 (11.5–18.75)	10 (7–18.5)	0.25
Postoperative mortality	1 (10%)	5 (13%)	1.00
Overall mortality (including liveborns who did not survive to OT)	8 (50%) n = 16	15 (31%) n = 49	0.41
GQ at one year	91.0 (76.5–93.0) n = 7	97.0 (86.0–102.0) n = 26	0.13
Length of admission (days)	30.5 (18.5–78.25)	21 (13.5–37)	0.26
Number of patients with related readmissions	6 (66.7%) n = 9	20 (55.6%) n = 36	0.71
Number of readmissions per patient	1 (0.75–1.25) n = 9	1 (0–1) n = 36	0.67
Number with recurrence of hernia	5 (50%)	6 (15%) (6 in 1 patient, twice in 2 patients, total 13 recurrences)	0.03
Number of patients with associated operations	7 (70%)	16 (41%)	0.44
Number of associated operations per patient	1.0 (0.25–2)	0 (0–1)	0.16
Length of follow-up (years)	5.1 (3.6–6.2) n = 9	3.0 (1.9–6.4) n = 31	0.39

In contrast, one of the more recent studies comparing right sided versus left sided CDH outcomes demonstrated a considerable trend toward increased survival rates in those infants with right sided CDH (94% vs. 70%, $p = 0.07$) [22]. The study found that left sided CDH was often associated with pulmonary hypertension resistant to therapy, including extracorporeal membrane oxygenation (ECMO), resulting in higher death rates. Although response rates to ECMO were better in R CDH, higher survival rates also conferred a higher incidence of chronic lung disease.

A 2013 study by Wynn et al. [4] found that right sided CDH did not necessarily predict worse outcomes. Although right sided CDH patients were more likely to require ECMO, they did not have more significant pulmonary hypertension at 1 or 3 months of age, and did not have reduced survival. None of our patients required preoperative ECMO and mortality in the R CDH group was not significantly higher than in the L CDH group.

Improved treatment methods have increased rates of survival for CDH, particularly those who are more severely affected. However, the challenge now arises to assess long term outcomes, critical for parent counseling in the perinatal period. Approximately half of CDH cases occur without associated malformations and normal brain development in utero. However neurodevelopment of CDH infants may be affected by hypoxic injuries as a direct result of the defect [10]. Data suggests that CDH patients do well in the immediate perioperative period with low rates of seizures, cerebral palsy and stroke [8]. However, survivors may go on to develop problems with fine and gross motor skills, visual motor integration, executive function, behavioral problems and learning disabilities [8]. Within the literature there is a vast heterogeneity in the neurodevelopment tools used to assess outcomes and the majority of studies have been performed retrospectively, making it difficult to properly determine outcomes. Our study uses an internationally validated tool, the Griffiths developmental assessment, to objectively quantify neurodevelopmental outcomes of CDH cases at one year of age, directly comparing L CDH with R CDH cases. We acknowledge that some high risk follow up centers are currently using the Bayley Scales of Infant and Toddler Development Third Edition (Bayley III). However, during the study period our center used the Griffiths developmental assessment and to maintain continuity through the study we have reported outcomes using this assessment tool.

In our study, CDH patients had median neurodevelopmental scores within the normal range for both L CDH and R CDH at one year of age. Our data is similar to findings from other Australian centers. Recent evidence [23] from the Children's Hospital at Westmead in Sydney used the Bayley III to assess development of CDH patients at 12 and 36 months of age. Compared with a control group of healthy infants, those with CDH had similar neurodevelopment scores. This suggests that during the first three years of life CDH is not necessarily associated with poorer neurodevelopmental outcomes. However, international studies have noted poorer neurodevelopmental outcomes for CDH. Danzer et al. [24], demonstrated significant neurodevelopmental morbidity in approximately half of the 41 CDH patients enrolled in a

follow up program, with the most common sequelae being neuromuscular hypotonicity (41%) and psychomotor dysfunction (23% mild, 31% severe). Interestingly, in their study, risk factors for borderline or delayed outcomes included right sided CDH ($p = 0.02$).

This group has subsequently published data on neurodevelopmental outcomes of 63 CDH patients that did not require ECMO [25]. Their data suggested poorer outcomes, with deficits in cognitive scores, a trend to higher risk of neuromuscular hypotonicity (not statistically significant) and R CDH carrying a higher risk for impaired neurodevelopmental outcome. Only 43% scored within the average range for all scores, with 44% demonstrating mild and 13% demonstrating severe delays in at least one domain (of cognitive, language and motor scores). In their experience, a high percentage of CDH children who did not require ECMO had below normal neurodevelopmental scores at one year of life. Compared to our cohort, their cohort likely had more severe CDH cases, as suggested by the large proportion of their cohort requiring patch repair and supplemental oxygen beyond 30 days.

The need for ECMO also seems to be related to a poorer neurological outcome. Data suggests that infants with R CDH are more likely to require ECMO. For example, in a study performed by Fisher et al. [26] of 267 cases of unilateral CDH, 40% of R CDH required ECMO compared to 15% of L CDH. This is supported by a study performed by Hedrick et al., in which 52% of R CDH required ECMO [27].

Our data demonstrating Griffiths developmental outcomes predominantly in the normal range in both R CDH and L CDH cases at one year is a reassuring finding, given the previous concerns about neurodevelopmental outcomes in CDH. It was pleasing to note that there was no significant difference between the left and right sided groups.

Our institute is the only tertiary referral center for pediatric surgery in our region, giving strength to our ability to collect and report reliable quantitative data, including the ability to provide data on long term follow up. We recognize the limitations of the small data sample, the retrospective nature and the relatively short period of formal neurodevelopmental follow up. Nonetheless, the data provides a valuable analysis of the outcomes for R CDH compared with L CDH and suggests that liveborns who survive to operation may have a significant risk of morbidity, but with current management techniques, have appropriate neurodevelopmental outcomes at one year.

4. Conclusions

We present data from PMH on the 113 cases of CDH that were recorded during our 10 year study period, particularly focusing on the 49 cases that were managed surgically. The demographics of the L CDH compared with the R CDH cases operated on at our institute during this time were comparable. Cases of R CDH were more likely to have large defects and require patch repair rather than primary closure. Furthermore R CDH cases were more likely to have recurrence and require reoperation. Despite this, and with current management techniques, survivors of R CDH did not have a significant difference at one year of

age in neurodevelopmental outcomes compared to L CDH, with both groups exhibiting normal median GQ scores.

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Chapter 5

Paraprobiotics

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Introduction

Probiotics are live organisms that when administered, confer health benefits to the host [1]. Studies have shown that probiotics have the potential to improve the outcomes of patients with ulcerative colitis [2,3], *Clostridium difficile*-associated diarrhea [4], antibiotic-associated diarrhea [5], traveler's diarrhea [6] and irritable bowel syndrome [7]. Probiotics have also been shown to be beneficial in the prevention of necrotizing enterocolitis (NEC) [8,9] and late-onset sepsis in preterm infants [10], ventilator-associated pneumonia [11], postoperative infections [12] and many other conditions [13].

In spite of the encouraging results, there is hesitancy among healthcare professionals to use probiotics, considering the occasional risk of serious infections due to the administered probiotic organism [14–22]. The other concerns are altered long-term immune responses and the possibility of development and spread of antibiotic resistance. Paraprobiotics on the other hand are inactivated organisms and hence unlikely to be harmful, while retaining the beneficial effects of live probiotics. They are incapable of growing in vitro, which can be verified by plating in adequate culture media [23–25]. Without the need for maintaining cold chain to preserve viability of the probiotic organisms, the costs associated with paraprobiotics are also expected to be lower.

Nomenclature

Paraprobiotics are also known as inactivated probiotics, nonviable probiotics, killed probiotics, ghost probiotics, modified probiotics, sterilized probiotics, and postbiotics. Taverniti et al. from Italy first proposed the term “paraprobiotic” for these agents [25]. The prefix “para” (from the ancient Greek, παρά) was chosen because of its meaning of “alongside of” or “atypical,” which can

simultaneously indicate similarity to and the difference from the conventional definition of a probiotic [25].

Methods of inactivation

Inactivation could be achieved using physical or chemical strategies, including heat treatment, γ or UV rays, chemical or mechanical disruption, pressure, lyophilization, or acid deactivation [25]. The excellent review by de Almada et al., covers the various methods for inactivation of probiotics [23] (Table 1).

Randomized controlled trials (RCTs)

Till date, nearly 60 RCTs have been conducted evaluating the efficacy and safety of paraprobiotics in various conditions in humans (Table 2).

Atopic dermatitis and allergic diseases

Harima-Mizusawa et al. examined whether citrus juice supplemented with heat-killed *Lactobacillus (L.) plantarum* YIT 0132 (LP0132) could alleviate the symptoms of perennial allergic rhinitis [26]. Patients consumed LP0132-fermented juice (n = 17) or unfermented citrus juice (placebo: n = 16) once a day for 8 weeks. The participants in the LP0132 group showed a significant reduction in the nasal symptom score and stuffy nose score. Significant attenuation of type 2 helper T cells (Th2 cells)/helper T cells, serum total immunoglobulin E (IgE), and eosinophil cationic protein (ECP), and augmentation of type 1 helper T cells (Th1 cells)/Th2 cells at 8 weeks of intervention was reported as well.

In the study by Inoue et al., 49 atopic dermatitis patients aged ≥ 16 years used heat-killed *L. acidophilus* L-92 [27]. Skin lesions were assessed using the SCORAD index. The L-92 group had significantly lower SCORAD scores

TABLE 1 Methods of inactivation of probiotics ^a.

Method	Key mechanisms	Key features
1. Thermal treatment		
a. Pasteurization: <100°C b. Sterilization: ≥100°C	Damage to cell membrane, protein denaturation, solute losses, enzyme inactivation, reduction of intracellular pH, conformational changes in ribosomes and nucleoids, disintegration of RNA filaments	Range of temperature and duration in various studies: 60–121°C for 15–60 min)
2. Nonthermal treatments		
a. Ionizing radiation b. Ultraviolet (UV) rays c. High pressure d. Sonication e. Pulsed Electric Field (PEF) technology f. Ohmic or Joule or resistive heating g. Supercritical CO ₂ technology h. Dehydration i. Modification of pH	Damage to nucleic acids Denaturation of proteins, formation of DNA photoproducts Damage to cell membranes Rupture of cell wall, damage to cell membrane, DNA damage, activation of intracellular esterases, inhibition of cell metabolism Electroporation (cell membrane damage) Thermal induced damage due to alternating electric current and electroporation. Induction of intracellular acidosis, cell membrane damage and depletion of vital constituents, enzyme inactivation, CO ₂ and HCO ₃ induced direct damage, intracellular electrolyte imbalance Damage to cell membrane, conformational changes to proteins, nucleic acids and ribosomes Damage to cell membranes, chemical denaturation of DNA and ATP and enzyme inactivation	Gamma irradiation; Cobalt 60 source for 20 h at 8.05 Gy/minute UV details not specified, UV for 30 min, UV for 20 min, UV for 5 min under 39 W germicidal lamp 400 or 600 MPa at 37°C for 10 min Low-frequency ultrasound (20 kHz) generated by submerging 10 mm diameter probe in 30 mL probiotic cell suspension. Sonic power: 300 W/cm ² . Ultrasonic power and irradiation time: 60 W/cm ² and 0–20 min. Suspension temperature maintained at 20 ± 1°C in ice water bath to prevent lethal thermal effect, Probiotic culture centrifuged at 13,000 rpm for 10 min and then sonicated for 5 min. Pulsed high voltage (40 kV/cm); applied to probiotic bacteria containing food products placed between two electrodes Based on Joule's law Use of CO ₂ at temperatures and pressures above their critical point values (T _c :31.1°C; P _c : 7.38 MPa) Lyophilization (Freeze drying): Water in the material to be dried is frozen first and then sublimed under vacuum conditions. Spray drying: Formation of powder by using an atomizer for pulverization of liquids and formation of small droplets in contact with hot air. Lowering pH of the medium to 3.0–4.0 was effective in inactivating probiotics; duration of exposure to low pH not specified
3. Combination treatments		
UV-C light and heat treatment	Denaturation of proteins, formation of DNA photoproducts, cell membrane damage, enzymatic denaturation	UV-C light (0-10.6 kJ/m ²) with heating at 50°C

^aBased on de Almada et al. [24].

TABLE 2 Paraprobiotics in various conditions and populations

1. Acute diarrhea (Prevention in children, treatment in adults and children)
2. Antibiotic associated diarrhea (Prevention in adults and children)
3. Atopic dermatitis and allergic diseases (Prevention and treatment in adults, children and neonates)
4. Chronic diarrhea (Treatment in adults)
5. Constipation (Treatment in female adults)
6. Dental diseases (Treatment in adults and prevention in children)
7. General Immunity (Treatment in healthy and elderly adults)
8. Helicobacter pylori infection (Treatment in children and adults)
9. Infantile colic (Treatment in infants)
10. Intestinal permeability (Prevention in healthy adults)
11. Irritable bowel syndrome (Treatment in adults)
12. Lactose malabsorption (Prevention in children)
13. Malignancies (As an adjuvant treatment in adults)
14. Multiple organ dysfunction (Prevention in adult intensive care unit patients)
15. Necrotizing enterocolitis (Prevention in preterm infants)
16. Neonatal sepsis (Prevention in preterm infants)
17. Obesity (Treatment in adults)
18. Ocular diseases (Prevention in healthy adults)
19. Pancreatitis (Treatment in adults)
20. Prevention of postoperative infections (Adults undergoing major abdominal surgery)
21. Sleep disturbances (Treatment in adults)
22. Traveler's diarrhea (Prevention in adult travelers)
23. Viral infections (Prevention in adults and children)

($p = 0.002$), decreased ratios of change for eosinophil count ($p = 0.03$), and increased ratios of change for serum TGF- β ($p = 0.03$).

Morisset et al. evaluated the impact of an infant formula containing heat-killed *Bifidobacterium (B.) breve* C50 and *Streptococcus thermophilus* 065 (HKBBST) in 129 infants at high risk of atopy [28]. Infants were given HKBBST or a standard infant formula since birth until one year of age, and were followed at 4, 12, and 24 months after birth. The use of HKBBST milk did not alter the proportion of cow's milk protein allergy (CMPA), but decreased the proportion of positive skin prick test to cow's milk (1.7% vs. 12.5%, $p = 0.03$), and the incidence of digestive (39.5% vs. 63%, $p = 0.01$) and respiratory "potentially allergic adverse events" (PAAE) (7% vs. 21%, $p = 0.03$) at 12 months, and that of respiratory PAAEs at 24 months (13% vs. 35%, $p = 0.01$).

In the investigation of Moroi et al., the clinical effect of a supplementary diet containing heat-killed lactic acid bacterium (LAB) *L. paracasei* K71 (LAB diet), was assessed in 34 adults with atopic dermatitis [29] who were being treated with conventional topical corticosteroids and tacrolimus. The skin severity scores decreased significantly from baseline at week 8 ($p < 0.05$) and at week 12 ($p < 0.01$). The consumption of topical therapeutics in the placebo group was 1.9-times greater compared to the LAB group, but the difference was not statistically significant.

Gotoh et al. conducted an RCT to determine whether the administration of heat-killed *L. gasseri* OLL2809 would

benefit patients with Japanese cedar pollinosis (JCP) [30]. Participants were randomly allocated to receive oral placebo, or tablets containing 100 mg of *L. gasseri* OLL2809 per day for 8 weeks during the pollen season. The results showed no obvious differences between the groups. Subgroup analysis revealed that the OLL2809 subgroups with CAP-RAST scores of 4 or 5, exhibited improvement in nasal symptoms scores and serum allergy-related items, including Japanese cedar pollen-specific IgE levels [30].

Peng et al. evaluated the efficacy of heat-killed *L. paracasei* 33 (LP33) for treating allergic rhinitis induced by house-dust-mite [31]. Ninety patients were assigned to one of the three treatment groups. Patients in groups A and B received two capsules per day of live or heat-killed LAB (5×10^9 colony forming units (CFU)/capsule), respectively, over a period of 30 days while Group C received a placebo. The overall quality-of-life score was significantly improved in groups A and B [31].

Negative results

Kirjavainen et al. [32] assessed the efficacy of oral supplementation with a viable and heat-inactivated probiotic (*L. rhamnosus* GG), in 35 infants with atopic eczema and CMPA. Treatment with heat-inactivated LGG was associated with adverse gastrointestinal symptoms and diarrhea; hence, the recruitment of patients was stopped early. Atopic eczema and subjective symptoms were significantly alleviated in all the intervention groups. The SCORAD

scores decreased from 13 (IQR 4–29) to 8 (0–29) units in the placebo group, from 19 (4–47) to 5 (0–18) units in the viable LGG group, and from 15 (0–29) to 7 (0–26) units in the heat-inactivated LGG group. The decrease in the SCORAD scores within the viable LGG group tended to be greater than within the placebo group. Treatments did not appear to affect the gut microbiota. The authors concluded that atopic eczema and CMPA could be potentially managed by supplementation of infant formulas with viable, but not heat-inactivated LGG [32].

Additional studies with paraprobiotic agents for prevention [33] or treatment of atopic dermatitis, with improvement in symptoms, can be found in the literature [34–36].

Irritable bowel syndrome

Symptoms (abdominal pain, bloating, number of stools per day, and stool consistency), impact on health-related quality of life (HRQOL), and consequences on anal continence, were evaluated in 297 patients with irritable bowel syndrome (IBS) [37] before and after 1 month of probiotic treatment with Lacteol (inactivated *Lactobacillus* LB plus fermented culture medium). The pain scores decreased from 4.46 ± 0.15 to 2.8 ± 0.14 ($p < 0.0001$). Bloating decreased from 4.49 ± 0.18 to 2.5 ± 0.15 ($p < 0.0001$). The HRQOL score, which is inversely correlated with quality of life, decreased from 5.99 ± 0.14 to 3.92 ± 0.16 ($p < 0.0001$). The mean number of stools per week decreased from 17.59 to 12.83 ($p < 0.0001$). Before treatment, 54% of patients had watery stools, and 46% had smooth stools; after completing the treatment, only 18.5% of patients had watery stools, and 34% had normal stools [37].

Guyonnet et al. [38], and Halpern et al. [39] have also reported symptomatic improvement with inactivated probiotics in adult patients with IBS.

Antibiotic-associated diarrhea

Aiming at prevention of antibiotic-associated diarrhea (AAD) [40], 87 hospitalized patients were randomized to receive a commercial probiotic milk drink fermented with LGG, *L. acidophilus* La-5 and *Bifidobacterium* Bb-12, or a milk drink with heat-killed bacteria as a placebo for 14 days. Sixty-three patients completed the study; two patients (5.9%) in the live probiotic group and eight (27.6%) in the heat-killed lactobacillus group developed AAD ($p = 0.035$). These results suggest that fermented milk with multistrain live probiotics, not the heat-killed supplement, may prevent AAD in hospitalized adult patients.

Merenstein et al. [41] reported no difference in the incidence of AAD (21.9% vs. 18%) in 125 children randomized to prophylactic modified or living probiotic.

Infections

Cow's milk (group A) or rice (group B) fermented with heat-inactivated *L. paracasei* CBA L74, or placebo (group C), were compared in children for 3 months during the winter season. The proportion of those who experienced at least one common infectious disease (CID) was lower in group A (51.8%) and B (65.9%), versus group C (80.3%). This included upper respiratory tract infections and acute gastroenteritis. A net increase of all fecal biomarkers of innate and acquired immunity was observed for groups A and B. The investigators concluded that dietary supplementation with cow's milk or rice supplemented with heat-killed *L. paracasei* CBA L74 prevents CIDs in children attending daycare, by enhancing their innate and acquired immunity [42]. In a substudy of 20 infants from the original RCT, Berni Canani [43] reported that the heat-killed lactobacillus group had higher fecal butyrate levels [43].

Salazar-Lindo et al. [44] (80 outpatients) and Bouloche et al. [45] (103 inpatients) reported the reduced mean duration of acute diarrhea in children, after treatment with inactivated probiotic. However, Khanna et al. [46] did not observe such a benefit in 98 in-patient children. Sharieff et al. [47] noticed no difference in the prevalence of diarrhea, in 50 children given prophylactic inactivated probiotic compared with placebo.

Experimental human rhinovirus infection (HRV) was induced by intranasal HRV A39 instillation in 59 adults, who had consumed juice enriched with live or heat-inactivated *Lactobacillus* LGG, or control juice in the 3 preceding weeks. There was a tendency toward the lowest HRV loads (\log_{10} copies/mL) in the LGG groups: 6.20 in live, 6.30 in inactivated LGG, and 7.25 in placebo group, $p = 0.57$ [48].

In contrast, Kumpu et al. [49] reported no significant differences in the incidence of rhinovirus infection in 60 adults given prophylactic live (74%) or inactivated LGG (90%), or placebo (90%).

Heat-killed *L. pentosus* b240 was administered to elderly adults [50]. Participants in the low-dose and high-dose b240 groups were given tablets containing 2×10^9 or 2×10^{10} cells, respectively, of heat-killed b240. The cumulative incidence rate of the common cold was 47.3%, 34.8% and 29.0% for the placebo, low-dose and high-dose b240 groups, respectively ($p = 0.012$). General health perception, as determined by the SF-36, increased in the b240 groups in a dose-dependent manner ($p < 0.025$) [50].

Hirose et al. [51] reported significant reduction in respiratory tract infections, in 78 adults with high levels of stress, after administration of inactivated *L. plantarum* L137 versus placebo (48.7% vs. 61.5%, $p < 0.05$).

Paraprobiotics plus culture medium

In a study, heat-killed *L. acidophilus* LB (10 billion CFU), plus 160 mg of spent culture medium was administered to infants with nonrotavirus diarrhea. Shortened recovery time by 1 day was documented. In conjunction with the simultaneously performed *in vitro* study, it was concluded that heat-killed *L. acidophilus* LB plus its culture medium, antagonizes the C1845-induced increase in paracellular permeability in intestinal Caco-2/TC7 cells, and produces a clinically significant benefit in children with nonrotavirus diarrhea [52].

Mitra et al. reported no significant difference in the median duration of *Vibrio cholerae* or *E. coli*-induced diarrhea, in 197 adults given inactivated or live *Streptococcus faecium* SF 68 [53].

One hundred and thirty-seven adult patients with chronic diarrhea were randomly allocated to receive either a 4-week course of Lacteol Fort (heat-killed *L. acidophilus* LB) twice a day or Lacidophilin (living lactobacilli) thrice daily. At the second and fourth week of treatment, mean bowel frequency was significantly lower in the Lacteol group ($p < 0.05$) [54].

As an adjunct to oral rehydration therapy in children (3–24 months) with acute diarrhea [55], the same Lacteol Fort (heat-killed *L. acidophilus* LB) or placebo was offered on admission and every 12-hourly for five doses. After 24 h of treatment, the number of rotavirus-positive children with watery stools was significantly lower ($p = 0.012$) in the *L. acidophilus* LB group, along with the mean duration of diarrhea [55].

Intestinal dysbiosis

Inactivated *L. Acidophilus* was prescribed to 63 children (<4 years), with infective diarrhea or extraintestinal infections. The patients were divided into three groups: the first and the second included children with acute diarrhea; the third included children with extraintestinal infections. The first group received inactivated *L. acidophilus* LB, the second (Control) group did not receive a probiotic, whereas the third group received antibiotic treatment plus inactivated *L. acidophilus*. Statistically significant clinical improvement was noted in the first, inactivated *Lactobacillus* group, while in the third group, antibiotic-induced gut dysbiosis was prevented [56].

In infants with rotavirus diarrhea, IgA enzyme immunoassay antibody responses were higher in infants treated with viable *L. casei* GG, versus inactivated *L. casei* GG.

There was a significant difference at convalescence, with rotavirus-specific IgA secreting cells found in 10/12 infants receiving viable, but in only 2/13 infants receiving inactivated *L. casei* GG. The authors concluded that viable *L. casei* GG stimulated rotavirus-specific IgA antibody responses, which played an important role in the prevention of reinfections [57].

On the other hand, Briand et al. [58] reported no difference in the incidence of traveler's diarrhea, in 348 adult travelers given inactivated prophylactic *L. acidophilus* versus placebo.

Immune functions

Elderly participants in two nursing homes were randomized to receive a jelly containing 10 billion heat-killed *L. paracasei* MCC1849 cells (LP group) or placebo, for 6 weeks [59]. Three weeks after starting the supplements, all participants were given an influenza vaccine. There were no significant differences in immune parameters between the groups, including in antibody responses against the vaccination. However, in the subgroup of those ≥ 85 years of age, the antibody responses to the A/H1N1 and B antigens were improved in the LP group [59].

Heat-killed *Lactobacillus gasseri* TMC0356 or placebo was orally administered to 28 healthy subjects aged 50–70 years for 4 weeks, at a dosage of 1.0×10^9 cfu/day. The number of CD8 (+) T cells significantly increased in the TMC0356 group ($p < 0.05$). The population of CD8 (+) CD28 (+) T cells and the amount of lymphocyte transformation both significantly decreased in the placebo group ($p < 0.05$), but not in the subjects who received TMC0356, indicating enhanced immunity in elderly people [60].

In healthy adults, the influence of heat-killed *L. plantarum* (HK-LP) on immune function and quality of life (QOL) was tested. Among the measured biomarkers, the percentage change in Concanavalin A-induced proliferation ($p = 0.036$) and the Th1:Th2 ratio ($p = 0.002$) in the HK-LP group was greater. The degree of improvement in QOL was higher in the HK-LP group at week 8 ($p = 0.049$), and tended to be higher at week 12 ($p = 0.092$) [34].

Infantile colic

Vandenplas et al. [61] studied the efficacy and safety of APT198K (xyloglucan plus heat-killed *L. reuteri* SGL01 and *B. brevis* SGB01), versus a lactase dietary supplement, for infantile colic. On day 8, the mean duration of crying per episode was significantly shorter in the APT198K group (9.14 ± 5.34 vs. 13.22 ± 5.29 min; $P = 0.014$), and remained so until the end of the observations, on day 11 [61].

Dental diseases

Thirty-nine patients undergoing supportive periodontal therapy (SPT) were randomly assigned to receive a capsule containing 10 mg of heat-killed *L. plantarum* HK L-137 or a placebo, daily for 12 weeks. The bleeding on probing, and number of teeth or sites with probing depth (PD) ≥ 4 mm were significantly reduced in both groups, while there was significantly greater PD reduction ($p < 0.05$) at site(s) with PD ≥ 4 mm at baseline, in the experimental group at week 12 [62].

Incidence of dental caries was the end point in 245 seven-year-old treated children [63,64]. They were randomized to chewable tablets containing pyridoxine and heat-killed lactic acid bacteria (LAB), or placebo for 16 weeks. A consistent reduction in the incidence of dental caries was observed in the heat killed lactic acid bacteria group. After 2 years of follow up, a 42% reduction in the incidence of dental caries was confirmed [63,64].

Helicobacter pylori infection

Mehling et al. conducted an RCT in 22 *Helicobacter pylori* positive, asymptomatic adults, with nonviable *L. reuteri* DSMZ 17648 (Pylopass). A significant reduction of *H. pylori* was observed in the nonviable *Lactobacillus* group [65]. *H. pylori*-positive patients ($n = 120$) were randomly assigned in another protocol, to a 7-day triple therapy based on rabeprazole, clarithromycin, and amoxicillin (RCA: $n = 60$), or to RCA regimen supplemented with a lyophilized and inactivated culture of *L. acidophilus* (RCAL: $n = 60$). Eradication was successful in 42/60 (70%) of patients in the RCA versus 52/60 (87%) patients in the RCAL group ($p = 0.02$) [66].

Negative results

Gotteland et al. conducted a multicentre RCT in 295 asymptomatic children (6–16 y) who tested positive for *H. pylori* by (13) C-urea breath test (UBT) [67]. Participants were allocated to four groups: cranberry juice/*L. johnsonii* La1 (CB/La1), placebo juice/La1 (La1), cranberry juice/heat-killed La1 (CB), and placebo juice/heat-killed La1 (control). The *H. pylori* eradication rates were 1.5% in the control group versus 14.9%, 16.9%, and 22.9% in the La1, CB, and CB/La1 groups, respectively ($p < 0.01$) [67]. The control group, i.e., heat-killed La1 plus placebo juice, had the lowest clearance rates, suggesting that heat-killed La1 was not beneficial.

A cohort of 326 asymptomatic children (9.7 ± 2.6 y) was screened for *H. pylori* by the (13) C-UBT, and *H. pylori*-colonized children were randomized to receive the following: live La1 or ST11 (groups 1 and 3),

heat-killed La1 or ST11 (groups 2 and 4), and placebo (group 5) once a day for 4 weeks. A moderate but significant difference was detected in children receiving live La1 (-7.64 per 1000; 95% confidence interval: -14.23 to -1.03), whereas other groups showed no differences [68].

Preterm infants

Infant formula containing heat-inactivated *B. breve* C50 and *Streptococcus thermophilus* 065⁶⁹, was assessed in 58 preterm infants with gestation 30–35 weeks during their hospital stay. Fecal calprotectin was significantly lower in infants fed fermented formula for 2 weeks, and secretory IgA increased with both mother's milk and the fermented formula. The study formula was well tolerated and did not significantly modulate the bacterial colonization, but had benefits on inflammatory and immune markers [69].

In a neonatal unit, 60 infants received LP (*L. acidophilus*), 60 received KP (killed probiotic), and 30 received a placebo on day one of life. Both LP and KP reduced the risk of NEC/necrotizing enterocolitis (absolute risk reduction (ARR): 16%, 15%, respectively), and LOS/length of stay (ARR: 18%) [70].

Lactose malabsorption

Thirty-nine children were randomized to receive live probiotic and another 40 to killed probiotic. The mean hydrogen levels on BHT/breath hydrogen test decreased from baseline, after administration of live probiotic as well as killed probiotic ($p < 0.001$). The mean hydrogen levels at 120 min was similar between the live and killed probiotic groups ($p = 0.453$) [71].

Surgical conditions

In an RCT by Rayes et al. [72–74], the incidence of bacterial sepsis was compared in 172 patients following major abdominal surgery or liver transplantation. The interventions included: (a) conventional parenteral or enteral nutrition, (b) enteral nutrition with fiber and live *L. plantarum* 299, or (c) enteral nutrition with fiber and heat-inactivated lactobacilli. The incidence of postoperative infections was 31% in the conventional group compared to 4% in the lactobacillus-group, and 13% in the heat inactivated group. In the liver transplant recipients ($n = 95$), 13% of group-b patients developed infections compared to 48% of group-a, and 34% of group-c patients. The duration of antibiotic therapy was significantly shorter in both live as well as heat inactivated *Lactobacillus*-groups [72–74].

Acute pancreatitis

Keckés et al. [75] and Olah et al. [76] studied the effect of *L. plantarum* 299 in reducing acute pancreatitis related morbidity and mortality. Patients with acute pancreatitis were randomized to receive a freeze-dried preparation containing 10^9 live *L. plantarum* 299 (N = 22) or heat-inactivated *L. plantarum* 299. Supplementation was continued for a week two-times-a-day. Infected necrosis and abscesses occurred in 1/22 (4.5%) in the live *L. plantarum* versus 7/23 (30%) in the heat inactivated group ($p = 0.023$). The length of stay was not significantly different in the treatment versus the placebo group (13.7 vs. 21.4 days, respectively) [75].

Malignancies

LC9018 (heat-killed *L. casei* YIT9018) was employed in 228 patients with Stage IIIB cervical cancer undergoing radiotherapy [77,78]. It enhanced tumor regression ($p < 0.1$) by radiation. The combination therapy also prolonged survival and the relapse-free interval ($p < 0.05$), compared to radiation alone. Radiation-induced leukopenia was less severe ($p < 0.05$) in the LC9018 group [77,78].

Ocular diseases

The effects of heat-killed *L. paracasei* KW 3110 in ocular disorders and symptoms of eye fatigue, were investigated among healthy subjects [79]. The Visual Display Terminal (VDT) load-induced reduction of critical flicker frequency, tended to be milder in the *L. paracasei* KW3110 versus the placebo group, during the fourth week. Subgroup analysis showed a significant benefit of *L. paracasei* KW3110 supplementation, in subjects with high-level eye fatigue ($p = 0.020$). The in vitro study found that *L. paracasei* KW3110 suppressed blue light-induced retinal pigment epithelial cell death [79].

Sleep disturbances

Seventeen male volunteers (41–69y) with insomnia (Athens Insomnia Scale (AIS) score ≥ 6) were given placebo or heat-killed *L. brevis* SBC8803 capsules for 10 days. The sleep journals revealed an improvement in “waking” ($p = 0.047$) SBC8803 group, and there was a marginally significant effect on “drowsiness during the following day” ($p = 0.067$). Effects on the EEG delta power value ($\mu V(2)/\text{min}$) were revealed by a stratified analysis based on age, AIS, and the Beck Depression Inventory (BDI). Specifically, beneficial effects were found among subjects in their 40s who consumed the SBC8803 capsules ($p = 0.049$) and ($p = 0.045$) [80]. The authors concluded that a beneficial

effect on sleep due to consumption of heat-killed *L. brevis* SBC8803 was found in subjects with slightly challenged sleep.

Other conditions

Forty-four female students with a tendency for constipation (20.2 ± 3.3 yr) were asked to consume 30 g test pickles daily for 2 weeks. They were divided into 3 groups: viable-cell group (n = 14, viable LAB), dead-cell group (n = 15, heat sterilized LAB), and placebo groups (n = 15) [81]. Marked enhancement of NK-cell activity and improved bowel symptoms were observed in subjects consuming pickles containing dead LAB cells.

Gotteland et al. [82] evaluated the ability of probiotic LGG to protect the gastrointestinal mucosa against indomethacin-induced alterations of permeability. They carried out four gastrointestinal permeability tests in 16 healthy volunteers: (i) basal; (ii) after indomethacin; (iii) after 5 days of living LGG ingestion before indomethacin administration; (iv) after 5 days of heat-killed LGG ingestion before indomethacin administration. Heat-killed LGG did not modify the indomethacin-induced increase of gastrointestinal permeability, while live bacteria significantly reduced the alteration of gastric but not indomethacin-induced intestinal permeability [82].

Higashikawa et al. [83] reported significant reduction in body mass index, in 62 obese adults treated with inactivated or live *P. pentosaceus* LP 28, or placebo [$-0.06 (\pm 0.12)$ versus $0.07 (\pm 0.1)$ versus $0.4 (\pm 0.15)$, $p < 0.05$]. Alberda et al. [84] reported no difference in multi-organ dysfunction scale, in 28 adult ICU patients given prophylactic inactivated or living VSL#3 compared to placebo.

Systematic review

Zorzela et al. have reported a systematic review evaluating the efficacy and safety of modified (heat-killed or sonicated) probiotics [85]. Modified probiotics were not significantly more or less effective than the living probiotic in 86% of the preventive trials, and 69% of the treatment trials. Modified probiotic strains were significantly more effective in 15% of the treatment trials [85].

Ongoing trials

Hammerman et al. (NCT02796703) from Israel plan to recruit 450 preterm neonates of very low birth weight (< 1500 gm), to receive daily supplementation with a heat-inactivated probiotic or placebo, starting with the initiation of feeds [9]. Supplements will be continued until the infant tolerates enteral feeds of 100 mL/kg/day, or reaches

35 weeks postconceptional age. Biotikid, a probiotic mixture (*L. rhamnosus*, *L. casei*, *B. infantis*, *B. bifidum*, *B. longum*, *L. acidophilus*, and *S. thermophilus*) will be heated to 100°C for 10 min. The hypothesis is that prophylaxis with heat-inactivated probiotics will reduce the incidence of NEC in preterm infants. The investigators will also measure intestinal barrier integrity, as reflected by levels of I-FABP in urine and fecal Calprotectin. It is not clear if the trial has begun recruiting participants as yet.

Jape et al. [86] from Western Australia are conducting a pilot RCT (n = 70, ACTRN12618000489291p) comparing the efficacy and safety of paraprotiotics in preterm infants with gestation <32 weeks. The intervention arm will receive a heat-inactivated mixture of 3 bifidobacteria strains (*B. breve* M-16V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536). The control arm will receive mixture of the three live bifidobacteria. The supplement will be commenced with enteral feeds and continued till at least 37 weeks corrected gestational age. Primary outcomes include safety and tolerance of the heat-inactivated preparation and fecal calprotectin levels. Secondary outcomes include fecal 16srRNA analysis, fecal cytokine, and short chain fatty acid levels, as well as the assessment of gut barrier integrity and intestinal transit time. Clinical outcomes such as NEC, all-cause mortality, time to full enteral feeds, duration of parenteral nutrition, and hospital stay will be secondary outcomes of interest.

The future of paraprotiotics

Adequately powered RCTs evaluating the efficacy and safety of paraprotiotics are essential to explore this new frontier [87]. The potential benefits of paraprotiotics with regards to probiotic sepsis, antibiotic resistance, better shelf life, and possibly lesser costs, are important, but consideration of their safety must be taken into consideration. Heat-killed probiotics are able to act as biological response modifiers. While moderate stimulation of proinflammatory cytokines could be beneficial in maintaining a good immunological balance and increasing resistance to infections, excessive stimulation is undesirable. Experts have warned that dead probiotic cells have the potential to cause harmful effects, and hence, careful screening and selection of strains as well as safety monitoring is highly essential [24].

The RCTs in this field should evaluate paraprotiotics against a placebo, in conditions where probiotic therapy is not a standard of care. Assuring follow up of participants in such trials is important to assess the long term safety and efficacy of paraprotiotics. Paraprotiotics could also be studied in conditions where probiotics are contraindicated. Last but not least, assessing the cost-benefit ratio of different methods of inactivating probiotics is essential.

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RESEARCH ARTICLE

Benefits of *Bifidobacterium breve* M-16V Supplementation in Preterm Neonates - A Retrospective Cohort Study

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Abstract

Background

Systematic reviews of randomised controlled trials report that probiotics reduce the risk of necrotising enterocolitis (NEC) in preterm neonates.

Aim

To determine whether routine probiotic supplementation (RPS) to preterm neonates would reduce the incidence of NEC.

Methods

The incidence of NEC \geq Stage II and all-cause mortality was compared for an equal period of 24 months 'before' (Epoch 1) and 'after' (Epoch 2) RPS with *Bifidobacterium breve* M-16V in neonates <34 weeks. Multivariate logistic regression analysis was conducted to adjust for relevant confounders.

Results

A total of 1755 neonates (Epoch I vs. II: 835 vs. 920) with comparable gestation and birth weights were admitted. There was a significant reduction in NEC \geq Stage II: 3% vs. 1%, adjusted odds ratio (aOR) = 0.43 (95%CI: 0.21–0.87); 'NEC \geq Stage II or all-cause mortality': 9% vs. 5%, aOR = 0.53 (95%CI: 0.32–0.88); but not all-cause mortality alone: 7% vs. 4%, aOR = 0.58 (95% CI: 0.31–1.06) in Epoch II. The benefits in neonates <28 weeks did not reach statistical significance: NEC \geq Stage II: 6% vs. 3%, aOR 0.51 (95%CI: 0.20–1.27), 'NEC \geq Stage II or all-cause mortality', 21% vs. 14%, aOR = 0.59 (95%CI: 0.29–1.18); all-cause mortality: 17% vs. 11%, aOR = 0.63 (95%CI: 0.28–1.41). There was no probiotic sepsis.

Conclusion

RPS with *Bifidobacterium breve* M-16V was associated with decreased NEC \geq Stage II and 'NEC \geq Stage II or all-cause mortality' in neonates <34 weeks. Large sample size is required to assess the potential benefits of RPS in neonates <28 weeks.

Introduction

Necrotising enterocolitis (NEC) continues to have significant mortality and morbidity including long-term neurodevelopmental impairment in very preterm neonates with gestation <32 weeks [1,2]. The outcomes are worse if surgical intervention is required, especially in extremely preterm neonates with gestation <28 weeks [3]. Despite decades of research, the pathogenesis of NEC is still not clear [4–6]. Excessive intestinal inflammatory response from an immature innate immune system and toll like receptors (TLR4) are currently considered to play an important role in its pathogenesis [7–10]. Having had no success in developing effective strategies for prevention of preterm birth, there have been limited options to reduce the risk of NEC. These included antenatal glucocorticoids, postnatal early and preferential breastmilk feeding, and standardised feeding protocols to minimise variations in feeding practice that have been epidemiologically linked to NEC [11–14].

Probiotics are live microorganisms that when administered in adequate amounts, confer benefits to the host [15]. Systematic reviews of randomised controlled trials (RCT) have shown that probiotics reduce the risk of NEC (\geq Stage II) and all-cause mortality significantly and facilitate enteral feeding in preterm very low birth weight (VLBW) neonates [16–21]. None of the trials reported adverse effects such as probiotic sepsis. There is broad consensus that probiotic effects are strain-specific [22–24]. Therefore despite the results from various meta analyses there has been a reluctance to adopt this intervention considering the heterogeneity of probiotic strains and protocols, population characteristics, type of feeds (milk/formula) and the trial settings [25–30]. However experts point out that clinical data to support strain-specific effects of probiotics are limited and the consistently decreased risk of NEC in RCTs using variable probiotic regimens suggests protection by different strains by shared beneficial pathways [31–33]. The number of reports on routine probiotic supplementation (RPS) indicates that clinical practice is changing in favour of probiotics in preterm neonates [34].

Ours is one of the largest neonatal intensive care units in the southern hemisphere (30 level III and 70 level II beds) that annually admits ~ 500 neonates with gestation <34 weeks including 100 to 120 with gestation <28 weeks. Considering the evidence in totality we decided to introduce RPS with *Bifidobacterium breve* M-16V (*B. breve* M-16V) for preterm neonates <34 weeks' gestation in our unit. Both, the evidence supporting the use of this product in preterm neonates, and the results of our independent assessment of its quality including effect on fecal bifidobacteria, have been reported earlier [35].

Aim

We aimed to assess if RPS with *B. breve* M-16V was associated with reduced incidence of NEC \geq Stage II in preterm neonates born <34 weeks' gestation [36].

Hypothesis

We hypothesised that introduction of RPS would significantly reduce NEC \geq Stage II [36].

Materials and Methods

This was a retrospective cohort study comparing data from before (Epoch I: December 2008 to November 2010, $n = 835$) versus after (Epoch 2: June 2012 to May 2014, $n = 920$) introducing RPS with *B. breve* M-16V (Morinaga Milk Industry Co., Ltd, Japan). The data from the *B. breve* M-16V trial period between the two epochs was excluded [35].

Ethics considerations

The study was approved by the Research Governance Committee, Women and Newborn Health Service (WNHS), Western Australia, based at King Edward Memorial Hospital for Women. Approval was also obtained from the Therapeutic Goods Administration (TGA, Canberra), under the Authorised Prescriber Pathway [37]. Written informed parental consent was obtained in the format approved by the WNHS Research Governance Committee and TGA, Canberra, Australia.

Eligibility criteria

All preterm neonates born <34 weeks' gestation were eligible for RPS. Those with major congenital malformations, chromosomal aberrations, and contraindications for enteral feeding, and those where no informed consent was available were excluded.

Primary outcome

Incidence of NEC \geq Stage II [36].

Secondary outcomes

All-cause mortality, 'NEC \geq Stage II or all-cause mortality', blood culture positive late onset sepsis (LOS) after 72 hours of life, and postnatal age at full feeds (150 ml/kg/day).

All outcomes were monitored till discharge or death during initial hospitalisation.

The diagnosis of pneumatosis intestinalis by the attending neonatologist was verified independently by the radiologist on call. In case of disagreement, consensus was reached by group discussion between the neonatal and radiology team during the weekly grand rounds and subsequently the final diagnosis was used for coding in the database.

Probiotic protocol

When ready for enteral feeds, neonates were supplemented with the freshly reconstituted contents of the probiotic sachets every day, and continued until the corrected age 37 weeks [35]. Breast milk (first choice) or sterile water for injection was used for reconstitution of the dry powder in the 1gram sachets. The dose was 3×10^9 (3 billion) cfu/day (1.5 ml of the reconstituted solution), given as a single dose via the orogastric feeding tube. For neonates <28 weeks the daily dose was 1.5×10^9 cfu/day until reaching feeds of 50 ml/kg/day. It was then increased to 3×10^9 cfu/day. The probiotic supplementation was stopped when feeds were stopped by the attending neonatologist for indications such as sepsis and NEC. Safety was assessed by monitoring for blood culture positive sepsis by *B. breve* M-16V. The automated blood culture system used by our laboratory detects *B. breve* M-16 V within the routine 5 day incubation period. We used the BACTEC™ PEDS PLUS™/F Medium blood culture vials with incubation monitored in the Bactec 9120 system [38]. Adherence to probiotic protocol was ensured by checking the medication charts of all eligible neonates.

Sample size estimation

Since our baseline incidence of NEC \geq stage II was 3–4%, a total sample of 1800, or \sim 2 years of data from before and after introducing RPS, based on annual admission rates of \sim 500, was considered to be adequate to achieve 80% power to observe an effect size of 60% with an alpha error of 0.05. The desired effect size was based on the previous systematic reviews [16–21].

Study infants were identified by interrogating our Neonatal Database. Clinical details of all admissions to our unit are entered into this database by trained, dedicated staff. The database is used by the Australia and New Zealand Neonatal Network (ANZNN), for publishing annual reports [39]. The ANZNN conducts regular audits to ensure accuracy of the recorded data.

Statistical considerations

Descriptive data were summarised using medians, interquartile ranges (IQR) and ranges (R) for continuous outcomes, and frequency distributions for categorical outcomes. Univariate comparisons for continuous data were made using Mann Whitney tests and for categorical data using Chi-square or by using exact inference. The duration of respiratory support measures such as ventilation, continuous positive airway pressure (CPAP) and oxygen was summarised using Kaplan-Meier survival estimates and compared between epochs using the log rank test. Neonatal outcomes of NEC, mortality, LOS and age at full feeds were analysed using multiple logistic regression with adjustment for gestational age $<$ 28 weeks and intra-uterine growth restriction (IUGR: Birth weight $<$ 10th centile for gestation). Characteristics that differed between epochs and other parameters considered to influence neonatal outcomes (e.g. maternal antenatal antibiotics) were also assessed during modelling. The effects of epochs were summarised as unadjusted (OR) and adjusted odds ratios (aOR) with 95% confidence intervals (CI). The analysis was conducted on all neonates $<$ 34 weeks' gestation, and in a subset of neonates $<$ 28 weeks who are at a higher risk for NEC. Adjustment for multiple testing was not utilized for the subgroup analysis as insufficient statistical power was considered likely. All tests were two-sided, and a p-value $<$ 0.05 was considered statistically significant. The analysis was performed using IBM SPSS 20.0 for Windows (IBM, Armonk, NY) and StatXact 8.0 (Cytel Inc, MA).

Reporting

The STROBE checklist for reporting observational studies was used [40].

Results

A total of 1755 preterm neonates born $<$ 34 weeks (Epoch I vs. II: 835 vs. 920) were admitted to the nursery over the two epochs (Fig 1). A total of 57/835 (6.8%) and 42/920 (4.6%) infants from Epoch 1 and 2 respectively, were transferred to another hospital for ongoing care. Complete information from all infants (discharged home or transferred to another hospital) was available with no loss to follow up. Their median gestation and birth weight were comparable (Table 1). The frequency of maternal antenatal antibiotic (Erythromycin or Benzyl penicillin) use and gestation at birth $<$ 28 weeks was lower in Epoch II (Table 1). Most mothers received antenatal steroids; Epoch I: 717 (93%) vs. Epoch II: 791 (91%); 197 (26%) vs. 193 (22%) single dose, 313 (41%) vs. 320 (37%) complete course, 207 (27%) vs. 278 (32%) $>$ 7 days from last dose to delivery ($p = 0.021$). In Epoch II, there was an increased use of CPAP, oxygen support and oxygen at 36 weeks, and reduced incidence of intraventricular hemorrhage (IVH) (Table 1) [41]. A statistically non-significant increase in the incidence of retinopathy of prematurity (ROP) was noted in Epoch II (Table 1) [42].

Epoch I: December 2008 to November 2010 (*Before RPS**)

- All admissions to Level III unit: 1663
- ↓
- Total number of admissions <34 weeks: 871
- ↓
- Infants <34 weeks with congenital malformations/surgical conditions: 36
- ↓
- Infants <34 weeks who did not receive probiotic: 835

Epoch 2: June 2012 to May 2014 (*After RPS*)

- All admissions to Level III unit: 2034
- ↓
- Total number of admissions <34 weeks: 938
- ↓
- Infants <34 weeks with congenital malformations/surgical conditions: 18
- ↓
- Infants <34 weeks who received RPS: 920

*RPS: Routine probiotic supplementation

Fig 1. Patient flow diagram.

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Outcomes for neonates <34 weeks

NEC \geq Stage II was significantly reduced in Epoch II after adjustment for gestation <28 weeks, IUGR, maternal antenatal antibiotics, CPAP and oxygen support (Table 2). In Epochs I and II respectively, there were 25 (3%) NEC \geq Stage II (10 Stage III including 3 surgical cases) and 12 (1%) NEC \geq Stage II (5 Stage III with no surgical cases) ($p < 0.001$), of which 17 (68%) and 11 (92%) survived to discharge ($p = 0.220$).

The composite outcome of 'NEC \geq Stage II or all-cause mortality' was significantly reduced in Epoch II, but not all-cause mortality as an individual outcome (Table 2). There were 56 and 37 deaths in Epochs I and II, of which 25 (45%) and 15 (38%) were within 72 hours of birth respectively.

Postnatal age at full feeds and incidence of LOS were reduced in Epoch II (Table 2).

The incidence of patent ductus arteriosus (PDA: Left atrium to aortic root ratio > 1.4 or ductal diameter > 1.5 mm with a left to right shunt) requiring treatment was not significantly different between epochs (Table 1).

Table 1. Pregnancy and neonatal characteristics.

Characteristics	Epoch I N = 835 N (%)	Epoch II N = 920 N (%)	p-value
Maternal			
PIH	157 (19%)	186 (20%)	0.455
APH	233 (28%)	228 (25%)	0.138
Chorioamnionitis	80 (10%)	109 (12%)	0.126
Antibiotics	478 (57%)	371 (40%)	<0.001
Glucocorticoids	717 (93%)	791 (91%)	0.099
PPROM	278 (33%)	273 (30%)	0.103
Inborn	790 (95%)	864 (94%)	0.531
Gestation (w)*	30 (27–32;23–33)	30 (28–32;23–33)	0.101
Gestation <28 w	250 (30%)	220 (24%)	0.004
Mode of delivery			
Vaginal	349 (42%)	366 (40%)	0.402
Caesarean section	486 (58%)	553 (60%)	
Neonatal			
Birth weight (g)*	1340 (925–1670;293–2980)	1340 (1000–1696;330–2560)	0.145
Male gender	458 (55%)	488 (53%)	0.448
Apgar <7 at 5 minutes	157 (19%)	150 (16%)	0.169
IUGR	83 (10%)	103 (11%)	0.393
Respiratory support			
Ventilation	517 (62%)	538 (59%)	0.142
CPAP	687 (82%)	800 (87%)	0.006
Oxygen	551 (94%)	705 (98%)	<0.001
Duration (h) [#]			
Ventilation	27 (11–229)	19 (10–122)	0.001
CPAP	128 (28–815)	168 (36–861)	0.444
Oxygen	56 (5–929)	58 (5–633)	0.349
Oxygen 36 weeks	85 (10%)	148 (16%)	<0.001
PDA			
Treated	157 (60%)	180 (66%)	0.108
IVH Grade III-IV	43 (5%)	25 (3%)	0.009
ROP Stage III-IV	14 (2%)	25 (3%)	0.095
Early onset sepsis	15 (2%)	13 (1.5%)	0.522
Received formula	34 (4%)	36 (4%)	0.865
Length of nursery stay (d) [#]	36 (21–64)	37 (19–63)	0.785
Discharge weight* (g)	2093 (1831–2439;545–4465)	2280 (1905–2784;606–5580)	<0.001

*Median (IQR, range)

[#]Median, IQR, Kaplan-Meier survival estimates PIH: Pregnancy induced hypertension, APH: Antepartum hemorrhage, PPRM: Preterm pre-labour rupture of membranes, IUGR: Intrauterine growth restriction, CPAP: Continuous positive airway pressure, PDA: Patent ductus arteriosus, IVH: Intraventricular hemorrhage, ROP: Retinopathy of prematurity

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Outcomes for neonates <28 weeks

There were 21% and 14% neonates <28 weeks gestation with 'NEC \geq Stage II or all-cause mortality' in Epochs I and II respectively (Table 3). On Univariate analysis, there was a significant reduction in 'NEC \geq Stage II or all-cause mortality' in Epoch II (OR 0.60, CI 0.37–0.98, $p = 0.042$), but the reduction was no longer significant after adjustment (Table 3). The

Table 2. Outcomes for neonates <34 weeks.

<34 weeks	Epoch I N = 835	Epoch II N = 920	Unadjusted OR (CI)	Adjusted aOR (CI)	p-value
NEC ¹	25 (3%)	12 (1%)	0.43 (0.21–0.86)	0.43 (0.21–0.87)	0.019
Mortality ²	56 (7%)	37 (4%)	0.58 (0.38–0.89)	0.58 (0.31–1.06)	0.078
NEC/Mortality ²	73 (9%)	48 (5%)	0.57 (0.39–0.84)	0.53 (0.32–0.88)	0.014
Late onset sepsis ³	120 (14%)	82 (9%)	0.58 (0.43–0.79)	0.57 (0.42–0.78)	0.001
Age at full feeds ⁴ (d)	10 (7–17)	7 (5–12)	HR: 1.61 (1.46–1.78)	HR: 1.79 (1.62–1.98)	<0.001

¹Adjusted for gestation<28w, IUGR, CPAP, oxygen support

²Adjusted for gestation<28w, IUGR, CPAP, oxygen support, maternal antenatal antibiotics, early onset sepsis, IVH

³Adjusted for gestation<28w, CPAP, oxygen support, PDA

⁴Data represents median (IQR) Kaplan-Meier estimates, hazard ratios (HR) and 95% confidence intervals (CI) from Cox Hazard regression modelling, adjusted for gestation<28w, IUGR, oxygen support, IVH, PDA

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individual outcomes of NEC \geq Stage II and all-cause mortality did not significantly differ between epochs. Postnatal age at full feeds and LOS were reduced in Epoch II (Table 3).

Safety

There were no adverse effects including probiotic sepsis and abdominal distension, vomiting, and diarrhea needing cessation of the supplementation.

Discussion

Our results indicate that RPS with *B. breve* M-16V was associated with lower incidence of NEC \geq Stage II in preterm VLBW neonates born <34 weeks. The incidence of NEC \geq Stage II was lower but not statistically significant in those born <28 weeks, probably because of the small numbers. Using the baseline rate of NEC (6%) in our study, a total sample of 1000, or the equivalent of about 5 years data before and after introducing RPS would be needed (100–125 admissions/year), to detect the desired effect size in neonates <28 weeks.

The benefit of RPS occurred in presence of high rates of breastmilk feeding in our unit that is supported by a human milk bank since 2006, and the low baseline incidence of \geq Stage II NEC that has remained stable over years. Since 2004 we have adopted a standardised feeding protocol for preterm neonates. To our knowledge no significant changes in clinical practices

Table 3. Outcomes for neonates <28 weeks.

<28 weeks	Epoch I N = 250	Epoch II N = 220	Unadjusted OR (CI)	Adjusted aOR (CI)	p-value
NEC ¹	16 (6%)	7 (3%)	0.48 (0.19–1.19)	0.51 (0.20–1.27)	0.148
Mortality ²	42 (17%)	24 (11%)	0.61 (0.35–1.04)	0.63 (0.28–1.41)	0.258
NEC/Mortality ²	52 (21%)	30 (14%)	0.60 (0.37–0.98)	0.59 (0.29–1.18)	0.135
Late onset sepsis ³	79 (32%)	44 (20%)	0.54 (0.35–0.83)	0.53 (0.35–0.82)	0.004
Age at full feeds ⁴ (d)	20 (15–27)	13 (10–17)	HR 2.23(1.81–2.73)	HR 2.44 (1.97–3.01)	<0.001

¹Adjusted for CPAP

²Adjusted for EOS, CPAP, IVH

³Adjusted for GA, CPAP

⁴Data represents median (IQR) Kaplan-Meier estimates, hazard ratios (HR) and 95% confidence intervals (CI) from Cox Hazard regression modelling, adjusted for GA, IUGR, CPAP, oxygen support

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have taken place over the pre and post RPS study period. The risk of bias in our results is minimised by multiple logistic regression controlling for confounders such as gestation and antenatal maternal antibiotics. Our results are supported by **Satoh et al** who also used *B. breve* M-16V for RPS in preterm neonates [43]. Results of RPS are important for assessing benefits of probiotics in real life situation as RCTs may underestimate the effects of probiotics due to cross colonisation of the control group neonates by as much as 44% [44–46].

The results of the multicentre RCT from UK (**PIPS**) are in contrast with benefits of RPS with *B. breve* M-16V in preterm infants [47]. This adequately powered ($n = 1310$) showed no improvement in any of the primary outcomes (NEC or LOS or mortality) in preterm infants <31 weeks gestation supplemented with *B. breve* BBG-001 or placebo [47]. There was no probiotic sepsis, further supporting the safety of probiotics in preterm infants. The reasons for the negative results include the 49% cross-colonisation of the placebo arm infants and an inadequate dose towards the end of the shelf life of the product due to loss of viable bacteria. We have discussed these issues in detail elsewhere.

Comparing our results with those from comparable units is important. **Janvier et al** have reported their cohort study in very preterm neonates [48]. All neonates <32 weeks' gestation received RPS with 0.5 g of a mixture of four bifidobacteria (*B. breve*, *bifidum*, *infantis*, and *longum*) and *Lactobacillus rhamnosus* HA-111 (2×10^9 cfu/day), starting with the first feed, and continued until reaching 34 weeks. Data from the first 17 months of RPS ($n = 294$) were compared with those from previous 17 months without RPS ($n = 317$). RPS was associated with a reduction in NEC \geq Stage II (from 9.8% to 5.4%, $p < .02$), a non-significant decrease in death (9.8% to 6.8%), and a significant reduction in the combined outcome of 'death or NEC' (from 17% to 10.5%, $p < .05$). The improvements [OR (95% CI)] remained significant after adjustment for gestation, IUGR, and sex [NEC: 0.51 (0.26–0.98); Death or NEC: 0.56 (0.33–0.93)]. RPS had no effect on LOS. Neonates with birth weight <1001 grams showed similar percentage reductions in NEC [Pre-RPS: 18 (17%) vs. RPS: 10 (10%)] and the combined outcome of death and NEC [Pre-RPS: 38 (35%) vs. RPS: 22 (22%)] but the numbers (Pre-RPS:109; RPS: 96) were small to reach statistical significance [48]. **Repa et al** have reported that probiotics may not overcome the adverse effects of formula feeding and that their benefits occur in breast milk fed preterm neonates at high risk of NEC [49]. VLBW neonates receiving RPS with a mixture of lactobacilli and bifidobacteria (2010–2012) were prospectively followed. Neonates from 2008 to 2009 without RPS served as controls. RPS had no significant impact on NEC [Controls: 24/233 (10.3%); RPS: 16/230 (7%); $p = 0.2$]. However, NEC was significantly reduced in RPS group neonates fed any breast milk [20/179 (11.2%) vs. 10/183 (5.5%); $p = 0.027$]. RPS was ineffective in those on exclusive formula feeding [4/54 (7.4%) vs. 6/44 (13.6%); $p = 0.345$]. Occurrence of severe NEC (Stage IIIb), time to full feeds, and gastric residuals were similar [49]. **Hartel et al** have reported a cohort of VLBW neonates stratified to prophylactic use of *Lactobacillus acidophilus/B. infantis* [50]. Within the observational period (1/9/2010–31/12/2012, $n = 5351$) participating centers were categorized into 3 groups based on their choice of probiotic use: (1) no prophylactic use; (2 a/b) changing from being nonuser to user during observational period; and (3) use before start of observation. In a multivariable logistic regression analysis, probiotics were protective for NEC surgery (OR: 0.58, 95% CI: 0.37–0.91; $p = .017$), any abdominal surgery (OR: 0.7, 95% CI: 0.51–0.95; $p = .02$), and the combined outcome 'abdominal surgery or death' (OR: 0.43; 95% CI: 0.33–0.56; $p < .001$). These findings are important considering the health burden of stage II NEC is primarily related to its progression to stage III [50]. Selection of 'any abdominal surgery' as the outcome minimises the risk of bias due to misclassification of spontaneous intestinal perforation as NEC. **Olsen et al** have recently reported a systematic review of observational studies reporting on RPS in preterm neonates [34]. Meta-analysis of data from 12 studies (Prophylactic probiotics: 5,144 vs. Controls: 5,656)

showed a significantly decreased incidence of NEC (RR: 0.55, 95% CI: 0.39–0.78; $p = 0.0006$) and mortality (RR: 0.72, 95% CI: 0.61–0.85; $p < 0.0001$). Late onset sepsis did not differ significantly between the two groups (RR: 0.86, 95% CI: 0.74–1.00; $p = 0.05$). The effect sizes were similar to findings in meta-analyses of RCTs. There were no adverse events including probiotic sepsis [34].

The **strengths** of our study include its large sample size, use of multivariate regression analysis, benefits of RPS in a setting with low baseline incidence of NEC and donor milk bank, and use of STROBE guidelines for reporting. The **limitation** is its retrospective design which makes it difficult to control for all confounders.

In summary our results indicate that RPS with *B. breve* M-16V was associated with significant reduction in \geq Stage II NEC in preterm VLBW neonates. Caution is warranted in generalising these results considering the variations in patient demographics and clinical practices across units. However, the report by Olsen et al is reassuring in this context [34]. The importance of probiotic quality control cannot be overemphasised considering the report of fatal Mucormycosis in a preterm neonate following the use of a contaminated probiotic product [51].

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Author Contributions

Conceived and designed the experiments: SKP. Performed the experiments: SKP SCR KNS ADK. Analyzed the data: DAD EAN. Contributed reagents/materials/analysis tools: SKP SCR KNS ADK DAD EAN. Wrote the paper: SKP.

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Probiotic Supplementation and Late-Onset Sepsis in Preterm Infants: A Meta-analysis

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abstract

CONTEXT: Late-onset sepsis (LOS) is a major cause of mortality and morbidity in preterm infants. Despite various preventive measures, its incidence continues to remain high, hence the urgent need for additional approaches. One such potential strategy is supplementation with probiotics. The updated Cochrane Review (2014) did not find benefits of probiotics in reducing the risk of LOS in preterm infants (19 studies, $N = 5338$). Currently there are >30 randomized controlled trials (RCTs) of probiotics in preterm infants that have reported on LOS.

OBJECTIVES: To conduct a systematic review including all relevant RCTs.

DATA SOURCES: PubMed, Embase, Cochrane Central Register of Controlled Trials, Cumulative Index of Nursing and Allied Health Literature, and E-abstracts from the Pediatric Academic Society meetings and other pediatric and neonatal conference proceedings were searched in June and August 2015.

STUDY SELECTION: RCTs comparing probiotics versus placebo/no probiotic were included.

DATA EXTRACTION: Relevant data were extracted independently by 3 reviewers.

RESULTS: Pooled results from 37 RCTs ($N = 9416$) using fixed effects model meta analysis showed that probiotics significantly decreased the risk of LOS (675/4852 [13.9%] vs 744/4564 [16.3%]; relative risk, 0.86; 95% confidence interval, 0.78–0.94; $P = .0007$; $I^2 = 35\%$; number needed to treat, 44). The results were significant even after excluding studies with high risk of bias.

CONCLUSIONS: Probiotic supplementation reduces the risk of LOS in preterm infants.

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Dr Rao conceptualized and designed the study, performed an independent literature search, selected studies for inclusion, extracted and interpreted data, assessed risk of bias of included studies, handled the meta-analysis software, and wrote the first and final drafts of the manuscript; Dr Athalye-Jape performed an independent literature search, selected studies for inclusion, contacted authors for additional information where necessary, extracted and interpreted the data, checked the data entered by S.C.R. on the meta-analysis software, assessed the risk of bias of included studies, and helped with the first and the final draft of the manuscript; Dr Deshpande performed an independent literature search, selected studies for inclusion, verified the extracted data, assessed risk of bias, interpreted data, and oversaw translation of manuscripts in the Chinese language; Dr Simmer supervised the project, interpreted the data, and supervised the first and final versions of the manuscript; Dr Patole supervised the project, acted as referee author in case of differences of opinion between the first 3 authors, interpreted the data, and supervised the first and final versions of the manuscript; and all authors approved the final manuscript as submitted.

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Late-onset sepsis (LOS) is a major cause of mortality and morbidity, including adverse long-term neurodevelopmental outcomes in preterm infants.¹⁻⁸ The burden of LOS is significant in developed^{2,8,9} and developing nations of the world.¹⁰⁻¹³ The incidence of LOS varies inversely with gestational age and birth weight.⁴ The important risk factors for LOS in preterm infants are intravascular catheters, delayed commencement of enteral feeds, prolonged use of parenteral nutrition, prolonged ventilation, and surgery.¹ Although the predominant organism causing LOS is coagulase-negative staphylococci, other organisms such as *Staphylococcus aureus*, Gram-negative bacteria, and fungi also are important.^{3,9,14-17} The cost-effective strategies for preventing LOS include antimicrobial stewardship, limited steroid use, early enteral feeding, limited use of invasive devices, standardization of catheter care practices, and meticulous hand hygiene.^{18,19} Despite these preventive measures, the incidence of LOS remains high in preterm infants.^{2,3,20} Therefore, additional approaches to reduce LOS are needed urgently.^{8,21} One such potential strategy that might reduce LOS is supplementation with probiotics.²²

Probiotics are defined as live microorganisms that when administered in adequate amounts may confer health benefits on people with specific illnesses.²³ Animal research and in vitro studies²⁴ have shown that probiotics improve gut barrier function,^{25,26} inhibit gut colonization with pathogenic bacteria,²⁷ improve colonization with healthy commensals,^{28,29} protect from enteropathogenic infection through production of acetate,³⁰ enhance innate immunity,³¹ and increase maturation of the enteric nervous system,³² all of which have the potential to decrease the risk of LOS in preterm infants. However, the recent Cochrane Review³³

(2014) concluded that probiotic supplementation did not result in statistically significant reduction of LOS in preterm infants (relative risk [RR] 0.91; 95% confidence interval [CI], 0.80–1.03; 19 studies, $N = 5338$). Another meta-analysis³⁴ (2015) also reported similar results on LOS (RR, 0.919; 95% CI, 0.823–1.027; $P = .137$; 17 randomized controlled trials [RCTs], $N = 5215$).

The meta-analyses done so far have included a maximum of 19 RCTs, whereas currently there are >30 RCTs of probiotic supplementation that have reported on LOS. Therefore, we decided to conduct a systematic review and meta-analysis to evaluate the role of probiotic supplementation in reducing the risk of LOS in preterm infants.

METHODS

Guidelines from the Cochrane Neonatal Review Group (<http://neonatal.cochrane.org/resources-review-authors>),³⁵ Centre for Reviews and Dissemination (<http://www.york.ac.uk/crd/guidance/>), and the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement³⁶ were followed for undertaking and reporting this systematic review and meta-analysis. Ethics approval was not required.

Eligibility Criteria

Types of Studies

Only RCTs were included in the review. Observational studies, narrative reviews, systematic reviews, case reports, letters, editorials, and commentaries were excluded but read to identify potential additional studies.

Types of Participants

Preterm neonates born at a gestational age <37 weeks, low birth weight (<2500 g), or both (same criteria as the Cochrane Review, 2014).

Intervention and Comparison

Enteral administration of probiotic supplement versus placebo or control.

Outcomes

LOS, defined as the presence of positive blood or cerebrospinal fluid culture on a sample collected 48 to 72 hours after birth^{2,8,20}

Search Strategy

The databases PubMed (www.ncbi.nlm.nih.gov, 1966–2015), Embase (Excerpta Medica dataBASE) via Ovid (<http://ovidsp.tx.ovid.com>, 1980–2015), Cochrane Central Register of Controlled Trials (www.thecochranelibrary.com, through August 2015), Cumulative Index of Nursing and Allied Health Literature via Ovid (<http://ovidsp.tx.ovid.com>, 1980–August 2015), and E-abstracts from the Pediatric Academic Society meetings (www.abstracts2view.com/pasall, 2000–August 2015) were searched in August 2015. A similar search was also done in June 2015. Abstracts of other conference proceedings such as Perinatal Society of Australia and New Zealand, European Academy of Pediatric Societies, and the British Maternal and Fetal Medicine Society were searched in Embase. Google Scholar was searched for articles that might not have been cited in the standard medical databases. Gray literature was searched through the national technical information services (<http://www.ntis.gov/>), Open Grey (<http://www.opengrey.eu/>), and Trove (<http://trove.nla.gov.au/>). The reference lists of eligible studies and review articles were searched to identify additional studies. Reviewers S.C.R., G.K.A.J., and G.C.D. conducted the literature search independently. No language restriction was applied. The non-English studies were identified by reading through the recently published systematic reviews of probiotic supplementation on the incidence of necrotizing

enterocolitis (NEC).^{37,38} Search of Embase also identified 1 non-English study. Full texts of all the non-English studies were obtained via the library of University of Sydney. A research officer from the University of Sydney translated the articles. Attempts were made to contact the authors for additional data and clarification of methods, but there was no response. Only published data were used for those studies, where available.

We searched PubMed for the following terms: (((“Infant, Newborn”[Mesh]) OR (“Infant, Extremely Premature”[Mesh] OR “Infant, Premature”[Mesh])) OR (“Infant, Low Birth Weight”[Mesh] OR “Infant, Extremely Low Birth Weight”[Mesh] OR “Infant, Very Low Birth Weight”[Mesh])) AND “Probiotics”[Majr]. We also searched for ((“Infant, Extremely Premature”[Mesh] OR “Infant, Extremely Low Birth Weight”[Mesh] OR “Infant, Very Low Birth Weight”[Mesh] OR “Infant, Small for Gestational Age”[Mesh] OR “Infant, Premature, Diseases”[Mesh] OR “Infant, Premature”[Mesh] OR “Infant, Newborn, Diseases”[Mesh] OR “Infant, Newborn”[Mesh] OR “Infant, Low Birth Weight”[Mesh])) AND (“Bifidobacterium”[Mesh] OR (“Lactobacillus”[Mesh]) OR “Saccharomyces”[Mesh])). The other databases were searched for similar terms.

Study Selection

Abstracts of the citations obtained from the initial broad search were read independently by 3 reviewers (S.C.R., G.K.A.J., and G.C.D.) to identify potentially eligible studies. Full-text articles of these studies were obtained and assessed for eligibility by 3 reviewers independently (S.C.R., G.K.A.J., and G.C.D.), under the predefined eligibility criteria. Differences in opinion were resolved by group discussion among all reviewers to reach consensus. Care was taken to ensure that multiple

publications of the same study were identified and excluded to avoid duplication of the data.

Data Extraction

Reviewers S.C.R., G.K.A.J., and G.C.D. extracted the data independently by using a data collection form designed for this review. The number of patients with LOS and the number of patients analyzed in each treatment group of each trial were entered into the form. Information about the study design and outcomes was verified by all reviewers. Discrepancies during the data extraction process were resolved by discussion and consensus among all reviewers. We contacted authors for additional information and clarifications when details on LOS were not available in published manuscripts. Such studies were excluded if there was no response from the authors.

Assessment of Risk of Bias

We assessed risk of bias (ROB) by using the Cochrane “Risk of Bias Assessment Tool.”³⁵ Authors S.C.R. and G.K.A.J. independently assessed the ROB in all domains including random number generation, allocation concealment, blinding of intervention and outcome assessors, completeness of follow-up, selectivity of reporting, and other potential sources of bias. For each domain, the ROB was assessed as low, high, or unclear risk based on the Cochrane Collaboration guidelines.

Data Synthesis

Meta-analysis was conducted in Review Manager 5.3 (Cochrane Collaboration, Nordic Cochrane Centre, Copenhagen, Denmark). A fixed-effects model (FEM) (Mantel-Haenszel method) was used. However, analysis using random effects model (REM) was also conducted to ensure that the results and conclusions were not influenced by the type of model used

for the meta-analysis. Effect size was expressed as RR and 95% CI.

Statistical heterogeneity was assessed with the χ^2 test and I^2 statistic and by visual inspection of the forest plot (overlap of CIs). A P value $<.1$ on the χ^2 statistic was considered to indicate heterogeneity. I^2 statistic values were interpreted according to the guidelines of Cochrane Handbook as follows: 0% to 40%, might not be important; 30% to 60%, may represent moderate heterogeneity; 50% to 90%, may represent substantial heterogeneity; 75% to 100%, considerable heterogeneity.³⁵ The risk of publication bias was assessed by visual inspection of the funnel plot.³⁹

Subgroup Analysis

Infants <28 weeks’ gestation or <1000 g.

Sensitivity Analysis

Considering the importance of random sequence generation and allocation concealment in RCTs,⁴⁰ we conducted sensitivity analyses by excluding studies that had high ROB in these 2 domains separately. Because the risk of LOS is higher in infants born at <32 weeks or <1500 g,^{3,4} we conducted sensitivity analysis by excluding RCTs where the inclusion criteria were ≥ 32 weeks or ≥ 1500 g.

Similar analyses were also conducted for studies where *Bifidobacterium* was or was not part of the supplement and studies where *Lactobacillus* was or was not part of the supplement, given the importance of these microorganisms in the neonatal gut flora.⁴¹

There is some evidence that multistrain probiotics may be more effective than single strains.⁴² We therefore conducted analyses separately for studies that used single-strain supplements and multistrain probiotics. Lastly, we also conducted analyses separately for

studies where LOS was the primary outcome of interest.

Summary of Findings Table

The key information about the quality of evidence, the magnitude of effect of the intervention, and the sum of available data on the main outcome was presented in the summary of findings table according to the Grades of Recommendation, Assessment, Development and Evaluation (GRADE) guidelines.⁴³

RESULTS

The literature search retrieved 1736 potential relevant citations, of which 1685 were excluded and 51 RCTs were considered eligible for inclusion. Finally, 37 RCTs were included in the systematic review and meta-analysis.^{44–80} The remaining 14 studies had to be excluded because of lack of information from the published manuscripts.^{81–94} The flow diagram of the study selection process is given in Fig 1.

Out of the 37 included studies, LOS was the primary outcome of interest in only 9 studies, whereas in the remaining 28 it was a secondary outcome. Single-strain probiotics were used in 23 studies, whereas 14 used multiple strains. *Lactobacillus* was part of the supplementation in 21 studies; *Bifidobacterium* was part of the supplementation in 22 studies. The detailed characteristics of the included studies including the dose and duration of supplementation are given in Table 1.

ROB of Included Studies

Of the 37 included studies, 28 (76%) were judged to have low ROB for the domain of “random sequence generation,” and 24 (65%) were considered to have low ROB for “allocation concealment.” Details of the ROB analysis are given in Table 2.

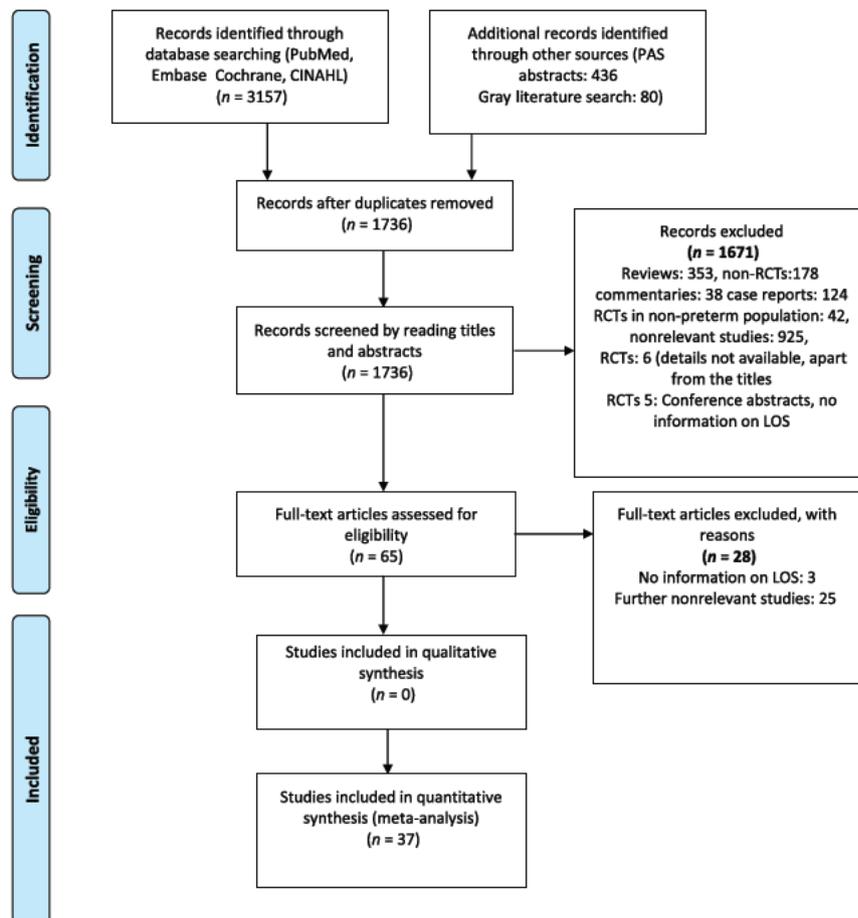


FIGURE 1

Flow diagram of search strategy and study selection. PAS, Pediatric Academic Societies meeting.

Outcome of Interest

The pooled meta-analysis (FEM) of 37 RCTs ($n = 9416$) that compared “probiotics” with “placebo” or “no probiotics” showed that probiotic supplementation resulted in a statistically significant reduction in the incidence of LOS [675/4852 [13.9%] vs 744/4564 [16.3%]; RR 0.86; 95% CI, 0.78–0.94; $P = .0007$; χ^2 statistic for heterogeneity $P = .02$; $I^2 = 35\%$; number needed to treat, 44] (Fig 2). The results were significant even when REM was used (RR 0.85; 95% CI, 0.75–0.95, $P = .007$; χ^2 statistic for heterogeneity $P = .02$, $I^2 = 35\%$). Visual inspection of the funnel plot suggested that there was no publication bias (Fig 3).

On sensitivity analysis (Table 3), the beneficial effects continued to be observed in studies that had

low ROB for random sequence generation and also for allocation concealment. The results were also significant in studies that included only infants with gestational age <32 weeks or birth weight <1500 g (24 studies, sample size 7175), studies where *Bifidobacterium* was part of the supplementation (22 studies, sample size 6069), studies where *Lactobacillus* was part of the supplementation (21 studies, sample size 4608), studies where single-strain probiotics were used (23 studies, sample size 5961), and studies where multiple-strain supplements were used (14 studies, sample size 3455); however, on REM, statistical significance was lost for many of these analyses. The overall evidence according to GRADE guidelines is provided as a summary of findings table (Table 4).

TABLE 1 Characteristics of the Included Studies

Study ID	Study Characteristics
1. Al Hosni 2012	<p>Participants: Preterm infants 501–1000 g</p> <p>Intervention: (<i>L. rhamnosus</i> GG 0.5×10^9 CFU + <i>B. infantis</i> 0.5×10^9 CFU) vs no probiotics</p> <p>Duration of supplementation: Once daily from the time of initiation of enteral feeds, until discharge or 34 wk PMA</p> <p>Sample size: 101 (probiotics 50, controls 51)</p> <p>Type of milk: EBM; Type of delivery: CD 44% vs 59%</p> <p>Primary outcome: % age of infants <10th centile at 34 wk PMA</p> <p>LOS: 13/50 (26%) vs 16/51 (31%)</p>
2. Awad 2010	<p>Participants: All neonates admitted to nursery, 28–41 wk and wt 1.1–4.3 kg</p> <p>Intervention: KP (<i>L. acidophilus</i>, 6×10^9 CFU) vs LP (<i>L. acidophilus</i>, 6×10^9 CFU) vs. placebo</p> <p>Duration of supplementation: Commenced on d1, duration NA</p> <p>Sample size: 150 (60 vs 60 vs 30), preterm 89 (37 vs 36 vs 16)</p> <p>Type of milk: Details NA; Type of delivery: Preterm CD: KP (57%) vs LP (56%) vs placebo (75%)</p> <p>Primary outcome: Incidence of neonatal sepsis and NEC in neonates and evaluating whether a KP would be equally efficacious</p> <p>LOS: Preterm: LP (18/36, 50%) vs KP (25/37, 68%) vs placebo (12/16, 75%)</p>
3. Bin-Nun 2005	<p>Participants: Preterm infants <1500 g</p> <p>Intervention: (<i>B. infantis</i> 0.35×10^9 CFU + <i>S. thermophilus</i> 0.35×10^9 CFU + <i>B. bifidus</i> 0.35×10^9 CFU) vs no probiotic</p> <p>Duration of supplementation: Once daily from the day of commencement of enteral feeds until 36 wk PMA</p> <p>Sample size = 145 (probiotics 72, controls 73)</p> <p>Type of milk: EBM/formula; Type of delivery: 78% vs 78%</p> <p>Primary outcome: Stage \geq II NEC</p> <p>LOS: 31/72 (43%) vs 24/73 (33%)</p>
4. Braga 2010	<p>Participants: Preterm infants 750–1499 g</p> <p>Intervention: (<i>L. casei</i> + <i>B. breve</i> vs 3.5×10^7 to 3.5×10^9 CFU once daily until 30 d of life) vs no probiotic</p> <p>Duration of supplementation: Once daily from the second day of life until d30</p> <p>Sample size: 231 (probiotics 119, controls 112)</p> <p>Type of milk: EBM/PDHM; Type of delivery: CD 53.8% vs 49.1%</p> <p>Primary outcome: \geq Stage II NEC</p> <p>LOS: 40/119 (33.6%) vs 42/112 (37.5%)</p>
5. Chrzanowska-Liszewska 2012	<p>Participants: Preterm infants <32 wk and birth wt >1000 g</p> <p>Intervention: <i>L. rhamnosus</i> 6×10^9 CFU vs placebo (maltodextrin)</p> <p>Duration of supplementation: Once daily from 0–3 d of life until d42</p> <p>Sample size: 47 (probiotics 21, placebo 26)</p> <p>Type of milk: Formula; Type of delivery: Spontaneous VD 23% vs 34%</p> <p>Primary outcome: Bacterial colonization of the gut</p> <p>LOS: 2/21 (9.5%) vs 3/26 (11.5%)</p>
6. Costalos 2003	<p>Participants: Preterm infants 28–32 wk</p> <p>Intervention: <i>S. boulardii</i> (1×10^9 CFU) vs placebo (maltodextrin)</p> <p>Duration of supplementation: Twice daily for a median duration of 30 d</p> <p>Sample size: 87 (probiotics 51, placebo 36)</p> <p>Type of milk: Formula; Type of delivery: CD 49% vs 38%</p> <p>Primary outcome: Tolerance to <i>S. boulardii</i> supplemented formula, fecal flora analysis, intestinal D xylose absorption, and fecal lipid excretion</p> <p>LOS: 3/51 (5.8%) vs 3/36 (8.3%)</p>
7. Dani 2002	<p>Participants: Infants <33 wk or birth wt <1500 g</p> <p>Intervention: <i>Lactobacillus</i> GG (6×10^9 CFU) vs placebo</p> <p>Duration of supplementation: Once a day until discharge, starting with the first feed</p> <p>Sample size: 585 (probiotics 295, placebo 290)</p> <p>Type of milk: Breast milk, formula; Type of delivery: CD 76.3% vs 82.4%</p> <p>Primary outcome: Urinary tract infection, bacterial sepsis, NEC</p> <p>LOS: 14/295 (4.7%) vs 12/290 (4.1%)</p>
8. Demirel, Erdeve 2013A	<p>Participants: Preterm infants \leq32 wk and \leq1500 g</p> <p>Intervention: <i>S. boulardii</i> 5×10^9 CFU vs no probiotic</p> <p>Sample size: 271 (probiotic 135, controls 136)</p> <p>Type of milk: EBM/formula; Type of delivery: CD 77.7% vs 83.0%</p> <p>Primary outcome: NEC \geq stage 2</p> <p>LOS: 20/135 (14.9%) vs 21/136 (15.4%)</p>

TABLE 1 Continued

Study ID	Study Characteristics
9. Dilli 2015	<p>Participants: VLBW infants with a gestation of <32 wk and birth wt <1500 g</p> <p>Intervention: <i>B. lactis</i> (5×10^9 CFU) vs placebo (maltodextrin)</p> <p>Duration of supplementation: From d 8 of life, once daily until discharge or a maximum of 8 wk</p> <p>Sample size: 200 (probiotic 100, placebo 100)</p> <p>Type of milk: EBM/formula</p> <p>Primary outcome: NEC (\geqStage 2)</p> <p>LOS: 8/100 (8%) vs 13/100 (13%)</p>
10. Dutta 2015	<p>Participants: Preterm infants 27–33 wk gestation</p> <p>Intervention: High dose (10 billion CFU: <i>L. acidophilus</i>, <i>L. rhamnosus</i>, <i>B. longum</i>, <i>S. boulardii</i>) vs low dose (1 billion CFU: <i>L. acidophilus</i>, <i>L. rhamnosus</i>, <i>B. longum</i>, <i>S. boulardii</i>) vs placebo (potato starch, maltodextrin)</p> <p>Duration of supplementation: High-dose long-course group + low-dose long-course group (21 d) vs high-dose short-course group (d1–d14 and d15–d21)</p> <p>Sample size: 149 (probiotic 114, placebo 35)</p> <p>Type of milk: EBM/formula; Type of delivery: Probiotic group vs placebo: Spontaneous VD (69% vs. 60%), CD: data NA</p> <p>Primary outcome: Stool colonization rates on d14, d21, and d28 with 3 different regimens of probiotic</p> <p>LOS: 10/114 (8.8%) vs 6/35 (17.1%)</p>
11. Fernandez-Carrocera 2013	<p>Participants: Preterm infants <1500 g</p> <p>Intervention: Multispecies probiotic product (<i>L. acidophilus</i> + <i>L. rhamnosus</i> + <i>L. casei</i> + <i>L. plantarum</i> + <i>B. infantis</i> + <i>S. thermophilus</i>) vs no probiotic</p> <p>Duration of supplementation: From the day of commencement of enteral feeds, once daily. Duration of supplementation: not clear</p> <p>Sample size: 150 (probiotics 75, controls 75)</p> <p>Type of milk: EBM/formula; Type of delivery: Data NA</p> <p>Primary outcome: \geqStage 2 NEC</p> <p>LOS: 42/75 (56%) vs 44/75 (58.7%)</p>
12. Hays 2015	<p>Participants: Preterm infants <32 wk and <1500 g</p> <p>Intervention: Probiotic group (3 subgroups receiving <i>B. lactis</i> only vs <i>B. longum</i> only vs <i>B. lactis</i> + <i>B. longum</i> 10^9 CFU of each strain) vs placebo (maltodextrin)</p> <p>Duration of supplementation: 4 wk if ≥ 29 wk and 6 wk if ≤ 28 wk gestation</p> <p>Sample size: 199</p> <p>Type of milk: EBM/PDHM/formula; Type of delivery: Probiotic group vs placebo group: CD (79.3% vs 75%)</p> <p>Primary outcome: Effect of probiotic supplementation on short-term postnatal growth and body composition</p> <p>LOS: 17/145 (11.7%) vs 19/52 (37%)</p>
13. Hikaru 2012	<p>Participants: Extremely low birth weight and VLBW infants</p> <p>Intervention: <i>B. breve</i> (0.5×10^9 CFU twice daily) vs no supplementation</p> <p>Duration of supplementation: From the day of birth until discharge from the NICU</p> <p>Sample size: 208 (probiotics 108, controls 100)</p> <p>Type of milk: EBM/formula; Type of delivery: NA</p> <p>Primary outcome: LOS: 10/108 (9.3%) vs 22/100 (22%), infection (ie, elevated C-reactive protein, irrespective of blood culture reports)</p>
14. Hua 2014	<p>Participants: Preterm <37 wk, admitted to NICU</p> <p>Intervention: Probiotic Jin Shuang Qi (<i>L. acidophilus</i>, <i>S. thermophilus</i>, <i>Bifidobacterium</i>) 5×10^7 CFU/d vs no probiotic</p> <p>Duration of supplementation: From the day of commencement of enteral feeds, once daily. Duration of supplementation: not clear</p> <p>Sample size: 257 (probiotics 119, controls 138)</p> <p>Type of milk: EBM/formula; Type of delivery: CD 55.5% vs 64.5%</p> <p>Primary outcome: Stool colonization by drug-resistant bacteria</p> <p>LOS: 2/119 (1.7%) vs. 8/138 (5.8%)</p>
15. Kitajima 1997	<p>Participants: VLBW infants</p> <p>Intervention: <i>B. breve</i> YIT4010 (0.5×10^9 CFU) vs distilled water</p> <p>Duration of supplementation: From the day of birth until d 28</p> <p>Sample size = 97 randomized, 91 analyzed 1 (probiotics 45, controls 46)</p> <p>Type of milk: EBM/formula; Type of delivery: Data NA</p> <p>Primary outcome: Gut colonization with <i>B. breve</i> BGG</p> <p>LOS: 1/45 (2.2%) vs 0/46 (0%)</p>
16. Lin 2005	<p>Participants: VLBW infants</p> <p>Intervention: <i>L. acidophilus</i> and <i>B. infantis</i> (minimum of 1 004 356 and 1 015 697 organisms, respectively), twice daily</p> <p>Duration of supplementation: After d 7 of life, from the time of commencement of enteral feeds</p> <p>Sample size = (probiotics 180, controls 187)</p> <p>Type of milk: EBM/PDHM; Type of delivery: CD 57.8% vs 53.5%</p> <p>Primary outcome: Incidence and severity of NEC</p> <p>LOS: 22/180 (12.2%) vs 36/187 (19.3%)</p>

TABLE 1 Continued

Study ID	Study Characteristics
17. Lin 2008	<p>Participants: VLBW preterm infants <34 wk gestation</p> <p>Intervention: <i>L. acidophilus</i> and <i>B. bifidum</i> (1×10^9 CFU each, twice daily) vs no probiotic</p> <p>Duration of supplementation: From the time of commencement of enteral feeds, for 6 wk</p> <p>Sample size = 434 (probiotics 217, controls 217)</p> <p>Type of milk: EBM/formula; Type of delivery: CD 69.6% vs 63.3%</p> <p>Primary outcomes: Death or NEC \geqStage 2</p> <p>LOS: 40/217 (19.8%) vs 24/217(11.5%)</p>
18. Manzoni 2006	<p>Participants: Preterm VLBW infants</p> <p>Intervention: <i>L. rhamnosus</i> GG (6×10^9 CFU once daily) vs no probiotic</p> <p>Duration of supplementation: From d 3 of life, for 6 wk or until discharge, if discharge occurred <6 wk</p> <p>Sample size: 80 (probiotics 39, controls 41)</p> <p>Type of milk: EBM/PDHM; Type of delivery: VD 30% vs 35%</p> <p>Primary outcome: Enteric fungal colonization</p> <p>LOS: 19 (47.8%) vs 22 (54.7%)</p>
19. Mihatsch 2010	<p>Participants: VLBW infants <30 wk gestation</p> <p>Intervention: <i>B. lactis</i> BB12 (2×10^9 CFU/kg, 6 times a day) vs placebo (HMF)</p> <p>Duration of supplementation: From the time of commencement of enteral feeds, for 6 wk</p> <p>Sample size: 183 (probiotics: 93, placebo: 90)</p> <p>Type of milk: EBM/formula; Type of delivery: VD 30% vs 31%</p> <p>Primary outcome: Incidence density of nosocomial infections, defined as periods of elevated C-reactive protein from 7 to 42 d after initiation of enteral feeding</p> <p>LOS: 28/91 (30.7%) vs 29/89 (32.6%)</p>
20. Millar 1993	<p>Participants: Preterm infants <33 wk</p> <p>Intervention: <i>Lactobacillus</i> GG 1×10^8 CFU twice daily</p> <p>Duration of supplementation: From the time of commencement of enteral feeds until discharge</p> <p>Sample size: 20 (probiotics 10, control 10)</p> <p>Type of milk: EBM/preterm formula</p> <p>Primary outcome: Gut colonization</p> <p>LOS: 0/10 vs 0/10</p>
21. Oncel and Sari 2014	<p>Participants: Preterm infants \leq32 wk and <1500 g</p> <p>Intervention: <i>L. reuteri</i> (DSM 17938) in oil-based suspension, 1×10^8 CFU/day vs placebo (oil-based suspension without probiotics)</p> <p>Duration of supplementation: From the time of first enteral feeds until discharge</p> <p>Sample size: 400 (probiotics 200, placebo 200)</p> <p>Type of milk: EBM/preterm formula; Type of delivery: CD 75% vs 76%</p> <p>Primary outcome: \geqStage 2 NEC</p> <p>LOS: 13/200 (6.5%) vs 25/200 (12.5%)</p>
22. Partty 2013	<p>Participants: Preterm infants (32–36 wk)</p> <p>Intervention: <i>L. rhamnosus</i> GG 1×10^9 CFU vs placebo (microcrystalline cellulose and dextrose anhydrate)</p> <p>Duration of supplementation: Once daily until d 30 and twice daily from d 31–60</p> <p>Sample size: 63 (probiotic 31, placebo 32)</p> <p>Type of milk: BM/formula; Type of delivery: VD 63% vs 81%</p> <p>Primary outcomes: Gut microbiota, fussing, and crying</p> <p>LOS: 0/31 vs 0/32</p>
23. Patole 2014	<p>Participants: Preterm infants <33 wk</p> <p>Intervention: <i>B. breve</i> M16V, 3×10^9/d vs placebo (Dextrin)</p> <p>Duration of supplementation: Ready to commence or on enteral feeds for <12 h</p> <p>Sample size: 159 (probiotics 79, placebo 80)</p> <p>Type of milk: EBM/PDHM; Type of delivery: CD 75% vs 65%</p> <p>Primary outcome: <i>B. breve</i> fecal counts</p> <p>LOS: 17/74 (23%) vs 12/66 (18%)</p>
24. PiPS 2014	<p>Participants: Preterm infants <31 wk</p> <p>Intervention: <i>B. breve</i> BBG-001 (2.1 to 5.3×10^8 CFU) once daily vs placebo (freeze-dried cornstarch)</p> <p>Duration of supplementation: Commenced within 48 h of birth, until 36 wk PMA</p> <p>Sample size: 1310 (probiotics 650, placebo 650)</p> <p>Type of milk: NA; Type of delivery: NA</p> <p>Primary outcome: \geqStage 2 NEC, LOS, death</p> <p>LOS: 73 (11.2%) vs 77 (11.7%)</p>

TABLE 1 Continued

Study ID	Study Characteristics
25. ProPreams 2013	<p>Participants: Preterm infants <32 wk gestation and <1500 g</p> <p>Intervention: <i>B. infantis</i> + <i>S. thermophilus</i> + <i>B. lactis</i> (1×10^9 organisms in total) vs placebo (maltodextrin)</p> <p>Duration of supplementation: until discharge from hospital or till term corrected age</p> <p>Sample size: 1099 (probiotic 548, placebo 551)</p> <p>Type of milk: EBM/formula; Type of delivery: CD 65.5% vs 68.4%</p> <p>Primary outcome: LOS</p> <p>LOS: 72 (13.1%) vs 89 (16.5%)</p>
26. Ren B 2010	<p>Participants: Preterm infants (exact gestation unclear)</p> <p>Intervention: <i>Bacillus clausii</i> (1×10^7 CFU) and <i>Clostridium (butyricum) San Chang Le Kang</i> (1×10^6 CFU) (Shandong Kexing Biological Products) 250 mg twice daily vs no probiotics</p> <p>Duration of supplementation: Until discharge</p> <p>Sample size: 70 (probiotic 35, controls 35)</p> <p>Type of milk: NA; Type of delivery: NA</p> <p>Primary outcome: Intestinal bacterial colonization rate</p> <p>LOS: 2 (6%) vs 9 (26%)</p>
27. Rojas 2012	<p>Participants: Preterm infants ≤ 2000 g</p> <p>Intervention: <i>L. reuteri</i> DSM 17938, 1×10^8 CFU, once daily vs placebo (oil-based suspension without probiotics)</p> <p>Duration of supplementation: Commenced within 48 h of life. Duration: NA</p> <p>Sample size: 750 (probiotics 372, placebo 378)</p> <p>Type of milk: EBM/formula; Type of delivery: VD noninstrumental 16% (probiotics) vs 17% (placebo), VD instrumental 0% (probiotics) vs 0.5%</p> <p>Primary outcome: Nosocomial infection and mortality</p> <p>LOS: 24 (6.5%) vs 17 (4.5%)</p>
28. Romeo 2011	<p>Participants: Preterm infants <37 wk and <2500 g</p> <p>Intervention: <i>L. reuteri</i> (1×10^8 CFU) vs <i>L. rhamnosus</i> (6×10^9) vs no probiotics</p> <p>Duration of supplementation: From within 72 h of life until discharge or for 6 wk</p> <p>Sample size: 249 (<i>L. reuteri</i> 83, <i>L. rhamnosus</i> 83, controls 83)</p> <p>Type of milk: EBM/formula; Type of delivery: CD 94% (<i>L. reuteri</i> group) vs 86% (<i>L. rhamnosus</i> group) vs. 93% (controls)</p> <p>Primary outcome: Enteric colonization by <i>Candida</i>, LOS, neurologic outcome at 12 mo corrected gestational age</p> <p>LOS: <i>L. reuteri</i> 1/83 (1.2%), <i>L. rhamnosus</i> 2/83 (2.4%), controls 9/83 (3.6%)</p>
29. Rougé 2009	<p>Participants: Preterm infants <32 wk and <1500 g</p> <p>Intervention: (<i>B. longum</i> + <i>L. rhamnosus</i> GG + maltodextrin [1×10^8 CFU], 4 times/d) vs placebo (maltodextrin)</p> <p>Duration of supplementation: From the day of commencement of enteral feeds until discharge</p> <p>Sample size: 94 (probiotics 45, placebo 49)</p> <p>Type of milk: EBM/formula; Type of delivery: CD 62.2% vs 71.4%</p> <p>Primary outcome: Percentage of infants receiving >50% of feeds via enteral route at d 14 of life</p> <p>LOS: 15 (33.3%) vs 13 (26.5%)</p>
30. Roy 2014	<p>Participants: Preterm infants <37 wk and birth wt <2500 g</p> <p>Interventions: Half of the 1-g sachet that contained <i>L. acidophilus</i> 1.25×10^9 + <i>B. longum</i> 0.125×10^9 + <i>B. bifidum</i> 0.125×10^9 + <i>B. lactis</i> 1×10^9 vs sterile water</p> <p>Duration of supplementation: Commenced within 72 h of birth for 6 wk or until discharge</p> <p>Sample size: 112 (probiotics: 56, placebo: 56)</p> <p>Type of milk: EBM; Type of delivery: CD 83.9% vs 76.8%</p> <p>Primary outcome: Enteric fungal colonization</p> <p>LOS: 55.4% vs 75%</p>
31. Saengtawesin 2014	<p>Participants: Preterm infants <34 wk and birth wt ≤ 1500 g</p> <p>Intervention: Infloran (<i>L. acidophilus</i> and <i>B. bifidum</i>, 1×10^9 CFU each) 125 mg/kg/dose twice daily vs no probiotic</p> <p>Duration of supplementation: From commencement of feeds until 6 wk or discharge</p> <p>Sample size: 60 (probiotics 31, controls 29)</p> <p>Type of milk: EBM/preterm formula; Type of delivery: CS 67.7% vs 62%</p> <p>Primary outcome: NEC</p> <p>LOS: 2 (6.45%) vs 1 (3.44%)</p>
32. Samanta 2009	<p>Participants: Preterm (<32 wk) and VLBW (<1500 g) infants</p> <p>Interventions: Probiotic mixture (<i>B. infantis</i> + <i>B. bifidum</i> + <i>B. longum</i> + <i>L. acidophilus</i>, each 2.5×10^9 CFU), administered twice daily vs no probiotic</p> <p>Duration of supplementation: NA</p> <p>Sample size: 186 (probiotics 91, controls 95)</p> <p>Type of milk: EBM; Type of delivery: CD 46.15% vs 49.47%</p> <p>Primary outcomes: NEC, death due to NEC, feed tolerance</p> <p>LOS: 13 (14.3%) vs 28 (29.5%)</p>

TABLE 1 Continued

Study ID	Study Characteristics
33. Sari 2011	Participants: Preterm infants <33 wk or birth wt <1500 g Intervention: <i>L. sporogenes</i> , 0.35×10^9 CFU, once a day vs no probiotic Duration of supplementation: From first enteral feeds until discharge Sample size: 221 (probiotics 110, controls 111) Type of milk: EBM/formula; Type of delivery: CD 67.3% vs 75.7% Primary outcomes: NEC \geq Stage II LOS: 29 (26.4%) vs 26 (23.4%)
34. Serce 2013	Participants: Preterm infants <32 wk and <1500 g Intervention: <i>S. boulardii</i> 0.5×10^9 CFU twice daily vs placebo (distilled water) Duration of supplementation: From the first enteral feed until discharge Sample size: 208 (probiotic 104, placebo 104) Type of milk: EBM/formula; Type of delivery: CD 80.8% vs 88.5% Primary outcomes: Stage ≥ 2 NEC LOS: 19 (18.3%) vs 25 (24.3%)
35. Stratiki 2007	Participants: Preterm infants of 27–37 wk gestation Intervention: Preterm formula supplemented with <i>B. lactis</i> (2×10^7 /g formula) vs preterm formula Duration of supplementation: Until discharge Sample size: 75 (study 41, controls 34) Type of milk: Preterm formula; Type of delivery: CD 36.5% vs 35% Primary outcomes: Intestinal permeability LOS: 0 vs 3 (8.8%)
36. Tewari 2015	Participants: Preterm infants <34 wk (2 groups: extremely preterm, 27–30 + 6 wk, and very preterm, 31–33 + 6 wk) Intervention: <i>Bacillus clausii</i> (2.4×10^9 spores/d) vs placebo Duration of supplementation: Commenced d5 in asymptomatic and d10 in symptomatic neonates and continued for 6 wk, discharge, death, or occurrence of LOS, whichever was earlier Sample size: 244 (study: extremely preterm 61, very preterm 62) vs (placebo: 121) Type of milk: EBM/PDHM; Type of delivery: CD: extremely preterm, 66% vs 59% and very preterm, 58% vs 60% Primary outcome: Incidence of definite and probable LOS in neonates <34 wk LOS: extremely preterm 6/61 (9.8%) vs 8/59 (13.6%) and very preterm 2/62 (3%) vs 3/62 (4.8%)
37. Totsu 2014	Participants: VLBW infants (<1500 g) Intervention: <i>B. bifidum</i> 1.25×10^9 CFU twice daily vs placebo (dextrin) Duration of supplementation: Commenced within 48 h of birth and continued until discharge Sample size: 283 (probiotic 153, placebo 130) Type of milk: EBM/formula; Type of delivery: CD 50.5% vs 79.2% Primary outcome: Postnatal day when enteral feed exceeding 100 mL/kg/d LOS: 6/153 (3.9%) vs 13/130 (10%)

For all outcomes, results in the probiotic group are given first. *B. Bifidobacterium*; CD, cesarean delivery; EBM, expressed breast milk; GG, Gorbach and Goldin; HMF, human milk fortifier; KP, killed probiotic; *L. Lactobacillus*; LB, live probiotic; NA, not available; PDHM, pasteurized donor human milk; PMA, postmenstrual age; *S. Saccharomyces*; VD, vaginal delivery; VLBW, very low birth weight.

Subgroup analysis of infants born at <28 weeks' gestation or <1000 g revealed no significant benefits of probiotic supplementation in reducing LOS (Fig 4).

DISCUSSION

Our systematic review of 37 RCTs ($N = 9416$) showed that probiotic supplementation leads to a statistically significant decrease in the risk of LOS in preterm infants born at <37 weeks or <2500 g. To our knowledge, this is the largest meta-analysis of probiotic supplementation in preterm neonates (4078 more than the previous ones). It is also the largest meta-analysis of RCTs for any

intervention in neonatal medicine so far.

Our results are in contrast to those of the latest meta-analyses^{33,34} (Alfaleh 2014 Cochrane review, 19 studies, $N = 5338$; Lau 2015, 17 studies, $N = 5215$) that did not find statistically significant benefit of probiotic supplementation in reducing LOS in preterm infants. The most likely reason for the difference between our meta-analysis and the previous ones is the sample size. The latest Cochrane Review³³ found a "trend" toward reduction in LOS with probiotic supplementation (RR 0.91; 95% CI, 0.80–1.03), but probably the sample size was inadequate to detect

a small but significant beneficial effect. Our systematic review has 4078 more preterm infants than the previous ones.^{33,34}

Our results are also in contrast to the recently concluded 2 large multicenter trials (ProPrems,⁶⁸ $N = 1099$; PiPS,⁶⁷ $N = 1310$). In the ProPrems trial, there was significant decrease in LOS in infants born at ≥ 28 weeks' gestation (probiotics, 5.5%; placebo, 10.8%; $P = .01$); however, in the overall group born at <32 weeks' gestation, there was no such benefit (probiotics, 13.1%; placebo, 16.2%; RR 0.81; 95% CI, 0.61–1.08; $P = .16$). The probable reason for nonsignificant results

TABLE 2 Assessment of the ROB of Included RCTs

Study	Random Sequence Generation	Allocation Concealment	Blinding of Participants and Personnel	Blinding of Outcome Assessment	Incomplete Outcome Data	Selective Reporting	Other Bias
Al Hosni 2011	Unclear risk	Unclear risk	Low risk	Low risk	Low risk	Low risk	Low risk
Awad 2010	Unclear risk	Low risk	Low risk	Unclear risk	Low risk	Low risk	Low risk
Bin-Nun 2005	Unclear risk	Unclear risk	Low risk	Low risk	Unclear risk	Low risk	Low risk
Braga 2010	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Chrzanoska 2012	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Costalos 2003	Unclear risk	Low risk	Low risk	Unclear risk	Low risk	Low risk	Low risk
Dani 2002	Unclear risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Demirel 2013	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Dilli 2015A	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Dutta 2015	Low risk	Unclear risk	Low risk	Low risk	Low risk	Low risk	Low risk
Fernandez-Carrocerca 2013	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Hays 2015	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Hikaru 2010	Unclear risk	Low risk	High risk	High risk	Low risk	Low risk	Unclear risk
Hua 2014	Unclear risk	Unclear risk	Low risk	Unclear risk	Low risk	Low risk	Unclear risk
Kitajima 1997	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Low risk	Unclear risk
Lin 2005	Low risk	Low risk	High risk	Unclear risk	Low risk	Low risk	Low risk
Lin 2008	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Manzoni 2006	Low risk	Unclear risk	Low risk	Low risk	Low risk	Low risk	Low risk
Mihatsch 2010	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Millar 1993	Low risk	Low risk	Low risk	Unclear risk	Low risk	Low risk	Unclear risk
Oncel and Sari 2014	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Partty 2013	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Unclear risk
Patole 2014	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
PiPS 2014	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
ProPrems 2013	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Ren B 2010	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk
Rojas 2012	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Romeo 2011	Low risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Low risk	Low risk
Rougé 2009	Low risk	Unclear risk	Low risk	Low risk	Low risk	Low risk	Low risk
Roy 2014	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Saengtawesin 2014	Low risk	Unclear risk	High risk	High risk	Low risk	Low risk	Unclear risk
Samanta 2008	Low risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Low risk	Unclear risk
Sari 2011	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Serce 2013	Low risk	Low risk	Unclear risk	Unclear risk	Low risk	Low risk	Low risk
Stratiki 2007	Low risk	Unclear risk	Low risk	Unclear risk	Low risk	Low risk	Low risk
Tewari 2015	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Totsu 2014	Low risk	Unclear risk	Low risk	Low risk	Low risk	Low risk	Low risk

is the small sample size, because to detect a statistically significant benefit for an RR reduction of 20%, a sample size of ~4500 (2250 in each arm) would be needed. The PiPS trial (of infants born at <31 weeks) also found no significant reduction in LOS in the probiotic group compared with the placebo group (11.2% vs 11.7%; adjusted RR 0.97; 95% CI, 0.73–1.29).⁶⁷ The ProPrems trial used a multistrain probiotic supplement at a dosage of 1.0×10^9 colony-forming units (1 billion CFUs), whereas the PiPS trial used a single-strain probiotic at a dosage of 2.1 to 5.3

$\times 10^8$ CFUs (0.2–0.53 billion CFUs) daily.

In our review, it was reassuring to note that for the main analysis of LOS in preterm infants, the benefits continued to remain significant even when REM was used (FEM $P = .0007$; REM $P = .007$). However, for many of the sensitivity analyses, statistical significance was lost when REM was used (Table 3). There is ongoing debate about the pros and cons of FEM and REM.^{95–98} In a detailed analysis of the Cochrane Reviews in perinatal medicine, Villar et al⁹⁵ found that the REM estimates showed wider CIs, particularly

in those meta-analyses showing heterogeneity in the trial results. Schmidt et al⁹⁸ compared the results of 68 meta-analyses in psychological medicine using REM and FEM. They reported that the published FE CIs around mean effect sizes were on average 52% narrower than their actual width, compared with the REM methods. They concluded that because most meta-analyses in the literature use FEM, the precision of findings in the literature has often been substantially overstated, with important consequences for research and practice. The Cochrane Neonatal Review Group recommends

the use of FEM (<http://neonatal.cochrane.org/resources-review-authors>, accessed August 10, 2015). Considering these issues, it is prudent to check the results with both FEM and REM to increase their reliability.

We conducted sensitivity analysis after excluding RCTs with high ROB because such studies are known to overestimate the effect size (by up to 30%),⁴⁰ which can lead to spuriously optimistic results. It was reassuring to note that the results were significant with both FEM and REM even after we excluded studies that had high ROB on random sequence generation and allocation concealment separately.

Subgroup analysis of extremely preterm infants (born at <28 weeks' gestation or <1000 g) revealed no significant benefits of probiotic supplementation in reducing LOS, but the sample size was small. On the other hand, sensitivity analysis of 24 studies ($n = 7175$) where the inclusion criteria were more mature preterm infants (born at <32 weeks or <1500 g) found probiotic supplementation to be beneficial in reducing LOS (FEM RR 0.88; 95% CI, 0.80–0.98, $P = .02$; REM RR 0.89; 95% CI, 0.79–1.00; $P = .06$). Unlike the NICUs of the developed world, where the focus of attention is extremely preterm infants (born at <28 weeks or <1000 g), the majority of NICUs around the world cater to the needs of more mature infants (born at <32 weeks or <1500 g). Therefore, the positive results of probiotic supplementation for more mature infants could have global implications.

The main strength of our systematic review is the large sample size and its exclusive focus on LOS (unlike the previous meta-analyses where the main attention was on NEC).

The limitations of our systematic review include the fact that LOS was

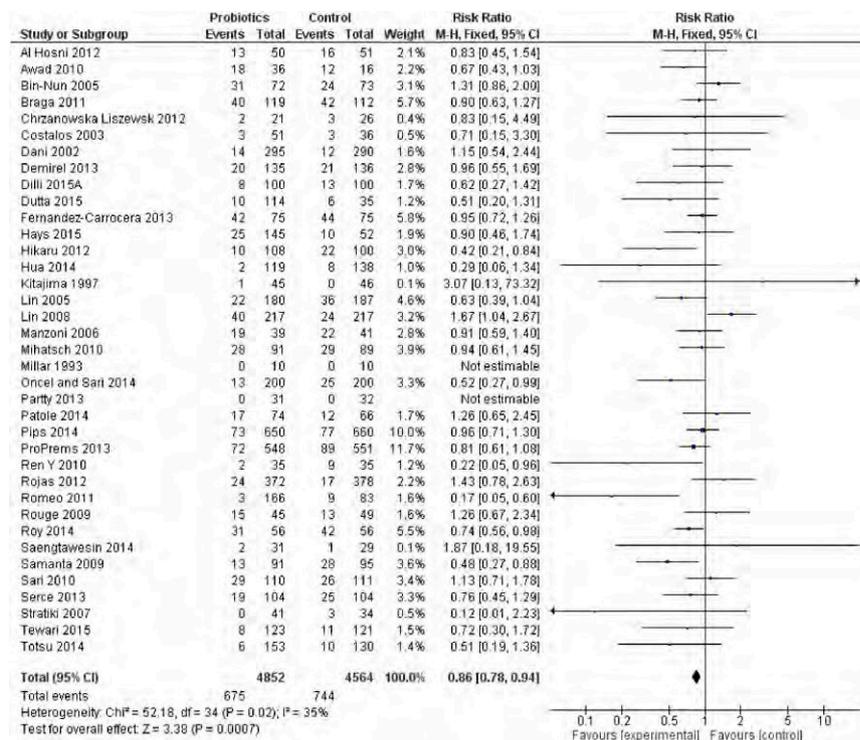


FIGURE 2

Forest plot: Probiotic supplementation to reduce LOS in preterm infants. M-H, Mantel–Haenszel.

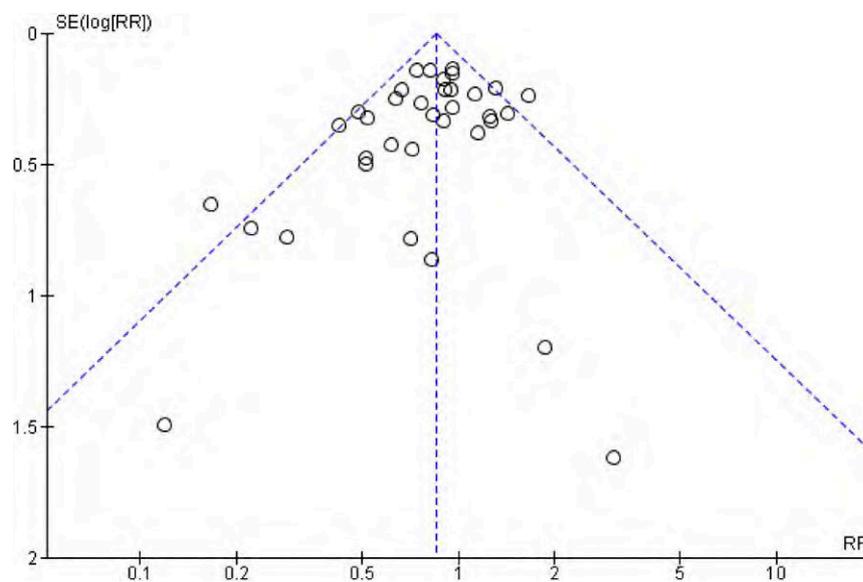


FIGURE 3

Funnel plot assessing publication bias.

a secondary outcome of interest in majority of the studies, we lacked information from 14 RCTs, and minimal information was available on extremely preterm or extremely low birth weight infants. Another limitation was the

fact that we could not objectively assess the effect of variables such as dosage and duration of supplementation on LOS in this review. These highly important questions are best addressed by head-to-head comparisons of

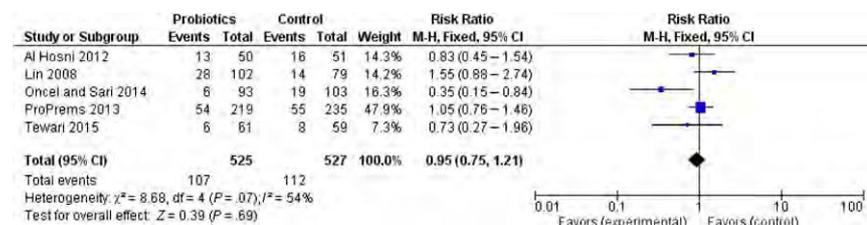
TABLE 3 Results of the Sensitivity Analyses

Item	Number of Studies	Sample Size	RR (95% CI) FEM	RR (95% CI) REM
Studies with low ROB on random sequence generation	28	7820	0.87 (0.79–0.96); $P = .005$	0.86 (0.76–0.99); $P = .02$
Studies with low ROB on allocation concealment	24	7576	0.89 (0.80–0.98); $P = .02$	0.87 (0.78–0.98); $P = .02$
Studies that included gestational age <32 wk or birth wt <1500 g	24	7175	0.88 (0.80–0.98); $P = .02$	0.89 (0.79–1.00); $P = .06$
Studies where <i>Bifidobacterium</i> was part of the supplementation	22	6069	0.87(0.78–0.96); $P = .007$	0.86 (0.75–1.00); $P = .004$
Studies where <i>Bifidobacterium</i> was not part of the supplementation	15	3347	0.82 (0.69–0.99); $P = .04$	0.80 (0.64–1.02); $P = .07$
Studies where <i>Lactobacillus</i> was part of the supplementation	21	4608	0.86 (0.76–0.97); $P = .01$	0.84 (0.70–1.00); $P = .05$
Studies where <i>Lactobacillus</i> was not part of the supplementation	16	4808	0.85 (0.74–0.97); $P = .02$	0.86 (0.73–1.01); $P = .07$
Multiple-strain supplementation	14	3455	0.86 (0.76–0.97); $P = .02$	0.86 (0.71–1.04); $P = .12$
Single-strain supplementation	23	5961	0.85 (0.74–0.97); $P = .02$	0.84 (0.71–0.98); $P = .03$
Studies where LOS was the primary outcome	9	4677	0.85 (0.74–0.99); $P = .04$	0.81 (0.63–1.03); $P = .09$

TABLE 4 Summary of Findings According to GRADE Guidelines⁴³

Outcome	Absolute Risk		Relative Effect, RR (95% CI)	Number of Participants	Quality of Evidence GRADE	Comment
	Estimate Without Probiotic Supplementation	Corresponding Risk Estimate With Probiotic Supplementation				
LOS	744/4564 (16.3%)	675/4852 (13.9%)	0.86 (0.78–0.94), $P = .0007$	9416	High	See below

The evidence was deemed high in view of the large sample size, low risk of bias in majority of the included studies, narrow CIs around the effect size estimate, very low P value for effect size estimate, and mild statistical heterogeneity.

**FIGURE 4**

Probiotic supplementation in infants born at <28 weeks or <1000 g. M-H, Mantel–Haenszel.

different doses or durations in future RCTs.

Now that our meta-analysis has shown that probiotic supplementation results in statistically significant benefits in reducing LOS, it is up to the individual units and clinicians to decide whether a 14% RR reduction or an absolute risk reduction of 2.4% is enough to warrant routine supplementation.

If the evidence is considered sufficient, this intervention can be adopted after the safety and quality of the probiotic product are ensured.^{99,100}

If clinicians and researchers are not convinced that the evidence is strong enough, the other option is to conduct a multicenter RCT. If one were to do a megatrial, to detect a statistically significant difference of ~14% RR reduction

in the incidence of LOS (from 16.3% to 13.9%), with a power of 80% and an α error of 0.05, a sample size of ~7152 preterm infants born at <37 weeks (3576 in each group) would be needed. To our knowledge, trials involving such large sample size have not been conducted in neonatal medicine so far. For the extremely preterm infants, the incidence of LOS is higher, and therefore the necessary sample size will be lower (to show a reduction in the incidence from 21% in the placebo to 17% in the probiotic group [20% RR reduction], with a power of 80% and an α error of 0.05, the total sample size needed is ~3000). Because the number of extremely preterm infants is also low, such an RCT will also need multicenter coordination.

CONCLUSIONS

Given the serious consequences of LOS in preterm infants, we believe that a strategy that has been shown by this largest neonatal meta-analysis to date is worth consideration by health care policymakers, clinicians, and, most importantly, the parents of preterm infants. Another important factor that must be considered is the fact that probiotic supplementation has been shown to reduce the risk of NEC in preterm

infants.^{33,34,68,101–103} If a simple intervention such as probiotic supplementation can reduce the risk of 2 of the most devastating conditions that affect preterm infants, it is worth paying attention.

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ABBREVIATIONS

CFU: colony-forming unit
 CI: confidence interval
 FEM: fixed-effects model
 GRADE: Grades of Recommendation, Assessment, Development and Evaluation
 LOS: late-onset sepsis
 NEC: necrotizing enterocolitis
 RCT: randomized controlled trial
 REM: random effects model
 ROB: risk of bias
 RR: relative risk

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Probiotic supplementation for preventing invasive fungal infections in preterm neonates – a systematic review and meta-analysis

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Summary

Invasive fungal infections (IFI) are associated with significant health burden in preterm neonates. The objective of this study was to systematically review effect of probiotic supplementation (PS) for preventing IFI in preterm neonates. We searched Cochrane Central Register of Controlled Trials, Medline, Embase, Cumulative Index of Nursing and Allied Health Literature, and proceedings of the Pediatric Academic Society meetings in August 2014. Study selection was performed on randomised controlled trials (RCT) of PS in neonates born <37 weeks. Primary outcome of this study was IFI (Isolation of fungus in blood/body fluids) and secondary outcome was fungal gut colonisation. Information on IFI/colonisation was available in 8 of 27 RCT. Meta-analysis (fixed effects model) showed that PS reduced the risk of IFI (RR: 0.50, 95% CI: 0.34, 0.73, $I^2 = 39\%$). Results were not significant with random effects model (RR: 0.64, 95% CI: 0.30, 1.38, $P = 0.25$, $I^2 = 39\%$). Analysis after excluding the study with a high baseline incidence (75%) of IFI showed that PS had no significant benefits (RR: 0.89; 95% CI: 0.44, 1.78). Of the five studies reporting on fungal gut colonisation, three reported benefits of probiotics; two did not. Current evidence is limited to derive firm conclusions on the effect of PS for preventing IFI/gut colonisation in preterm neonates.

Key words: Infants, neonates, preterm, probiotic supplementation, review.

Introduction

Invasive fungal infections (IFI) are associated with significant mortality and morbidity, including long-term neuro-developmental impairment (NDI) in preterm infants.¹ The incidence of IFI varies from centre to centre, ranging from 2.6 to 13.2% in very low birth weight (VLBW), and from 6.6 to 20.6% in extremely low birth weight (ELBW: Birth weight: <1000 g)

infants.² Risk factors for IFI include prematurity, VLBW/ELBW, central venous catheter, incubator humidity, endotracheal tube, antibiotic use and abdominal surgery.^{1–5} Nearly 70% of preterm ELBW infants have been reported to die or experience severe NDI after IFI despite treatment.⁶ Widespread use of antifungal prophylaxis and empirical treatment leading to antifungal resistance is a significant issue, and some species of candida may be inherently resistant to current antifungal agents.⁷ Strategies for prevention of IFI are thus important, considering the associated health burden. In a Cochrane Review, Austin *et al.* [8] have reviewed the effect of prophylactic oral/topical non-absorbed antifungal therapy on the incidence of IFI, mortality and morbidity in very preterm or VLBW infants. A total of four trials ($N = 1800$ infants) compared oral/topical non-absorbed antifungal prophylaxis (Nystatin or Miconazole) with placebo or no drug.

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These trials had methodological weaknesses including quasi-randomisation, lack of allocation concealment and lack of blinding of intervention and outcome assessment. The incidence of IFI was very high in the control groups in three out of the four included RCTs. Meta-analysis found a statistically significant reduction in the incidence of IFI [Relative risk (RR): 0.20, 95% Confidence interval (CI): 0.14–0.27]. There was no significant effect on mortality (RR: 0.87 (0.72–1.05)). Two trials ($N = 265$) assessed the effect of oral/topical non-absorbed vs. systemic antifungal prophylaxis. Meta-analyses did not find any statistically significant differences in the incidences of IFI or all-cause mortality.⁸ In another systematic review, Tripathi *et al.* [9] evaluated the safety and efficacy of fluconazole prophylaxis in preventing IFI and colonisation in preterm infants. They reported that a total of 645 ELBW and VLBW infants had been enrolled in four RCTs.^{10–13} Fluconazole (3–6 mg kg⁻¹ daily to twice weekly depending on postnatal age) decreased the incidence of colonisation and IFI, but had no effect on mortality.⁹ There were no significant adverse effects including impaired hepatic function.^{10–12} Benjamin *et al.* have recently reported the results of their large multicentre randomised, placebo-controlled trial evaluating the efficacy and safety of fluconazole (6 mg kg⁻¹ twice weekly for 6 weeks) in 361 ELBW infants.⁶ The composite primary end point of death/invasive candidiasis (IC) was 16% in the fluconazole vs. 21% in the placebo group (Odds Ratio: 0.73; 95% CI: 0.43–1.23). Neurodevelopmental impairment did not differ between the groups (fluconazole, 31% vs. placebo, 27%; $P = 0.60$). They concluded that their results did not support the universal use of prophylactic fluconazole in ELBW.⁶

In the RCT by Mersal *et al.* (not included in the Cochrane review), 57 preterm infants less than 30 weeks gestation were given either intravenous fluconazole or oral nystatin for 6 weeks.¹⁴ Rectal colonisation with candida occurred in 8% in the nystatin group and 12% in the fluconazole group. None of the infants in either group developed IC.¹⁴

Overall, the current evidence indicates that oral/topical nystatin may be better than placebo in reducing the risk of IFI. The evidence also indicates that oral/topical nystatin is as effective as systemically absorbed antifungals such as fluconazole. It is well known that IFI continues to occur in spite of the prophylactic use of topical nystatin or systemic antifungals.¹⁵ An important limitation of using systemically administered prophylactic antifungals is the risk of drug-resistant fungal infections.¹⁶ Recent studies have demonstrated an increasing incidence of fluconazole

resistant isolates, most likely related to increasing use of fluconazole in hospitalised patients.¹⁷ Hence, it is important to identify alternative strategies for prevention of fungal colonisation and IFI in preterm VLBW infants. Probiotics, defined as microorganisms that are beneficial to the host when provided in adequate amounts, may provide such an alternative.¹⁸ The results of the recent Cochrane review (24 RCTs, $N = 5500$) indicate that prophylactic enteral probiotic supplementation significantly reduces the incidence of mortality, necrotising enterocolitis (NEC) and facilitates early attainment of enteral feeds in preterm VLBW infants.¹⁹ However, there was no evidence of significant reduction in late-onset sepsis (LOS).¹⁹ This finding may relate to the fact that coagulase-negative staphylococci (CONS) are the commonest organisms in LOS in the developed nations. These organisms are known to secrete biofilms and adhere to the indwelling lines, tubes and catheters used frequently in this high-risk population. Probiotics which act mainly at the gastrointestinal level are therefore not expected to overcome the burden of LOS related to CONS. However, evidence indicates that probiotic supplementation has the potential to reduce the risk of fungal colonisation and IFI in preterm neonates.^{20,21} This evidence is supported by studies in mice indicating that probiotics may interfere with fungi in the enteric reservoir.²²

A recently published RCT in a paediatric intensive care set up showed that probiotic supplementation reduced the incidence of colonisation with candida in the gastrointestinal tract.²³ However, prevalence of candidaemia did not differ significantly between the two groups (Probiotic: 1.6% vs. Placebo: 6.35%; RR: 0.46; 95% CI: 0.08–2.74; $P = 0.39$). Given these data, we aimed to conduct a systematic review of the safety and efficacy of probiotic supplementation in reducing the risk of IFI and fungal colonisation in preterm infants.

Methods

Guidelines from the Cochrane Neonatal Review Group,²⁴ Centre for Reviews and Dissemination²⁵ and the PRISMA statement were followed for undertaking and reporting this systematic review and meta-analysis.²⁶

Types of studies

Only RCTs were included. Retrospective studies, prospective observational studies, narrative reviews, letters, editorials and commentaries were excluded, but read to identify potential studies.

Types of participants

Preterm neonates born at a gestational age (GA) under 37 weeks or with low birth weight (LBW <2500 g).

Intervention and comparison

Studies comparing enteral administration of any probiotic/s commenced within the first 7 days of life and continued for at least 6 weeks/discharge vs. placebo or controls were included. Studies comparing probiotics vs. nystatin were also eligible for inclusion. For RCTs where data on IFI or fungal colonisation of the gut were not available, authors were contacted. If there was no response, such studies were excluded.

Outcomes

Studies were included if they reported cases of IFI and/or fungal colonisation of the gut in the intervention and control group.

Search strategy

The Cochrane Central Register of Controlled Trials, MEDLINE (1946 2014), EMBASE (1980 2014),

CINAHL (Cumulative Index of Nursing and Allied Health Literature, 1980 2014) databases, and E-abstracts from the Paediatric Academic Society meetings (2000 2014) were searched in July 2014. The reference lists of identified studies and key review articles were also searched. No language restriction was applied. Medline was searched by using the MeSH terms ["infant, newborn" OR "infant premature" OR "infant" OR "infant, low birth weight"] AND ["probiotics" OR "lactobacillus" OR "bifidobacterium" OR "Saccharomyces"] AND ["Candida" OR "candidiasis" OR "fungal infections"].

Data extraction

All authors searched the literature independently and assessed the inclusion criteria. Inconsistencies were sorted out by discussion among all authors.

Assessment of risk of bias

Risk of bias (ROB) was assessed using the Cochrane 'Risk of Bias Assessment Tool'.²⁷ Two authors (SA, SR) independently assessed the ROB in all domains, including method of random number generation, allocation concealment, blinding of intervention and outcome

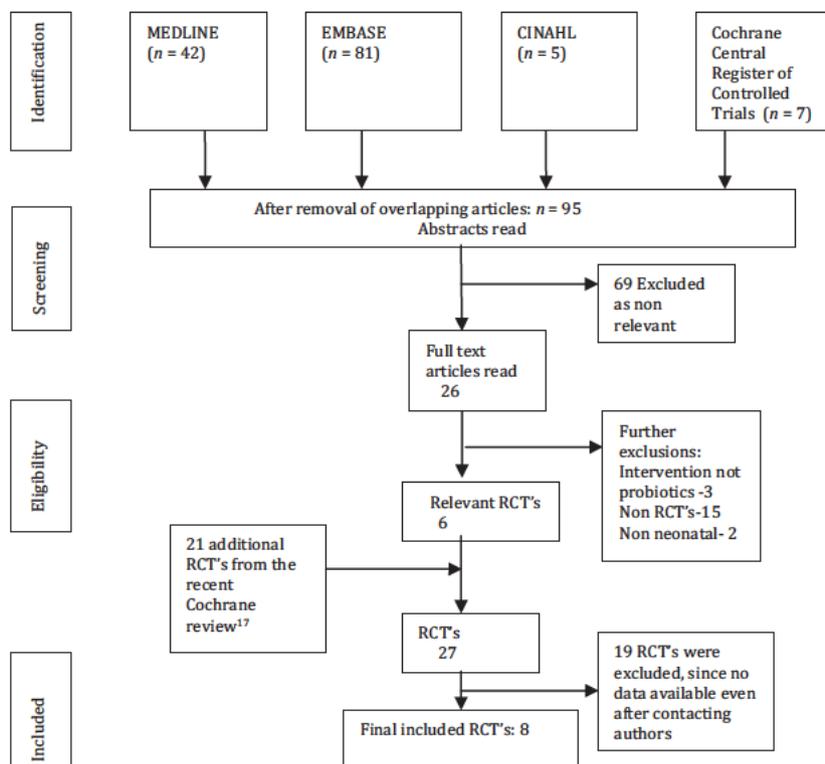


Figure 1 Flow diagram showing study selection process.

Table 1 Characteristics of trials included in study.

Source	Birth weight/ Gestation	Probiotic strain	Control	Dosage	Primary out come
Romeo <i>et al.</i> [20]	<2500 g/ <37 weeks	<i>L. rhamnosus</i> (ATCC53103) <i>L. reuteri</i> (ATCC55730)	Breast/Formula Milk without probiotics	6×10^9 cfu once daily <72 h to 6 weeks/discharge 1×10^8 cfu once daily <72 h to 6 weeks/discharge	<ul style="list-style-type: none"> • Gastrointestinal tract colonisation by candida species • Late onset sepsis • Clinical neurological outcome at 1 year
Manzoni <i>et al.</i> [21]	<1500 g	<i>L. Casei</i>	Human milk Without probiotics	6×10^9 cfu once Daily from third day to 6 weeks/discharge	Enteric fungal colonisation
Demirel <i>et al.</i> [31]	<1500 g <32 weeks	<i>Saccharomyces</i> <i>boulardii</i>	Oral nystatin 0.5 ml every 8 h (100 000 μ /ml)	5 billion cfu day ⁻¹ starting with first feed till discharge	Evaluate effectiveness of <i>S. boulardii</i> compared with Nystatin in prevention of fungal colonisation and invasive fungal infection
Roy <i>et al.</i> [32]	<2500 g/ 37 weeks <1000 g	<i>L. acidophilus</i> <i>B. longum</i> <i>B. bifum</i> <i>B. lactis</i>	Breast milk without probiotics	6×10^9 organisms once Daily from <72 h until 6 weeks/ discharge 1.5×10^9 organisms once daily from <72 h until 6 weeks/discharge	Enteric fungal Colonisation
Sari <i>et al.</i> [33]	<1500 g <33 weeks	<i>L. sporogens</i>	Formula/Breast milk without probiotics	35×10^7 cfu once daily From start of feeds till discharge	Death or stage 2 NEC
Al Hosni <i>et al.</i> [30]	500 1000 g \leq 14 days	<i>L. rhamnosus</i> GG <i>B. Inantis</i>	Milk without probiotics	500 million cfu each once daily from start of feeds until 34 weeks/discharge	<ul style="list-style-type: none"> • Improved growth in ELBW determined by decreasing percentage of wt <10 centile at 34 weeks • Improve feeding tolerance • Reducing antimicrobial days
Patole <i>et al.</i> [34]	32 week + 6 days <1500 g	<i>Bifidobacterium</i> <i>breve</i> M 16V	Milk/sterile water with dextrin	<ul style="list-style-type: none"> • 3×10^9 cfu day⁻¹ once daily until 37 weeks • <27 weeks <50 ml feeds 1.5×10^9 cfu day⁻¹ • >50 ml feeds 3×10^9 cfu day⁻¹ 	<i>B. breve</i> faecal counts
Oncel <i>et al.</i> [35]	<32 weeks 1500 g	<i>L. reutri</i> DSM 17938	Nystatin 1 ml (100 000 μ ml ⁻¹) 8 h	<ul style="list-style-type: none"> • 1×10^8 cfu day⁻¹ once daily from first day till discharge 	<ul style="list-style-type: none"> • Evaluate effectiveness of <i>L. reutri</i> with Nystatin • Prevention of Candidial colonisation and invasive candidiasis

assessors, completeness of follow-up and other sources of bias. Differences in opinion were resolved by consensus after group discussion involving all authors.

Data synthesis and statistical analysis

Meta-analysis was performed with the Review Manager (version 5.2, Cochrane Collaboration, Nordic Cochrane Centre, Copenhagen, Denmark). Based on the guidelines from the Cochrane Neonatal Review Group, the fixed effects model was preferred for meta-analysis.²⁴ Sensitivity analysis using the random effects model was planned if significant statistical heterogeneity was noted.²⁸

Heterogeneity and assessment of publication bias

Clinical heterogeneity was assessed and reported in the table of characteristics of included studies, by summarising the study population, the type, dose and duration of probiotic supplementation etc. Statistical heterogeneity was estimated using the I^2 statistic.²⁸ Publication bias was assessed using the funnel plot.²⁹

Results

Initial screening of the databases yielded 135 citations (Medline: 42, EMBASE: 81, CINAHL: 5,

Table 2 Characteristics of trials excluded from the study 1.

Sr no.	Source	Sample size (Intervention vs. Control)	Intervention and Control	Primary out come
1	Bin Nun (2005)	N 145 (72 vs. 73)	<i>B. infantis</i> + <i>S. thermophilus</i> vs. No Probiotic	NEC ≥ Stage II
2	Braga (2011)	N 231 (119 vs. 112)	Yakult LB (<i>L. casei</i> + <i>B. breve</i>) vs. No Probiotic	NEC ≥ Stage II
3	Costalos (2003)	N 87 (51 vs. 36)	<i>S. boulardii</i> vs. Maltodextrin	Tolerance to <i>S. boulardii</i> supplemented formula, faecal flora analysis, intestinal D xylose absorption and faecal lipid excretion
4	Dani (2002)	N 585 (295 vs. 290)	Lactobacillus GG vs. No Probiotics	Urinary tract infection, Bacterial sepsis, NEC
5	Demirel (2013)	N 271 (135 vs. 136)	<i>S. boulardii</i> vs. No Probiotic	NEC ≥ Stage II
6	Fernandez Carrocera (2013)	N 150 (75 vs. 75)	(<i>L. acidophilus</i> + <i>L. rhamnosus</i> + <i>L. casei</i> + <i>L. plantarum</i> + <i>B. infantis</i> + <i>S. thermophilus</i>) vs. No vs. No Probiotics	NEC (Stage 2A to 3B), mortality and combined NEC and Death.
7	Kitajima (1997)	N 91 (45 vs. 46)	<i>B. breve</i> YIT4010 (BBG) vs. Placebo (distilled water)	Gut colonisation with BBG
8	Li (2004)	N 30 (Group A/B: 10/10 vs. :10)	<i>B. breve</i> vs. No Probiotics	Colonisation rate
9	Lin (2005)	N 367 (180 vs. 187)	(<i>L. acidophilus</i> and <i>B. infantis</i>) vs. No Probiotics	Incidence and severity of NEC
10	Lin (2008)	N 434 (217 vs. 217)	(<i>L. acidophilus</i> + <i>B. bifidum</i>) vs. No probiotic	Death or NEC ≥ stage 2
11	Mihatsch (2010)	N 183 (93 vs. 90)	<i>B. lactis</i> vs. Placebo (Human milk fortifier)	Incidence density of nosocomial infections from D7 D42 of life after initiation of enteral feeding
12	Millar (1993)	N 20 (10 vs. 10)	LGG vs. placebo (un-supplemented milk)	(1) Gut colonisation with LGG (2) Colonisation impact on reduction of nosocomial pathogens (3) Colonisation have any effect on clinical progress and outcome
13	Mohan (2006)	N 69 (37 vs. 32)	<i>B. lactis</i> Bb12 vs. placebo (formula based)	Modification of gut microbiota to suppress the growth of potentially harmful bacteria
14	ProPrems (2013)	N 1099 (548 vs. 551)	<i>B. infantis</i> , Streptococcus thermophilus and <i>B. lactis</i> vs. placebo (maltodextrin)	The incidence of at least one episode of definite late onset sepsis before 40 weeks' postmenstrual age or discharge home, whichever occurred first
15	Reuman (1986)	45 (15 vs. 15 vs. untreated 15)	Lactobacillus vs. placebo (formula)	Colonisation of the gastrointestinal tract by lactobacillus and antibiotic resistant Gram negative bacteria
16	Rojas (2012)	N 750 (372 vs. 378)	<i>L. reuteri</i> vs. Placebo (oil base)	Death or nosocomial infections
17	Rouge (2009)	N 94 (45 vs. 49)	(<i>B. longum</i> + <i>L. rhamnosus</i> GG) vs. Placebo (maltodextrin)	Percentage of infants receiving >50% of feeds via enteral route at day 14.
18	Samanta (2008)	N 186 (91 vs. 95)	(<i>B. infantis</i> + <i>B. bifidum</i> + <i>B. longum</i> + <i>L. acidophilus</i>) vs. No Probiotics	Time to full feeds, length of stay, comorbidities: NEC, sepsis, death due to NEC/sepsis
19	Stratiki (2007)	N 75 (41 vs. 34)	<i>B. lactis</i> supplemented preterm formula vs. Preterm formula	Intestinal permeability

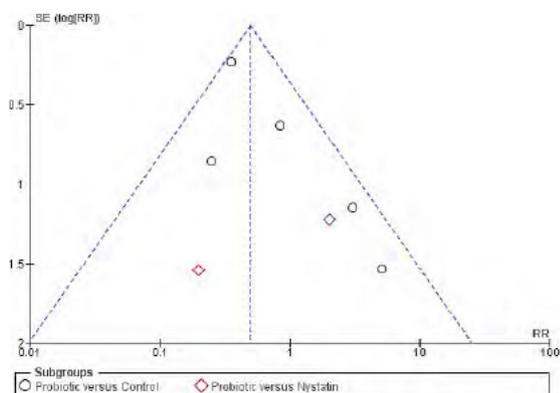
¹Data on fungal infection not available after contacting authors.

Cochrane Central Registry of Controlled Trials: 7). In addition, 21 RCTs of probiotic supplementation in preterm infants were identified from the recently published Cochrane review.¹⁹ After removing duplicates and non-relevant studies, 27 were considered to be eligible. However, there was no information regarding IFI or colonisation in majority of the

published RCTs. All authors were contacted to request this information but none responded. Hence, only Eight RCTs with full published manuscripts providing information on IFI or gut colonisation were included in the review.^{20,21,30–35} Details of the study selection process are given in Fig. 1. The characteristics of included studies are given in Table 1. The

Table 3 Risk of bias assessment.

Studies	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other bias
Manzoni <i>et al.</i> [21]	Low risk	Unclear risk	Low risk	Low risk	Low risk	Low risk	Low risk
Romeo <i>et al.</i> [20]	Low risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Low risk	Low risk
Roy <i>et al.</i> [32]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Demirel <i>et al.</i> [31]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Sari <i>et al.</i> [33]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Al Hosni <i>et al.</i> [30]	Unclear risk	Unclear risk	Low risk	Low risk	Low risk	Low risk	Low risk
Patole <i>et al.</i> [34]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Oncel <i>et al.</i> [35]	Low risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Low risk	Low risk

**Figure 2** Funnel plot to assess publication bias.

characteristics of excluded studies and reason for exclusion are given in Table 2.^{36–54}

Six out of the eight included trials (2006–2014) compared effects of probiotic supplementation against a control. The sample size of included studies ranged from 80 to 249. The remaining two trials ($N = 481$)

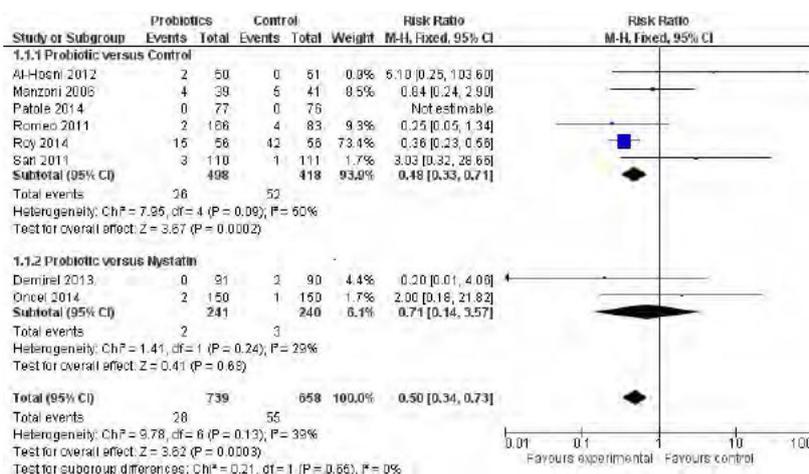
compared probiotics vs. nystatin.^{31,35} The study by Roy *et al.* had a very high incidence of IFI in the control group (75%), whereas it ranged from 0–12% among the other studies.

Risk of bias

The results of the ROB assessment are reported in Table 3. All but one trial (Al Hosni *et al.* [30]) used some form of random sequence generation method. Allocation concealment was not clear in four trials; and the ROB in allocation concealment was low in the remaining four. The ROB in outcome assessment was unclear in two, and low in six trials. The risk of selective reporting and other biases was low in all trials.

Publication bias

The funnel plot showed a somewhat asymmetrical distribution, suggesting that publication bias was likely (Fig. 2). The reason may be because a total of 26

Figure 3 Forrest plot using fixed effect model: Probiotic vs. Control (1.1.1), Probiotic vs. Nystatin (1.1.2).

RCTs have been published so far, but the information on IFI is available only from eight.

Data synthesis

Fixed effects model meta-analysis of data from the six RCTs that compared probiotics vs. control ($N = 916$) indicated that probiotic supplementation reduces the risk of IFI (RR: 0.48, 95% CI: 0.33, 0.71, $P = 0.0002$, $I^2 = 50\%$, Fig. 3).^{20,21,30–34} The two RCTs comparing probiotic supplementation against nystatin indicated no significant benefits (RR: 0.71, 95% CI: 0.14, 3.57, $P = 0.68$).^{31,35} Pooling the data from all eight RCTs ($N = 1397$) indicated that probiotic supplementation reduced the risk of IFI (RR: 0.50, 95% CI: 0.34, 0.73, $P = 0.0003$, $I^2 = 39\%$).

Given the statistical heterogeneity ($I^2 = 50\%$) we rechecked the results using the random effects model. Meta-analysis of data using random effects model from the six RCTs that compared probiotic supplementation vs. control showed no benefit (RR: 0.64, 95% CI: 0.26, 1.56, $P = 0.32$, $I^2 = 50\%$).^{20,21,30–34} The results continued to remain statistically not significant after pooling the data from all eight RCTs (RR: 0.64, 95% CI: 0.30, 1.38, $P = 0.25$, $I^2 = 39\%$).^{20,21,30–35}

Sensitivity analysis after excluding one study that had an extremely high baseline incidence of fungal infections (75%) showed that probiotic supplementation did not have significant benefits (RR: 0.89; 95% CI: 0.44, 1.78).

For the outcome of 'gut colonisation by the candida', meta-analysis could not be performed in view of the heterogeneous ways in which the results were reported in individual studies. Hence, the results have been synthesised and reported in a table format (Table 4).

Discussion

Preterm infants in the NICU frequently receive antibiotics that can destroy the normal commensal bacteria. In addition, delayed commencement of enteral feeds, living in the intensive care unit and lack of exposure to breast milk microbiota can cause intestinal dysbiosis and colonisation by pathogenic bacteria and fungi.^{55–57} Fungal colonisation of the gastrointestinal tract (GIT) is known to be associated with subsequent development of IFI in preterm infants.^{22,58–60} Probiotic supplementation has been shown to enhance gut colonisation with healthy bacteria, and hence has the potential to decrease the risk of fungal colonisation and IFI.^{22,61,62} In addition, probiotics are known to strengthen the intestinal epithelial barrier and hence have the potential to decrease IFI.²² We therefore analysed the effect of probiotic supplementation on both, the incidence of IFI as well as gut colonisation with candida.

Our systematic review found a limited number of studies evaluating the efficacy of probiotic supplementation to reduce IFI or fungal colonisation in preterm infants (eight trials, $N = 1397$).^{20,21,30–35} Even though analysis using the fixed effects model suggested a beneficial role of probiotics in reducing the incidence of IFI, one needs to be cautious because the results became statistically insignificant on using random effects model. The event rate for IFI was low in all except the trial by Roy *et al.* [32] (Probiotic: 26.8% vs. Control: 74.7%). On sensitivity analysis, once this study was excluded, the results were no longer significant.

Information on fungal colonisation of the GIT was available from only five studies.^{20,21,31,32,35} Three of which three reported beneficial effects on

Table 4 Effect of probiotics on fungal colonisation of the gut in preterm infants.

Study ID	Probiotics	Control	Effect size estimate and 95% CI	P value
Al Hosni <i>et al.</i> [30]	NA	NA	NA	NA
Manzoni <i>et al.</i> [21]	9/39 (23.1%)	20/41 (48.8%)	RR: 0.315 (95% CI: 0.12, 0.826)	0.01
Patole <i>et al.</i> [34]	NA	NA	NA	NA
Romeo <i>et al.</i> [20]	6×10^4 CFU in <i>L. reuteri</i> group, 9×10^4 CFU g^{-1} of stool in the <i>L. rhamnosus</i> group	19×10^4 CFU g^{-1} of stool	NA	<0.05 for <i>L. reuteri</i> vs. Control
Roy <i>et al.</i> [32]	$(3.06 \pm 2.33) \times 10^5$ CFU	$(3.0 \pm 1.5) \times 10^5$ CFU	NA	0.03
Sari <i>et al.</i> [33]	NA	NA	NA	NA
Demirel <i>et al.</i> [31]	29/91 (32.2%)	24/90 (27%)	NA	0.441
Oncel <i>et al.</i> [35]	28/150 (18.7%)	24/150 (16%)	NA	0.54

RR, relative risk; CI, confidence intervals; NA, not available; CFU, colony forming unit; *L.*, Lactobacillus.

colonisation,^{20,21,32} and two did not.^{31,35} It is therefore difficult to conclude whether probiotic supplementation does or does not reduce IFI and fungal colonisation of the GIT in preterm infants.

To our knowledge ours is the first systematic review to evaluate the efficacy of probiotic supplementation in reducing the risk of IFI or gut colonisation by *Candida* in preterm infants. The previous systematic reviews have addressed the issue of late onset sepsis, but did not focus specifically on IFI.^{19,63,64} An important strength of our review is the comprehensive review of literature and the use of both fixed and random effects model for meta-analysis. Since there is ongoing debate as to the indications and advantages of one model of meta-analysis over the other, we believe that it is safer to test the data with both methods.⁶⁵ Another strength of our systematic review is the fact that majority of included trials had low ROB.

The main limitation of our systematic review is the inability to obtain/clarify data specifically on IFI from 20/24 RCTs from the Cochrane review by Alfaleh *et al.* [19]. Another limitation is that 'IFI' was not the primary outcome of interest in majority of the included studies. Roy *et al.* [32] and Oncel *et al.* [35] had *Candida* colonisation and *Candida* sepsis as the primary outcomes, whereas colonisation was the primary outcome in Demirel *et al.*, [31] Manzoni *et al.* [21], and Romeo *et al.* [20]. The fungal sepsis-related outcomes were secondary in the studies by Patole *et al.* [34], Sari *et al.* [33] and Al-Hosni *et al.* [30].

Since probiotics are live organisms, there is a potential risk of sepsis due to probiotic organisms, especially in extremely preterm infants given the immaturity of their gut barrier and immune system. There are many reports of probiotic sepsis in preterm infants.^{66–68} While it is reassuring that none of the RCTs found sepsis caused by probiotic bacteria, the rarity of bifidobacterial sepsis in the literature could relate to failure to isolate these strains in blood culture by conventional culture-based techniques. Newer non-culture methods such as 16S rRNA may be a better option.⁶⁹ Recently, the Centers for Disease Control recommended the withdrawal of a probiotic product in November 2014 because of the death of a preterm infant due to gastrointestinal mucormycosis caused by *Rhizopus oryzae*. Testing of the same lot of unopened supplement revealed contamination with *Rhizopus oryzae*.⁷⁰ Hence stringent quality control as well as ongoing surveillance are essential if one were to use probiotics routinely in preterm infants.⁷¹

Conclusions

Current evidence is limited to derive firm conclusions on the effect of probiotics for prevention of IFI or fungal gut colonisation in preterm infants.

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Conflict of interest

None.

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Appendix 7

Papers published by the student before PhD enrolment and cited in thesis chapters

Updated Meta-analysis of Probiotics for Preventing Necrotizing Enterocolitis in Preterm Neonates

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KEY WORDS

neonates, necrotizing enterocolitis, preterm, probiotics, systematic review

ABBREVIATIONS

NEC—necrotizing enterocolitis

LOS—late-onset sepsis

VLBW—very low birth weight

RCT—randomized, controlled trial

CNRG—Cochrane Neonatal Review Group

TFF—time to full feeds

TSA—trial sequential analysis

RR—relative risk

CI—confidence interval

NNT—numbers needed to treat

TPN—total parenteral nutrition

NDI—neurodevelopmental impairment

CONS—coagulase-negative *Staphylococcus*

ELBW—extremely low birth weight

This work was presented in part at Perinatal Society of Australia and New Zealand meeting; April 19–22, 2009; Darwin, Australia (*J Pediatr Child Health*. 2009;45:S1–A029).

Dr Deshpande participated in the literature search, selection of trials, quality assessment of trials, contacting the authors of the trials for additional information, performing analysis, and writing the manuscript; Dr Rao did independent literature search, identified trials for inclusion and exclusion, assessed the methodologic quality of included trials, and extracted and also verified the data entered by Dr Deshpande in the RevMan software; Prof Bulsara was responsible for conducting the trial sequential analysis and interpreting its results and also contributed to the revised manuscript; and Dr Patole was responsible for the concept, design, interpretation of analysis, and writing of the final version of the manuscript that was seen and approved by all authors. All authors had full access to all of the data (including statistical reports and tables) in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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WHAT'S KNOWN ON THIS SUBJECT: Previous systematic reviews of RCTs indicated significantly lower risk for all-cause mortality and definite NEC and shorter time to full feeds after probiotic supplementation in preterm (<34 weeks' gestation) VLBW (birth weight <1500 g) neonates.



WHAT THIS STUDY ADDS: The results of this conclusive updated meta-analysis confirm the benefits of probiotic supplements in reducing death and disease in preterm neonates. Given the totality of the evidence, additional placebo-controlled trials are unnecessary if a suitable probiotic product is available.

abstract



OBJECTIVE: Systematic reviews of randomized, controlled trials (RCTs) indicate lower mortality and necrotizing enterocolitis (NEC) and shorter time to full feeds after probiotic supplementation in preterm (<34 weeks' gestation) very low birth weight (VLBW; birth weight <1500 g) neonates. The objective of this study was to update our 2007 systematic review of RCTs of probiotic supplementation for preventing NEC in preterm VLBW neonates.

METHODS: We searched in March 2009 the Cochrane Central register; Medline, Embase, and Cinahl databases; and proceedings of the Pediatric Academic Society meetings and gastroenterology conferences. Cochrane Neonatal Review Group search strategy was followed. Selection criteria were RCTs of any enteral probiotic supplementation that started within first 10 days and continued for ≥ 7 days in preterm VLBW neonates and reported on stage 2 NEC or higher (Modified Bell Staging).

RESULTS: A total of 11 ($N = 2176$), including 4 new ($n = 783$), trials were eligible for inclusion in the meta-analysis by using a fixed-effects model. The risk for NEC and death was significantly lower. Risk for sepsis did not differ significantly. No significant adverse effects were reported. Trial sequential analysis showed 30% reduction in the incidence of NEC ($\alpha = .05$ and $.01$; power: 80%).

CONCLUSIONS: The results confirm the significant benefits of probiotic supplements in reducing death and disease in preterm neonates. The dramatic effect sizes, tight confidence intervals, extremely low P values, and overall evidence indicate that additional placebo-controlled trials are unnecessary if a suitable probiotic product is available. *Pediatrics* 2010;125:921–930

Mortality and morbidities such as necrotizing enterocolitis (NEC), late-onset sepsis (LOS), and feeding difficulties as a result of immature bowel function are a major issue in preterm, especially extremely preterm (<28 weeks' gestation) neonates worldwide. Probiotics may prevent NEC by promoting colonization of the gut with beneficial organisms, preventing colonization by pathogens, improving the maturity and function of gut mucosal barrier, and modulating the immune system (eg, TLR4 receptor, nuclear factor- κ B, inflammatory cytokines) to the advantage of the host.^{1,2} Many clinical trials have evaluated the safety and benefits of probiotic supplementation in preterm very low birth weight (VLBW) neonates. Deshpande et al³ first reported a systematic review of randomized, controlled trials (RCTs) of probiotic supplementation in preterm VLBW neonates. The results of their systematic review and meta-analysis were based on 7 trials that involved 1393 preterm VLBW neonates with gestation <33 weeks. The risk for all-cause mortality and NEC was reduced by 53% and 64%, respectively, in neonates who received probiotic supplementation compared with control group neonates. The time to achieve full milk feeds was also significantly less (by an average of ~3 days) in those who received probiotic supplementation.³ These significant results were subsequently confirmed by 2 more systematic reviews that indicated the tremendous potential of probiotic supplementation in saving preterm neonates from death and disease.^{4,5} Expert bodies such as the Cochrane Neonatal Review Group (CNRG) have concluded that except for those who weigh <1000 g (because of lack of specific data in this high-risk population), a change in practice is supported by the data.⁵ Individual experts have also commented that on the basis of current data, those who wish to of-

fer probiotic supplementation as a routine therapy for preterm neonates cannot be faulted.⁶ Subsequent to these systematic reviews, 4 more RCTs (including 1 multicenter trial) that involved 783 preterm neonates have been reported.^{7–10} Given the global burden related to death, NEC, LOS, and feeding difficulties in preterm VLBW neonates and the reported very significant benefits of this low-cost, simple, and easily available intervention, we aimed to update our systematic review³ of probiotic supplementation of preterm VLBW neonates and study the implications of its results to the current clinical practice and research.^{11–13}

METHODS

Guidelines from CNRG,¹⁴ Centre for Reviews and Dissemination,¹⁵ and the QUOROM statement were followed for this systematic review and meta-analysis.¹⁶ The following prespecified criteria, similar to our previous systematic review, justified inclusion of any trial in the analysis: (1) RCT involving preterm VLBW neonates (<34 weeks' gestation and birth weight <1500 g) and reporting on stage 2 NEC or higher (Modified Bell staging criteria)^{17,18} and (2) enteral administration of any probiotic commenced within the first 10 days of life and continued for at least 7 days. The details of the search strategy and approach to analysis are given in the Appendix, which is published as supplemental information at www.pediatrics.org/content/full/125/5/921.

The primary outcome was efficacy of probiotic supplement in preventing stage 2 NEC or higher, safety in terms of blood culture–positive sepsis including that by the organism(s) in the probiotic supplement, and any other adverse events reported by the authors. The secondary outcomes included the time to reach full feeds (TFF;

120–150 mL/kg per day enteral feeds or as per the prestated definitions by authors) and duration of hospital stay. Trial sequential analysis (TSA) was recently reported as a useful tool to establish when firm evidence is reached in a cumulative meta-analysis.¹⁹ We therefore conducted TSA to evaluate whether the findings of our updated meta-analysis are conclusive.^{2,20,21} Cumulative *z* curves, information size, and sequential monitoring boundaries were estimated on the basis of risk for type I error of 5% and type II error of 20%. An intervention effect of 30% for the prevention of NEC was regarded as clinically significant. Moderate heterogeneity was assumed, and a heterogeneity correction factor of 1.33 was applied. Cumulative *z* curve of each cumulative meta-analysis was calculated with a fixed-effect and a random-effect model. The monitoring boundaries were calculated by using the method reported by Reboussin et al.²²

RESULTS

A total of 38 potentially relevant citations were obtained by our search method. An additional citation was identified by using text words and searching related articles.¹⁰ The selection process details are given in Fig 1. Four new trials were finally included in the updated analysis after extraction of data from the publications,^{7–10} and receipt of additional data for preterm VLBW neonates who were <34 weeks' gestation.⁷ One trial (*N* = 20) was subsequently excluded because of unavailability of necessary data from the author.²⁵ Search of other databases mentioned already did not identify any additional eligible studies. Characteristics of the 11 trials (4 new and the 7 from our previous report³) that were included in the analysis (*N* = 2176) are summarized in Table 1.^{7–10,27–33} The quality of the trials assessed by Jadad score is sum-

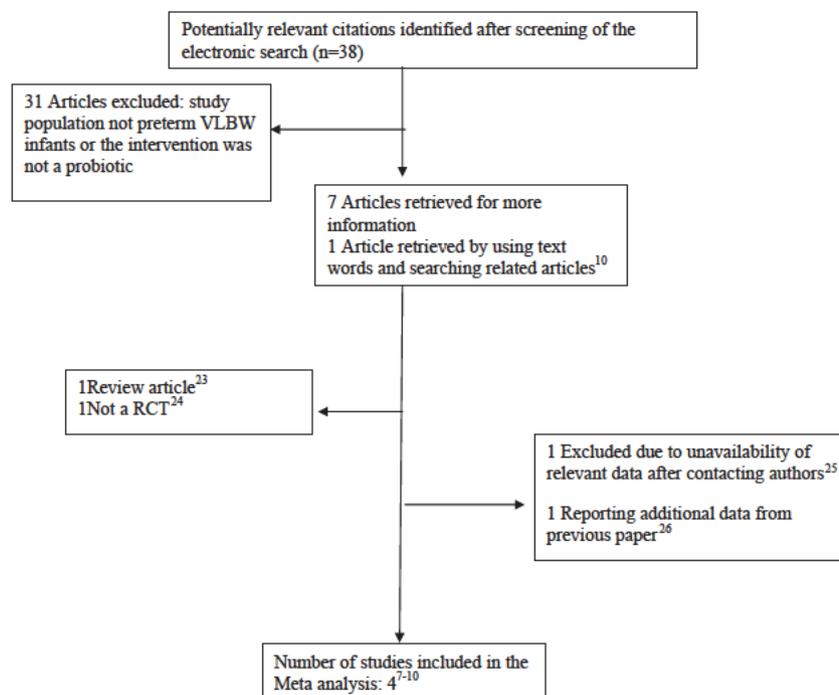


FIGURE 1
Flow diagram of the study selection process after screening of electronic search.

marized in Table 2.³⁴ The results of quality assessment were similar by using CNRG guidelines.

Effect of Probiotics on Stage 2 or Higher (Definite) NEC

Data on definite NEC were reported by all 11 trials involving 2176 neonates^{7-10,27-33} (Fig 2). A higher proportion of neonates in the control group (no probiotics) developed definite NEC compared with the probiotics group (71 [6.56%] of 1082 vs 26 [2.37%] of 1094). Meta-analysis of data by using a fixed-effects model estimated a lower risk (relative risk [RR]: 0.35 [95% confidence interval (CI): 0.23–0.55]; $P < .00001$) of NEC in the probiotic group. There was no significant heterogeneity ($I^2 = 0\%$, $P = .57$) among the trials. Individually, only 4 trials reported significantly higher risk for NEC in the control group.^{8,9,29,30} The numbers needed to treat (NNT) with probiotics to prevent 1 case of NEC was 25 (95% CI: 17–34).

Effect of Probiotics on Blood Culture–Positive Sepsis

Meta-analysis of data from 10 trials^{7-10,28-33} ($N = 2138$) estimated no significant difference in the risk for sepsis between the probiotics and control group neonates (RR: 0.98 [95% CI: 0.81–1.18] $P = .80$; Fig 3); however, there was significant heterogeneity ($I^2 = 52.1\%$, $P = .03$) among the trials. Only 1 trial reported significantly lower risk for sepsis in the probiotic group,⁷ and another reported higher risk for sepsis in the probiotic group, which was not significant after adjustment for gestation and birth weight.⁶

Effect of Probiotics on Mortality

Pooling of data from 9 trials,^{7-10,28-30,32,33} ($N = 2051$) showed a reduced risk for death from all causes in the probiotic versus the control group (RR: 0.42 [95% CI: 0.29–0.62]; $P < .00001$; Fig 4). No significant heterogeneity was noted between the trials ($I^2 = 0\%$, $P = .86$). The NNT to prevent 1 death from all

causes by treatment with probiotics was 20 (95% CI: 14–34). Pooling of data ($N = 1335$) from 5 trials,^{8,28,30,32,33} showed no significant difference in the risk for mortality as a result of NEC in the probiotic versus the control group (RR: 0.30 [95% CI: 0.08–1.08]). There was no significant heterogeneity among these trials ($I^2 = 0\%$, $P = 0.53$).

Effect of Probiotics on TFF

Meta-analysis of data ($N = 936$) from 5 trials^{8,9,30,32,33} showed significant reduction in TFF in the probiotic versus the control group (weighted mean difference: -5.03 days [95% CI: -5.62 to -4.44]; $P < .0001$). There was significant heterogeneity ($I^2 = 83.3\%$, $P < .0001$) among the trials. This difference was not significant after using the random-effects model (weighted mean difference: -2.39 days [95% CI: -5.53 to 0.75]; $P = .14$).

Sensitivity Analysis

Only 5 of 11 included trials had a primary outcome of NEC or of death and NEC.^{8,9,28-30} A sensitivity analysis of these 5 trials ($N = 1717$) showed significant reduction of NEC in the probiotic group (0.29 [95% CI: 0.17–0.49]; $P < .00001$) and reduction in mortality (0.39 [95% CI: 0.25–0.59]; $P < .00001$). There was no heterogeneity among all 5 trials ($I^2 = 0\%$, $P = .71$).^{8,9,28-30} Four of these 5 trials with primary outcome of NEC or of death and NEC showed significant reduction of NEC in the probiotic group^{8,9,29,30}, however, no difference in sepsis was noted. All trials included in the meta-analysis had Jadad quality score ≥ 3 (Table 2). A sensitivity analysis based on Jadad score < 3 vs ≥ 3 therefore was not required. The roughly symmetrical funnel plot (Fig 5) suggests that publication bias was unlikely. All results except TFF remained similar by using the random-effects model.

TABLE 1 Characteristics of Trials Included in the Analysis

Source	Birth Weight/GA	Probiotic Agent/s	Dosage and Duration	Type of Milk	Primary Outcome
Kitajima et al, ³³ 1997	<1500 g	BB	0.5 × 10 ⁹ organisms once daily from first feed for 28 d	MM or FM	Gut colonization by BB
Dani et al, ²⁸ 2002	<33 wk or <1500 g	LB-GG (Dicloflor)	6 × 10 ⁹ CFU once daily from first feed until discharge	MM, DM, or FM	UTI, sepsis, NEC
Costalos et al, ³¹ 2003	28–32 wk	SB	10 ⁹ /kg twice daily from first feed for 30 d	FM	Gut function and stool colonization
Bin Nun et al, ³⁰ 2005	<1500 g	BI, ST, BBB	BI 0.35 × 10 ⁹ CFU, ST 0.35 × 10 ⁹ CFU, and BBB 0.35 × 10 ⁹ CFU once daily from first feed to 36 wk corrected age	MM or FM	NEC
Lin et al, ²⁹ 2005	<1500 g	LB-A, BI	LB-A 1004356 and BI 1015697 organisms twice daily from day 7 until discharge	MM or DM	NEC or death
Manzoni et al, ³² 2006	<1500 g	LB-C (Dicloflor)	6 × 10 ⁹ CFU once daily from day 3 of life to 6 wk or discharge from NICU	MM or DM	Gut colonization by <i>Candida</i> species
Mohan et al, ²⁷ 2006	<37 wk ^a	BB-L	1.6 × 10 ⁹ CFU once daily from day 1 to day 3 4.8 × 10 ⁹ CFU once daily from day 4 to day 21	FM	Gut colonization by BB-L and enteric pathogens
Stratiki et al, ⁷ 2007	27 to 37 wk ^a	BB-L	Preterm formula 1 × 10 ⁷ CFU/g started within 48 h to 30 d	FM	Intestinal permeability
Lin et al, ⁸ 2008	<34 wk and <1500 g	BBB, LB-A	2 × 10 ⁹ CFU/d for 6 wk	MM or FM	NEC or death
Samanta et al, ⁹ 2009	<34 wk and <1500 g	BBB, BB-L, BI, LB-A	2.5 × 10 ⁹ CFU/d until discharge	MM or FM	NEC, TFF, sepsis, death, and hospital stay
Rougé et al, ¹⁰ 2009	<32 wk and <1500 g	BB-LG, LB GG	1 × 10 ⁸ CFU/d until discharge	MM, DM, or FM	Enteral feed intake at day 14

GA indicates gestational age; BB, *Bifidobacterium breve*; LB GG, *Lactobacillus GG*; SB, *Saccharomyces boulardii*; BI, *Bifidobacteria infantis*; ST, *Streptococcus thermophilus*; BBB, *Bifidobacterium bifidus*; LB A, *Lactobacillus acidophilus*; LB-C, *Lactobacillus casei*; BB-L, *Bifidobacterium lactis*; BB-LG, *Bifidobacterium longum*; CFU, colony-forming units; MM, mother's milk; DM, donor milk; FM, formula milk; UTI, urinary tract infection.

^a Data for <34 weeks and <1500 g obtained by contacting the authors.

Results of TSA

TSA results showed evidence to support a 30% reduction in the incidence of NEC ($\alpha = .05$; power: 80%; Fig 6). The cumulative z curve crossed the monitoring boundary. The conclusion was unchanged when $\alpha = .01$ was used. Adjusting for 1 interim analysis (first meta-analysis)³ did not change the conclusion, because the boundary was still crossed. The results were similar for random- and fixed-effect models.

DISCUSSION

The results of our update confirm those of the previous systematic reviews while improving their precision and further reducing the role of chance alone. The dramatic benefits in terms of reduced risk for all-cause mortality and definite NEC are sustained; however, despite the addition of 4 new trials ($N = 783$) to the existing

data, there is still no evidence that probiotic supplementation reduces the risk for LOS.

The incidence of NEC (5%–6% of VLBW neonates) has not changed significantly despite advances in neonatal intensive care.¹² Definite NEC (stage 2 or higher) continues to be a potentially disastrous illness in preterm neonates, with significant mortality (at least 20%–25%) and morbidity, including need for surgery and survival with short bowel syndrome and its consequences such as recurrent sepsis and dependence on total parenteral nutrition (TPN).^{12,35} Surgical NEC is associated with prolonged (>6 months) hospitalization and long-term neurodevelopmental impairment (NDI).^{36–38} Results of a systematic review of observational studies indicated that the risk for long-term NDI was significantly higher in the presence of at least stage

2 NEC versus no NEC (odds ratio: 1.82 [95% CI: 1.46–2.27]) in preterm VLBW neonates. Those who required surgery were at higher risk for NDI than were those who were treated medically (odds ratio: 1.99 [95% CI: 1.26–3.14]).³⁸ On the basis of the length of stay, the estimated annual additional hospital charges for NEC have been reported to be as high as \$216 666 per survivor in the United States.³⁹ Given the significant burden of NEC, the benefits of probiotics in this area are extremely significant.

Feeding difficulties that lead to prolonged deprivation of enteral feeds and dependence on TPN are a major issue in extremely preterm neonates. The lack of scientific guidelines for defining and managing signs of “feed intolerance” and the fear of NEC are associated with frequent stoppage of enteral feeds in this high-risk popula-

TABLE 2 Jadad Score for Assessment of Trial Quality

Parameter	Kitajima et al. ³⁵ 1997	Dani et al. ²⁶ 2002	Costalos et al. ³¹ 2003	Bin Nun et al. ³⁰ 2005	Lin et al. ²⁸ 2005	Manzoni et al. ³² 2006	Mohan et al. ²⁷ 2006	Stratiki et al. ⁷ 2007	Lin et al. ⁸ 2008	Samantia et al. ⁹ 2009	Rougé et al. ¹⁰ 2009
1. Randomization	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
A. Method to generate randomization was clear and appropriate	Yes	Yes	Yes	NA	Yes	Yes	Yes	Yes	Yes	Yes	Yes
2. Double blind?	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
A. Was method for blinding appropriate?	N/A	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NA	Yes
3. Description of withdrawal or dropout	Yes	No	Yes	No	No	No	No	Yes	Yes	No	Yes
Total score	3	4	5	3	4	4	4	5	5	3	5

Yes = 1 point; No = 0 points; scores: 0 = worst, 5 = best. NA indicates not available; N/A, not applicable.

tion.¹³ Despite the aggressive approach to enteral and parental nutrition, postnatal growth restriction continues to remain a significant issue in this population.^{40,41} Given the importance of optimal enteral nutrition in early postnatal life, the benefits of probiotics (eg, improved gastric emptying and gut barrier function, reduction in TFF) have significant implications in improving the overall prognosis of this high-risk population.^{7,25} The change in results, from significant (fixed-effects model) to no significant (random-effects model) reduction in TFF, may relate to significant heterogeneity in the feeding protocols in various trials. Given the lack of specific data on neonates who are fed exclusively breast milk/formula/mixed milk feeds, evaluation of the benefits of probiotics in the presence of different types of milk feeds is difficult. It is important to note that despite its significant benefits, preferential use of breast milk alone has not eliminated the risk for NEC significantly in preterm VLBW neonates. Although specific data are not available from all trials included in our systematic review, the reduction in all-cause mortality may relate to the significant reduction in the incidence of definite NEC and possibly TFF and severe sepsis, leading to an improvement in the general well-being.^{8,42}

The reasons for the lack of reduction in the risk for LOS need to be discussed. Colonization of the gut by aberrant flora and its translocation play an important role in LOS in preterm neonates.^{11,43} Probiotic microorganisms are expected to colonize the gut, compete with pathogens, improve the gut barrier function and permeability, and modulate immune function.^{7,25} The gastrointestinal tract is reported to be the main reservoir of coagulase-negative *Staphylococcus* (CONS), the most frequent organism responsible for LOS in extremely preterm neonates.⁴⁴ The in-

ability of probiotics alone to overcome the burden of LOS may thus relate to the presence of not only a single (gut) but also multiple (eg, endotracheal tubes, central venous catheters, TPN solutions, lipid infusions) sources of various pathogens (CONS, Gram-negative, fungi) in the presence of frequent exposure to broad-spectrum antibiotics, prolonged deprivation of enteral nutrition, and an immature immune system in this high-risk population.⁸ Mortality from CONS sepsis is low, whereas that related to virulent pathogens (eg, Gram-negative organisms) is high.⁴⁴⁻⁴⁶ It is not known whether the immunomodulating effects of probiotics are different in CONS versus non-CONS organisms.⁴⁷ Benefits of probiotics may thus depend on the type of microorganisms responsible for LOS and, as with any intervention, on the baseline incidence of LOS in various settings. Although probiotic sepsis has been reported in immunocompromised hosts and neonates,⁴⁸⁻⁵⁰ it is reassuring to know that no significant adverse events, especially probiotic sepsis, have been reported in any of the trials included in our analysis despite the diversity of the populations and the settings of the trials. Nevertheless, we emphasize the need for careful surveillance not only for probiotic sepsis but also for the development of antibiotic resistance and altered immune responses in the long-term.^{51,52} Although we do not have the specific data to support this, the risk for translocation of probiotic bacteria across a compromised gut barrier followed by sepsis may be higher in critically sick and/or extremely low birth weight (ELBW) neonates.

Overall, the results of our updated systematic review and meta-analysis (11 good-quality RCTs and $N = 2176$) confirm the dramatic benefits of probiotic supplements in reducing the risk for death and for definite NEC in preterm

Review: Probiotics for prevention of necrotizing enterocolitis
 Comparison: 01 NEC
 Outcome: 01 Definite NEC

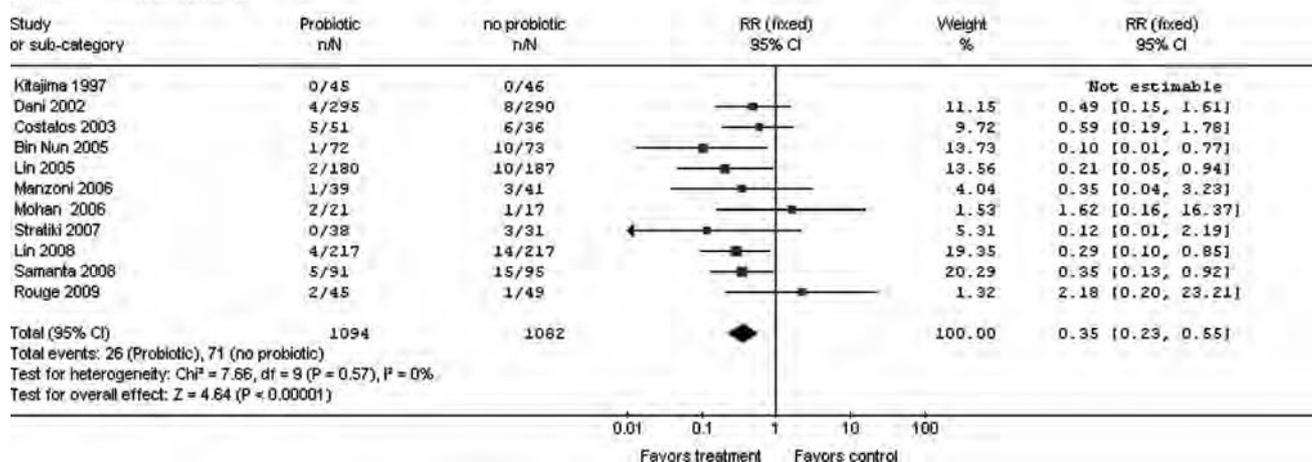


FIGURE 2

Effect of probiotics on NEC.

Review: Probiotics for prevention of necrotizing enterocolitis
 Comparison: 02 SEPSIS
 Outcome: 01 Blood culture positive Sepsis

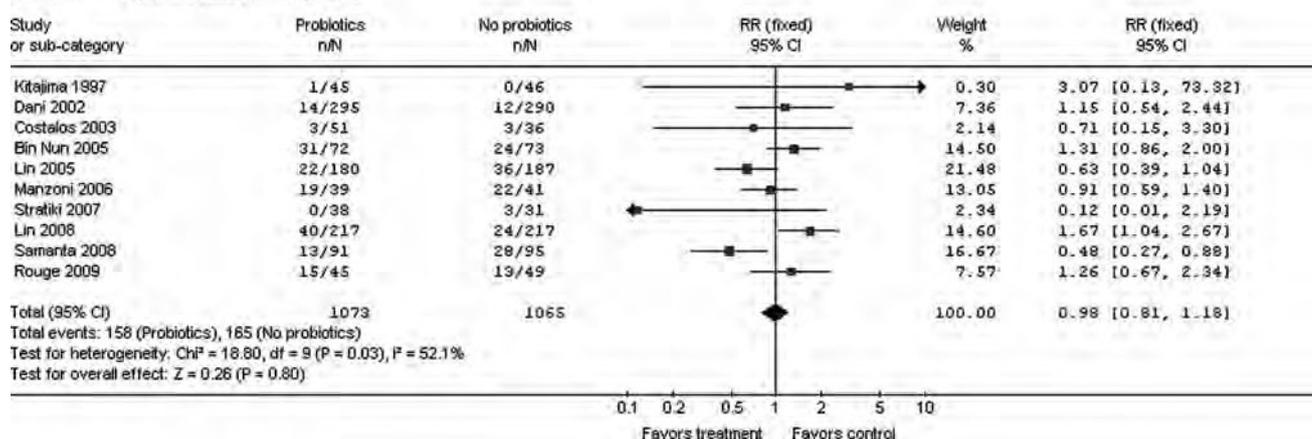


FIGURE 3

Effect of probiotics on blood culture-positive sepsis.

VLBW neonates. There is no evidence of a reduced risk for LOS. The significant effect size, precision, consistency of the results across all trials, extremely low P values almost ruling out the role of chance alone, low risk for publication bias, no statistical heterogeneity, critical areas of benefit, and the TSA conclusive of at least 30% reduction in the incidence of NEC all indicate that withholding probiotics from high-risk neonates is now almost unethical.⁵³

Our findings will have a significant impact on recruitment in the current/

planned placebo-controlled trials of probiotics in preterm neonates, because parents have the right to complete and up-to-date information on this topic in a transparent manner. On the basis of our results, we believe that it will be very difficult to justify the need for additional placebo-controlled trials in this population given the significant reduction in definite NEC and all-cause mortality. Moreover, given the sample size and power (Table 3) of the ongoing/planned placebo-controlled trials, it is unlikely that their

individual or cumulative results will affect our results significantly. We anticipate that the lack of specific data on extremely preterm (<28 weeks' gestation) neonates and on long-term outcomes and the need to reproduce these results in a setup with possibly a higher standard of care and lower baseline incidence of death and definite NEC could still be pushed forward as the basis for more placebo-controlled trials; however, the data reported by Satoh et al⁴² indicate the safety and efficacy of probiotics in ex-

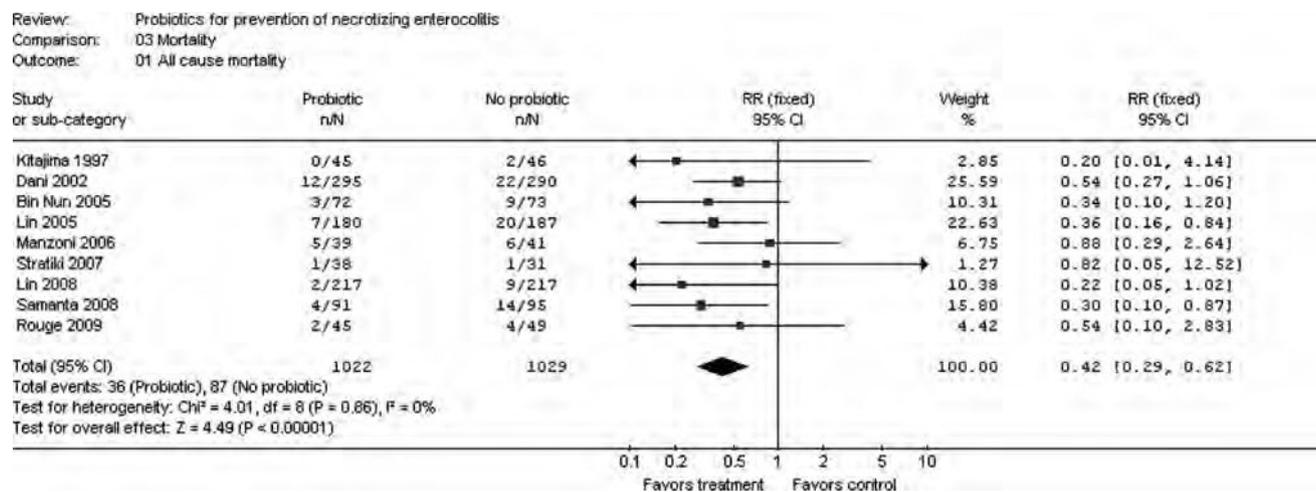


FIGURE 4

Effect of probiotics on all-cause mortality.

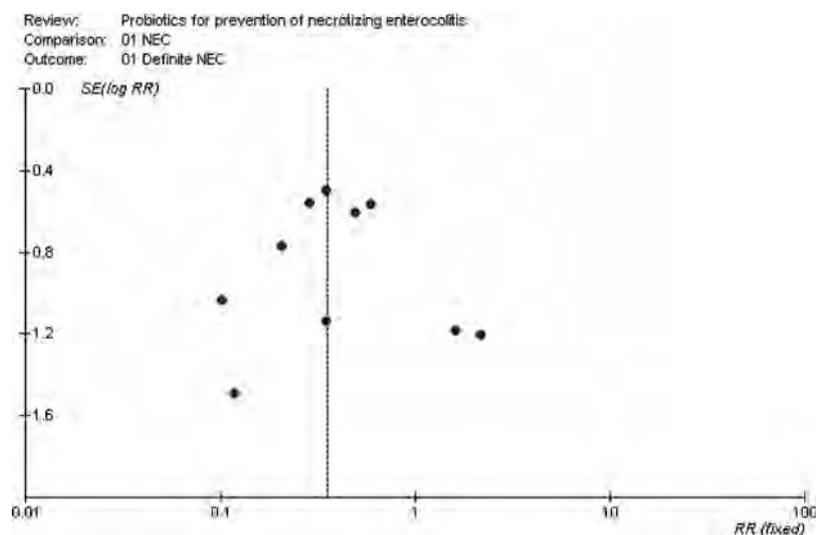


FIGURE 5

Funnel plot.

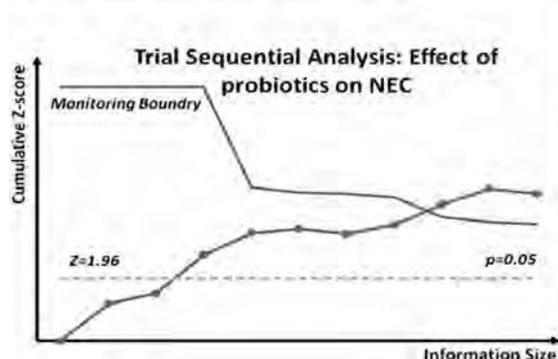
tremely preterm neonates. This observational study from Tokyo compared the data before and after introducing probiotics as a routine therapy in preterm neonates (epoch I [1994–1998, no probiotic]: ELBW = 101 of 226; epoch II [1999–2003, supplementation with *Bifidobacteria*]: ELBW = 220 of 338). *Bifidobacteria breve* (1 billion) supplementation was mixed in milk/formula and started as early as 7.2 hours of age and continued until discharge (37 weeks or 2.3 kg). There was significant reduction in the incidence of NEC, sepsis, and sepsis in death in

epoch II versus I (NEC: 6 [2.6%] vs 0 [0%]; sepsis: 65 [28.8%] vs 70 [20.7%]; sepsis in death: 9 [13.8%] vs 2 [0.6%]). The significant benefit in NEC occurred despite the low baseline incidence of the condition. Manzoni et al⁵⁴ also recently reported the safety of routine use of probiotics in VLBW neonates ($N = 743$; mean birth weight: 1056 ± 88 g; mean gestation: 29.5 ± 1.1 weeks) during a 6-year period.

Given that probiotics reduce all-cause mortality significantly, it is important to know whether this benefit

comes at the cost of an increased number of survivors with long-term NDI. Chou et al⁵⁵ recently reported the long-term neurodevelopmental outcomes of neonates (<32 weeks' gestation) in their RCT of oral probiotics for NEC. A total of 83.1% of neonates (probiotics: 153; placebo: 148) from their trial were assessed by Bayley infant developmental assessment tool (BSID-II) at 24 months' corrected age; 1 of 153 and 4 of 148 had died after discharge. There were no significant differences in growth (head circumference, length, and weight), cerebral palsy, blindness, deafness, Mental Developmental Index (<70), and Psychomotor Developmental Index (<70). Given the importance of this issue, it is critical that authors of all trials in this area report long-term neurodevelopmental outcomes of the enrolled neonates. Definite NEC and sepsis both are associated with higher risk for long-term NDI in preterm VLBW neonates.^{38,56} Given the significant reduction in definite NEC and possibly severe sepsis⁸ after probiotic supplementation, it is difficult to hypothesize that probiotic exposure in early postnatal life will be associated with long-term NDI in preterm neonates. A

A : Alpha 0.05 and power 80%



B: Alpha 0.01 and power 80%

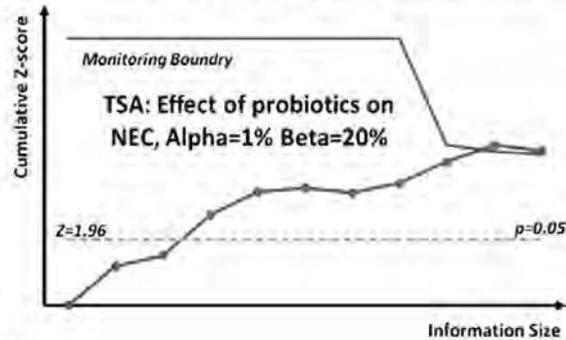


FIGURE 6

Trial sequential analysis.

TABLE 3 Characteristics of Ongoing Placebo-controlled Trials of Probiotics

Study	Primary Outcome	Inclusion Criteria	Estimated Completion	Sample Size	Effect Size	Power
Braga et al (Brazil) ⁶² ISRCTN67165178	NEC	750–1500 g	Completed	630	NA	NA
Costeloe et al (UK) ⁶³ ISRCTN05511098	Sepsis, NEC, or death	<31 wk	2013	1300	NA	NA
Lozano et al (Colombia) ⁶⁴ NCT00727363	Death or neonatal sepsis	<2000 g	2011	1110	NA	NA
Tobin et al (Australia) ⁶⁵ ACTRN12607000144415	LOS	<32 wk	NA	1100	33%	90%

NA indicates not available.

^a Completed recruitment results awaited.

significantly large sample size (Table 4) would be required to document the smallest but clinically significant benefit in centers with higher standards of care and lower baseline incidence of definite NEC and death. The potentially preventable number of deaths and cases of NEC in the placebo arm will be an ethically challenging issue in conducting such trials while ignoring the totality of evidence.

CONCLUSIONS

Considering the robustness of the evidence provided and the very signifi-

cant benefits in critical areas that outweigh the potential adverse effects, we believe that probiotics should now be offered as a routine therapy for preterm neonates and that additional placebo-controlled trials are not warranted; however, selection of a safe and suitable product with documented probiotic properties and close monitoring of the target population is a must before offering this therapy as a routine in this high-risk but most deserving population.^{57,58} Consistent benefits despite significant variations in pro-

biotic strains and protocols indicate that probiotics “in general” are beneficial in this high-risk population in the context of the broader perspective of meta-analysis.⁵⁹ It is important to note that the effect of a probiotic bacterium is strain-specific and cannot be extrapolated even to other strains of the same species.⁶⁰ Other important but as yet unanswered questions (eg, product/strain(s), dosage, duration, practicalities of administration) could easily be addressed by well-designed and tightly controlled prospective, observational studies or head-on trials of various strains/combinations/dosages/protocols etc in collaboration with the industry and regulatory agencies.^{2,61} Rigorous evaluation of an available and potentially suitable product that has not been tested in this high-risk population may possibly be the only role for additional

TABLE 4 Estimated Sample Sizes for Various Primary Outcomes in ELBW Neonates

Primary Outcome	Incidence in Control Group (%)	Incidence in Probiotic Group (%)	% Reduction	Power	α	Sample Size
Definite NEC	6.0 ^a	4.2	30	0.8	.05	4908
	10.7 ^b	7.5	30	0.8	.05	2658
Death or definite NEC	30.0 ^b	21.0	30	0.8	.05	740
	30.0 ^b	25.5	15	0.8	.05	2520

^a Figures based on Luig et al.⁶⁵

^b Figures based on Hintz et al.⁶⁶

placebo-controlled trials in this area. Current evidence makes it unlikely that parents would opt for a

50% chance of their infant's being allocated to a placebo if a suitable probiotic product were available.

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ORIGINAL ARTICLE

Ward reduction of gastroschisis in a single stage without general anaesthesia may increase the risk of short-term morbidities: Results of a retrospective audit

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Background: Ward reduction of gastroschisis in a single stage without the need for general inhalational anaesthesia (ward reduction) has been reported by some authors to be effective and safe. We introduced this practice to our neonatal unit 2 years ago.

Aim: To compare the short-term outcomes of this new practice with the standard procedure of reduction under general anaesthesia (GA).

Methods: Retrospective case series of all infants with gastroschisis between January 2004 and January 2008.

Results: Twenty-seven infants were managed with the traditional approach and 11 infants underwent ward reduction without GA. Infants in the ward reduction group had an increased frequency for all the three major adverse events (ischemic necrosis of bowel: 27.3% vs. 3.7%, odds ratio (OR) 10.72, 95% confidence interval (CI): 0.72, 159.6; need for total parenteral nutrition (TPN) more than 60 days: 18% vs. 3.7%, OR 4.13, 95% CI: 0.28, 61.55; and unplanned return to theatre: 27.3% vs. 7.4%, OR 3.88, 95% CI: 0.44, 34.08), although none of these events reached statistical significance. There were no significant differences between the groups for the outcomes of time to reach full feeds, duration of hospital stay and number of days on antibiotics.

Conclusions: These results raise concerns over the role of ward reduction of gastroschisis in a single sitting without the use of GA. Randomised trials with appropriate design and sample size are needed before embracing this method as a standard practice.

Key words: audit; gastroschisis; general anaesthesia.

Gastroschisis is a congenital anterior abdominal wall defect through which the intraperitoneal abdominal contents protrude to the exterior. The organs that may protrude through the defect include the stomach, small and large intestine, liver, spleen and bladder,¹ although most typically it is the bowel that is exteriorised. The majority of cases are diagnosed antenatally with prenatal ultrasound, facilitating antenatal management and delivery in centres equipped to care for the foetus with gastroschisis.²⁻⁴ The initial postnatal management of these

infants involves prevention of hypothermia secondary to heat loss from exposed viscera, protecting the exposed viscera from trauma and infection and fluid resuscitation.⁵ The goal of surgical treatment is to reduce the herniated viscera into the abdomen and close the fascia as well as skin to create a solid abdominal wall with a relatively normal umbilicus.⁶ There is considerable debate over the best means of treating the herniated bowel,⁷ with many units advocating and practicing primary reduction and closure under general anaesthesia (GA).⁸⁻¹⁰ Intraoperatively, various strategies such as stretching of the abdominal wall, evacuating the contents of stomach and small bowel, irrigating the meconium from the intestines, and enlarging the defect have been used to facilitate primary closure.^{10,11} To avoid the problems associated with GA and mechanical ventilation, reduction of the exteriorised contents in the neonatal unit without GA was advocated to be feasible and safe by Bianchi *et al.*¹² Successful outcomes have been reported using this method by some units.^{13,14} In addition, one study also showed this approach to be more cost effective.¹⁵ However, there have been no randomised controlled trials published comparing ward reductions with traditional reduction under GA.¹⁶

Traditionally in our unit, the primary treatment of gastroschisis was reduction under GA in the operating theatre. After reviewing the literature regarding the safety and efficacy of ward reductions, it was thought reasonable to try this approach

Key Points

- 1 Ward reduction of gastroschisis in a single stage without the use of general anaesthesia is practised by some units.
- 2 Our audit found that such a procedure may increase the risk of abdominal compartment syndrome and subsequent morbidities.
- 3 Randomised controlled trials (RCTs) are needed to evaluate the efficacy and safety of ward reduction of gastroschisis.

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in selected cases. This was an attractive option because it would not only avoid the short-term adverse effects of GA like ventilation, delay in initiation of feeding, etc., but also could prevent any potential long-term adverse effects of GA on the developing brain.¹⁷ Hence, the practice of ward reduction was introduced to our unit in January 2006. Gastroschisis with no bowel perforation, no discoloured or ischemic gut and absence of obvious intestinal atresia and 'excessive peel' were considered suitable for ward reduction. In addition to these criteria, selection was also based on the clinical opinion of the attending surgeon. In the past 2 years, 11 infants were managed with this new approach (2 out of 13 in 2006 and 9 out of 11 in 2007).

The aim of this study was to compare the short-term outcomes of this new procedure to the previous practice of reduction under GA in the operating theatre. The primary outcomes of interest were mortality before hospital discharge, development of bowel ischemia secondary to compartment syndrome within 72 h of primary procedure, need for total parenteral nutrition (TPN) for more than 60 days and any unplanned return to the operating theatre within 1 week of the primary procedure.

Materials and Methods

This was a retrospective study of all liveborn infants with gastroschisis, born between 1 January 2004 and 10 January 2008. Cases were identified from the foetal and neonatal databases at the King Edward Memorial Hospital (KEMH) for Women and Princess Margaret Hospital for Children, Perth, Western Australia. The study was approved by the Institutional Ethics Committee.

All women with a prenatal diagnosis of foetal gastroschisis in Western Australia are provided with antenatal care and delivery management at King Edward Memorial Hospital, the sole tertiary obstetric unit in the state. A uniform antenatal management strategy is provided for pregnancies complicated by foetal gastroschisis. The timing and mode of delivery for foetal gastroschisis is based on obstetric indications. Immediately after delivery, gastroschisis is managed in a standard way by protecting the herniated viscera using a sterile bowel bag, decompressing the stomach using a large bore nasogastric tube, administering prophylactic antibiotics, fluid resuscitation with normal saline boluses as required and transferring them to Princess Margaret Hospital, which is the only level 3 neonatal surgical intensive care unit in Western Australia. Any infant with post-natally diagnosed gastroschisis in the state is transferred to Princess Margaret Hospital. The pre-operative management was consistent throughout the study period except for fluid therapy. Prior to June 2006, all infants received the standard maintenance fluid therapy with (10% dextrose with 0.22 N Sodium Chloride) at the rate of 60–80 mL/kg/day. Normal saline boluses (10–20 mL/kg) were given as necessary to improve skin perfusion and correct metabolic acidosis. Since June 2006, these infants were administered continuous normal saline infusion at the rate of 15–20 mL/kg/h until the primary reduction is performed. In addition, they also received maintenance fluids (10% dextrose with 0.22 N Sodium Chloride) at the rate of 120 mL/kg/day. This amendment was made because of the concerns that at the time of reduction, the bowel of these infants

appeared to be dehydrated and the infants had a metabolic acidosis, possibly secondary to fluid deficiency.

Routine pre-operative blood indices, blood gas and glucose and electrolyte monitoring were carried out in all patients. Infants in the GA group underwent primary reduction in the operating theatre under general inhalational anaesthesia. Some infants underwent surgical extension of the defect to facilitate the reduction. If any time during the procedure in theatre the abdomen was felt to be too tight or if the infant developed respiratory compromise, the bowel contents were brought outside and a silo was fashioned. Once gradual reduction of the entire bowel was achieved, the silo was removed and the abdominal wall defect was closed in the operating theatre under GA. Some infants also underwent few revisions of the silo under GA before complete reduction was achieved. Postoperatively, all infants were managed in the neonatal intensive care unit until their discharge.

Infants in the ward reduction group underwent reduction of gastroschisis without GA; the viscera were reduced using gentle traction under the cover of intravenous paracetamol or low-dose intravenous morphine infusion.

Postreduction, infants in both groups were monitored in a similar way for the development of abdominal compartment syndrome, that is, systemic blood pressure, urine output, metabolic or mixed acidosis, lactate levels, discolouration and mottling of lower limbs and abdomen, excessive pain necessitating the administration of high dose narcotic analgesics, need for excessive ventilator pressures, etc. Formal measurement of intra-abdominal pressure, that is, bladder pressure or intragastric pressure, was not monitored in both groups of infants.

In both groups, feeds were started as soon as the clinical condition was considered stable. The unit policy is to begin enteral feeds as soon as possible, preferably within 48 h after the reduction because early commencement of enteral feeds possibly reduces the length of hospital stay and the duration of TPN.¹⁸

Statistical analysis

Statistical analysis was performed with Stata 10 software (Stata-Corp LP, 4905 Lakeway Drive, College Station, Texas, USA). For comparing the baseline characteristics, two-tailed Student's *t*-test was used for data with normal distribution. Mann-Whitney rank sum test was used for data with skewed distribution. For the dichotomous outcomes of interest, logistic regression analysis was used to calculate odds ratios (ORs). For the outcomes with continuous data that were not normally distributed, Mann-Whitney rank sum test was used to find differences between the two groups. Cox regression analysis was used to estimate Hazard ratios for censored outcomes. Poisson regression analysis was used to calculate incidence rate ratios (RRs) for count outcomes. For all these estimates, 95% confidence intervals (CIs) were also calculated. Since adverse events were more common in male infants, the analyses were adjusted for gender. However, all cases of short gut syndrome and of ischemic necrosis were in males, so gender adjustment was not possible for these outcomes. Analysis was also adjusted for 'fluid therapy before primary reduction' because there were significant differences between the two groups regarding this baseline character.

Table 1 Comparison of baseline characteristics between the two groups

	No GA (n = 11)	GA (n = 27)	P-value
Females (%)	4/11 (36%)	11/27 (41%)	0.55
GA (weeks)	36.4 (36.2, 37.1)	36.3 (35, 37.1)	0.59
BW (g)	2622 ± 136	2513 ± 103	0.56
Apgar at 1 min	8 (6, 9)	8 (7, 9)	0.42
Apgar at 5 min	9 (9, 9)	9 (9, 9)	0.28
Arterial cord pH	7.29 (7.29, 7.33), n = 8	7.32 (7.27, 7.36), n = 14	0.63
Base deficit	3.3 (2.25, 4.9), n = 8	2.25 (1, 4), n = 14	0.30
Lactate prior to reduction	4.5 (2.9, 5), n = 6	2.4 (2.05, 3.3), n = 12	0.07
CRP prior to reduction	2 (2, 9), n = 11	6 (2, 7), n = 26	0.23
Maximum CRP within 72 h	35 (26, 134), n = 10	53 (18, 79), n = 27	0.87
Age at reduction (min)	183 (155, 240)	240 (180, 300)	0.23
Fluids till the primary reduction (mL/kg/h)	22.3 (13.4, 32.4)	8.1 (6.7, 16.01)	0.003
Fluids within first 24 h of life (mL/kg)	156 (122, 207)	175 (142, 223)	0.18
Fluids at 25–48 h of life	113 (104, 138)	110 (88, 123)	0.25
Fluids 49–72 h of life	131 (105, 139)	106 (94, 129)	0.17
Bolus fluids within first 24 h (mL/kg)	41 (13, 95)	54 (44, 100)	0.19

Mean and standard deviation have been reported for data with normal distribution and median and interquartile range have been reported for those with skewed distribution. BW, birth weight; CRP, C Reactive Protein; GA, general anaesthesia.

Table 2 Primary outcomes

	No GA	GA	Adjusted odds ratio (95% CI)	P-value
Bowel necrosis after primary reduction	3/11 (27.3%)	1/27 (3.7%)	10.72 (0.72, 159.6)	0.085
Need for TPN > 60 days	2/11 (18%)	1/27 (3.7%)	4.13 (0.28, 61.55)	0.303
Unplanned return to theatre within 7 days of primary treatment	3/11 (27.3%)	2/27 (7.4%)	3.88 (0.44, 34.08)	0.22

CI, confidence interval; GA, general anaesthesia; TPN, total parenteral nutrition.

Results

Thirty-nine cases of gastroschisis were admitted to the neonatal intensive care unit at Princess Margaret Hospital between 1 January 2004 and 10 January 2008. One infant was extremely preterm (25-week gestation at birth) and hence excluded from the analysis. He died at 6 months of age (corrected age 3.5 months) because of gut-related complications and chronic lung disease. All others were included in the analysis. One infant in the GA group died at 8 months of age because of complications of severe congenital non-ischemic short gut syndrome and prolonged TPN. He was included in the analysis.

All except one infant were diagnosed antenatally and managed at KEMH during pregnancy, delivery and immediate 2–3 h after birth. During entire study period, there was one stillbirth and one termination of pregnancy.

Twenty-seven infants were managed with the traditional approach of reduction under GA. Eleven infants underwent ward reduction. Infants in ward reduction group received more intravenous fluids during the period from birth until the beginning of primary reduction compared with GA group. This was

expected because the high fluid volume protocol was introduced almost at the same period (July 2006) as the introduction of the practice of ward reductions (January 2006). Apart from that, the baseline characteristics were not different between the two groups (Table 1).

Primary outcomes

A total of 3 out of 11 infants in the ward reduction group suffered from one or more of the major adverse events compared to 3 out of 27 in the GA group (OR: 3.0; 95% CI: 0.3226898, 6.42249 p = 0.2153). The ORs for all the three major adverse events (ischemic necrosis, need for TPN more than 60 days and unplanned return to theatre within 7 days) were higher for the infants undergoing ward reduction (Table 2). However, none of these reached statistical significance. The results remained similar even after adjusting for the amount of fluid received (mL/kg/h) from birth till the beginning of primary reduction.

Since the increased fluid therapy regimen had begun almost at the same time as the introduction of ward reduction policy, we carefully further analysed the 'fluid intake until beginning of

Table 3 Secondary outcomes

	No GA (median and IQR)	GA (median and IQR)	Hazard ratio (95% CI)	P-value
Time to full feed	17 (13, 49)	18 (13, 24)	1.05 (0.43, 2.54)	0.913
Duration of hospital stay	20 (15, 49)	23 (18, 30)	1.21 (0.50, 2.97)	0.67

CI, confidence interval; GA, general anaesthesia; IQR, interquartile range.

primary procedure' to see if that was the possible etiological factor rather than the ward reduction itself.

Nineteen out of the 38 study infants received the increased fluid regimen. Out of them, 10 underwent ward reduction without GA, and the remaining nine underwent reduction in theatre under GA. During this period, the important complication of ischemic necrosis of bowel occurred in three cases of ward reduction and but in no case of GA reduction. The only one case of ischemic necrosis of bowel in the GA group occurred before the introduction of the new fluid regimen. Hence, it is unlikely that new fluid regimen was the etiological factor for this complication.

In addition, among the infants who underwent ward reduction, there was no statistically significant difference in the fluid intake between the three infants who developed ischemic necrosis of bowel versus the remaining eight infants who did not (13.4, 13.3 and 44.4 mL/kg/h vs. median 23.2, interquartile range (IQR) 19.3, and 30.3 mL/kg/h; *P*-value: 0.68, Mann-Whitney rank sum test).

Hence, it is reasonable to be certain that fluid therapy prior to undergoing primary reduction was not the risk factor for the adverse outcomes.

Secondary outcomes

Infants in the GA group spent more number of hours on ventilator compared to those who underwent ward reduction (Median 72 h (IQR: 45, 140) vs. median 0 (IQR 0, 120); *P*-value: 0.006). There was no difference in the number of days on antibiotics (Median 10 days (IQR 8, 14) vs. 12.5 days (IQR 6, 16); *P*-value: 0.97) or the number of episodes of sepsis between the two groups (RR 0.66, 95% CI 0.11, 3.78, *P* = 0.65). In addition, there was no difference in the time taken to attain full enteral feeds and the duration of hospital stay between the two groups (Table 3).

Discussion

Our data raise some concerns over the role of ward reduction of gastroschisis in a single sitting without the use of GA. Even though there were no statistically significant differences, the short-term complications were more common in the infants undergoing ward reduction. However, these results need to be interpreted with caution because of the retrospective nature of the study as well as small groups of unequal sizes. In addition, reluctance to accept 'failure of reduction' and hesitation in responding promptly to early warning signs when the abdomen is 'tight' may be the main risk factors rather than the procedure of ward reduction *per se*.

Similar concerns about ward reduction were also expressed by Dolgin *et al.*,¹⁹ who reported severe complications in three out of four cases managed with this 'minimal intervention' strategy.

Ward reduction is theoretically attractive because it avoids GA and possibly reduces the time to reach full feeds and duration of hospital stay. However, the results of our study did not show any significant benefit of the new procedure compared to the standard approach.

Interestingly, the incidence of compartment syndrome and its complications were rare in those who underwent reduction under GA. Since this procedure also involves the reduction of entire bowel in a single sitting, it may be argued that the incidence of compartment syndrome should not be different from those undergoing ward reductions. The possible explanation may be that under GA, the surgeons have more time to look at the bowel and the defect size in more detail. It is also possible that if abdominal distension occurs after reduction, the anaesthetists will notice the worsening of lung compliance and difficulty in ventilation, bringing this to the attention of surgeons. The surgeons in turn will take measures such as extending the incision, bringing out the bowel in a silo or applying a patch. All these safety mechanisms may not be available for infants undergoing ward reduction.

The fact that five out of the 27 infants (18%) that went to theatre with the intention of primary closure came back with partial reduction and silo supports this argument. In addition, during reduction under GA, the surgeon is able to look at the mesentery in detail and undertake rectifying measures such as broadening of the narrow mesentery, suturing of the defect in the mesentery, etc., thereby preventing potential postreduction complications like herniation of the gut through the mesenteric defect, decreased blood supply secondary to the narrow mesentery, etc.

Another interesting observation of this study was the C Reactive Protein (CRP) levels in these infants. Considering that gastroschisis is associated with significant perivisceritis secondary to contact between the bowel and amniotic fluid, we had expected that at least some infants will have elevated CRPs at birth. However, all infants had a normal initial CRP (<10 mg/L). The CRP was elevated (more than 20 mg/L) in 28 out of the 38 cases in the first 72 h after completion of primary reduction. Similar findings were observed by Bölke *et al.*,²⁰ who found normal CRP levels pre-operatively and elevated CRP levels after surgery, reaching a peak levels on postoperative day 3. Guibourdenche *et al.*,²¹ in a case control study found normal levels of CRP but elevated levels of interleukin-6 (IL-6) and interleukin-8 (IL-8) in the amniotic fluid of animal and human foetuses with gastroschisis.

In light of the results of this audit, it was agreed to be more cautious in the use of ward reduction of gastroschisis. But at the same time, considering the possible adverse effects of GA on the developing brain,¹⁷ the consensus was that it is preferable to avoid GA whenever possible. Hence, various other options were explored by thorough searching of literature. In the last few years, many studies have been published supporting the safety and efficacy of preformed silos and gradual reduction of the viscera over few days, without the use of GA for initial reduction, and with or without the need for general anaesthesia for the final fascial closure.^{22–26}

Hence, it was agreed that infants with gastroschisis would be preferably managed with preformed silos followed by gradual reduction over 3–5 days. This approach has the advantage of avoiding GA as well as potential to prevent abdominal compartment syndrome.

While amending the protocol after completion of this audit, the clinicians (both neonatologists and surgeons) 'opined' that the higher fluid regimen in the new protocol might have been unnecessary and hence decided to reduce it. It was a decision based on collective clinical opinion rather than on the results of the audit. In the new protocol, maintenance fluids (10% dextrose) will be administered at 80–100 mL/kg/day. In addition, normal saline will be infused at the reduced rate of 10 mL/kg/h until completion of primary reduction. The new approach will be prospectively audited.

Conclusions

The results of our retrospective study raise some concerns over the role of ward reduction of gastroschisis in a single stage without the use of GA. However, some units have reported favourable outcomes using this approach. Randomised trials with appropriate design and sample size are needed before embracing this method as a standard practice. Gradual reduction of the viscera with the help of preformed silos has the potential to avoid GA and also prevent the development of abdominal compartment syndrome and need to be further studied in randomised controlled trials.

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Growth and Developmental Outcomes of Infants with Hirschsprung Disease Presenting in the Neonatal Period: A Retrospective Study

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Objectives To describe the presentation and progress over the first year of life of neonates with Hirschsprung disease, to describe their physical and developmental outcomes at 12 months of age, and to compare the outcomes of infants with short- vs long-segment Hirschsprung disease.

Study design A retrospective study of neonates born with Hirschsprung disease in Western Australia between January 1, 2001, and December 31, 2010, to review their presentation, progress, growth, and development at 12 months of age.

Results Fifty-four infants were identified (40 with short and 11 with long segment and 3 with total colonic aganglionosis); 9 infants had a recognized syndrome and 1 infant died, unrelated to Hirschsprung disease. A primary pull-through procedure was performed in 97% and 21% of neonates with short- and non-short-segment Hirschsprung disease, respectively; 17 (31%) infants developed anal stenosis requiring dilatations. Enterocolitis occurred in 14 (26%) infants. Griffiths Mental Development Scale scores (1 year) were available in 31 of 45 nonsyndromic survivors: mean general quotient (94.2, SD 8.89) was significantly less than the population mean ($P = .007$), but the number of infants with developmental delay was within the expected range. Physical growth, except length, appeared adequate in nonsyndromic infants. There were no significant differences in the outcomes of infants with short- vs non-short-segment Hirschsprung disease.

Conclusions At 1 year of age, many infants with Hirschsprung disease have ongoing gastrointestinal problems. Their overall growth appears satisfactory, and most infants are developing normally; however, their mean general quotient appears shifted to the left. Longer-term studies will better define developmental outcomes. (*J Pediatr* 2014;165:73-7).

Evidence that genetic factors contribute to Hirschsprung disease derives from the observation of an increased risk to siblings (2%-49%) compared with the population incidence.^{1,2} Also, there is a dominant pattern of inheritance in several pedigrees of Hirschsprung disease and the frequent association with chromosomal abnormalities, such as Down syndrome, and other syndromes, such as Waardenburg syndrome.²⁻⁴

Studies have assessed the long-term clinical outcome and bowel function of patients with Hirschsprung disease,⁵⁻⁸ but there is limited information available on developmental outcomes.⁹ Hirschsprung disease is frequently associated with central nervous system anomalies, and there is evidence that similar neural growth factors govern both brain and enteric nervous system development.¹⁰ This may put infants with Hirschsprung disease at higher risk of adverse developmental outcomes. Surgery and general anesthesia in the neonatal period may also contribute to adverse neurodevelopmental outcomes.¹¹⁻¹³

Tsuij et al¹⁴ found that 25% of 5-year-olds with Hirschsprung disease were at <2nd percentile for weight. Suboptimal growth has also been linked to poor development.¹⁵

Therefore, our study aims were to describe the clinical characteristics and 12-month progress of a regional cohort (Western Australia) of neonates presenting with Hirschsprung disease, to describe their physical growth and developmental outcomes at 12 months of age, and to compare the outcomes of infants with short- vs long-segment disease.

Methods

This was a retrospective audit of all infants born in Western Australia who were diagnosed with Hirschsprung disease in the neonatal period from January 1, 2001, to December 31, 2010. Princess Margaret Hospital for Children (PMH) is the only institution in Western Australia capable of diagnosing and managing neonatal Hirschsprung disease. Therefore, interrogation of the PMH histopathology and neonatal databases identified all neonatal cases of Hirschsprung disease. Relevant clinical details during the initial hospital stay

ASQ	Ages and Stages Questionnaire
GQ	General quotient
HAEC	Hirschsprung associated enterocolitis
PMH	Princess Margaret Hospital for Children

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and until 1 year of age were obtained by reviewing the medical records of the cases.

Hirschsprung disease was classified as: (1) short segment if the aganglionic region was limited to the rectum and sigmoid colon; (2) long segment if it extended proximal to the sigmoid colon but not the entire colon; and (3) total colonic aganglionosis if the entire colon was involved but not >50 cm proximal to the ileocecal valve.¹⁶⁻¹⁹ The preferred primary procedure throughout the study period was a laparoscopic assisted endorectal pull through. The Duhamel operation was used in 4 infants with long-segment Hirschsprung disease, after an initial ostomy, at several months of age.

All neonates in Western Australia who undergo major surgery are routinely enrolled in a formal developmental follow-up program and are seen at 4, 8, and 12 months' corrected age. At the 12-month corrected gestational age visit, development is formally assessed using the Griffiths Mental Development Scales.²⁰⁻²³ Where a Griffiths assessment was not available, 2 other sources of developmental outcomes were used: the Ages and Stages Questionnaire (ASQ)²⁴ or informal developmental information obtained from the infant's local pediatrician.

The Griffiths Mental Development Scales assess development in 5 separate areas: locomotor, personal and social, hearing and speech, eye and hand coordination, and performance. The 5 subscales are assessed and scored separately and then combined to provide an overall general quotient (GQ) reflecting the child's developmental performance level relative to the general population.²⁵ All 5 subscales are combined to form a total scale, which has a mean score of 100.2 and an SD of 12.8.^{26,27} The majority of Griffiths assessments were conducted by a single developmental pediatrician (J.M.).

The ASQ is a parent-completed screening tool that uses parent observation to assess child development and behavior. The ASQ questionnaire at each age point contains 6 questions in each of 5 domains of development—communication, fine motor, gross motor, problem solving, and personal social—for a total of 30 questions. Answer options for each question include “yes,” “sometimes,” and “not yet.” A “yes” response receives 10 points, “sometimes” receives 5 points, and “not yet” receives 0 points. Each of the 5 domains is scored separately. A score of ≥ 2 SDs below the mean in any 1 of the domains is considered a “fail” on the ASQ.²⁴ It is a validated screening tool for identifying neurosensory disability.²⁸⁻³⁰

For our study, the main outcome of interest was suboptimal long-term developmental outcome, defined as a GQ >2 SDs below the mean (ie, GQ <75) or cerebral palsy, blindness (visual acuity of <6/60 in the better eye), or sensorineural deafness requiring hearing aids.

Other outcomes of interest were mild developmental delay defined as GQ between 1 and 2 SDs below the mean (76-88) or mild delay as assessed by the infant's local pediatrician, physical growth at 1 year of age, the incidence of rectal/anal strictures, or stenosis and Hirschsprung-associated enterocolitis (HAEC).

Because chromosomal syndromes and septo-optic dysplasia with panhypopituitarism can affect physical growth and neurodevelopmental outcomes, such patients were excluded from these 2 analyses. However, their data relating to gastrointestinal outcomes were included in the analysis.

The conduct of this study was approved by the PMH Institutional Audit Committee. Parental consent was deemed not necessary, considering the retrospective chart review nature of the study.

Statistical Analyses

Statistical analyses were performed using Stata 12.0 (Stata-Corp LP, College Station, Texas). Mean and SD values were calculated for normally distributed data. The mean GQ of this study sample was compared with the published healthy population mean using the *t* test, and the magnitude of this difference was evaluated using Cohen *d*, where *d* is the difference between the study and population means divided by the population SD. A *d* of .2 is considered a small effect; .5, a medium effect; and .8, a large effect size.^{9,31} The published SD for the normal population was used in calculating this effect size because this would reflect the extent to which our sample compared with the variation found in the normal population. Median, IQR, and range values were calculated for continuous data with non-normal distribution. Wilcoxon matched-pairs sign rank test was used to compare the percentiles at birth with percentiles at 1 year of age. Mann-Whitney 2-sample rank sum test was used to explore the differences between short-segment and long-segment Hirschsprung disease groups for nonparametric data; for comparison of categorical variables between the 2 groups, Fisher exact test was used. For all results, a value of *P* < .05 was considered statistically significant.

Results

We identified 54 cases of Hirschsprung disease during the 10-year study period. **Figure 1** (available at www.jpeds.com) provides a flow diagram of the patients' outcomes over the first year of life. There were 21 (39%) female and 33 (61%) male patients (**Table I**). There were 9 (17%) infants with syndromes: 7 with chromosomal anomalies (Down syndrome, 6; isodicentric chromosome 15, 1), 1 with panhypopituitarism and septo-optic dysplasia, and 1 with cerebral dysgenesis with severe hydrocephalus, who did not survive (his death was not related to Hirschsprung disease). Family history of Hirschsprung disease was present in 2 (4%) infants. All infants had >1 of the following presenting symptoms: abdominal distention (70%); vomiting (61%), most of which was bilious vomits or aspirates; delayed passage of meconium (56%); and a sepsis-like illness such as lethargy and poor feeding (20%).

The majority had short-segment disease (40, 74%); 11 (20%) had long-segment disease, and 3 (6%) had total aganglionosis of the colon with the transition zone in the terminal ileum. For this audit, all infants with non-short-segment disease were classified as having long-segment disease. A

Table I. Clinical characteristics of study population (N = 54)

Clinical characteristics	Median	IQR	Range
Gestational age, wk	39	38-40	31-41
Birth weight, g	3420	3114-3770	1450-4570
Birth head circumference, cm	34.5	34-35	30-40
Birth length, cm	51	48-53	43-55
Apgar at 5 min	9	9-9	5-10
Age at presentation, h	48	40-72	18-168
Age at primary surgery, d	10	6-14	3-63
Neonatal inpatient days	17	12-23	8-163
Total inpatient days, first year	21	15-31	9-175
GQ in 31 nonsyndromic infants with Griffiths assessments	Mean 94.2	SD 8.89	
Major developmental delay in the 43 nonsyndromic infants, n*	1/43 (2.3%)	-	-
Minor developmental delay in the 43 nonsyndromic infants, n*	6/43 (14%)	-	-

*Developmental information was available on 43 nonsyndromic infants.

primary endorectal pull-through procedure (all Soave) was performed in 39 (97%) of 40 infants with short-segment disease and 3 (21%) of 14 infants with long-segment disease. An initial colostomy or ileostomy was performed in the remaining infants.

Infants with short- and long-segment disease did not significantly differ in terms of their short- or long-term gastrointestinal outcomes, growth, or developmental outcomes, although the long-segment group included more infants with minor developmental delay (Table II).

Fourteen (26.5%) of 53 surviving infants developed HAEC during the first year of life—4 (7.5%) during their initial hospitalization, and 10 (19%) required either oral or parenteral antibiotics later in their first year of life. Seventeen (32%) infants developed anal strictures/stenosis requiring recurrent

anal dilatation at home or in hospital during first few months of life.

Griffiths assessments at 1 year of age were available in 31 (69%) of 45 nonsyndromic infants. The mean GQ was 94.2 (SD 8.89), which was significantly less than the population mean of 100.2 ($P = .007$); however, only 1 (3.2%) of 31 infants had a GQ >2 SDs below the population mean (major delay) and 4 (12.9%) of 31 infants had GQs between 1 and 2 SDs below mean (minor delay). The proportion of infants with major and mild developmental delay in this sample is close to the proportion expected from any random population sample. The magnitude of the difference between the mean GQ of Hirschsprung disease infants and the known general population mean GQ using Cohen d was .47, representing a medium effect size. In the remaining nonsyndromic infants, for whom a Griffiths assessment was not available, 5 had ASQ reports (all were normal) and 7 had a local pediatrician's assessment (2 with mild motor delay), giving a total of 6 (14%) of 43 infants with minor delay. No infant had cerebral palsy or significant visual or hearing loss. We could not find any developmental information on 2 infants.

The median GQ in those infants who had anal strictures/stenosis was 92.5 (IQR 89-94) vs 100 (IQR 90-101; $P = .235$) in those without complications.

The physical growth measurements over the first year of life in nonsyndromic infants varied as follows: weight percentiles were similar at birth and 1 year of age ($P = .585$, Figure 2; available at www.jpeds.com). The median length percentile at 1 year of age had fallen to approximately the 50th percentile, statistically lower than the birth percentile ($P = .019$, Figure 3; available at www.jpeds.com). The median head circumference percentile at 1 year was statistically higher than that at birth ($P = .009$, Figure 4; available at www.jpeds.com).

Table II. Clinical characteristics and outcomes of infants with short- vs long-segment disease*

Clinical characteristics and outcomes	Short-segment disease (n = 40)	Long-segment disease (n = 14)	P value
Gestational age, wk, median (IQR)	39 (38-40)	40 (37-41)	.779
Birth weight, g, median (IQR)	3430 (3240-3767)	3320 (2380-3820)	.825
Male:female, No.	26:14	7:7	.355
Apgar at 5 min, median (IQR)	9 (9-9)	9 (9-9)	.648
Syndromes, No.	6 (15%)	1 (7%)	.662
Deaths, No.	0	1 (7%)	1.000
Family history of Hirschsprung disease, No.	1 (2.5%)	1 (7%)	.455
Age at presentation, h, median (IQR)	48 (38-72)	48 (42-72)	.591
Primary endorectal pull through, No.	39 (97%)	3 (21%)	.001
HAEC (birth to 12 mo of age), No.	10/40 (25%)	3/14 (21%)	1.000
Length of neonatal stay, d, median (IQR)	15 (11-23)	18 (16-30)	.087
Strictures/stenosis, No.	13 (32%)	4 (28%)	1.000
Total inpatient days in the first year, median (IQR)	19 (13-28)	26 (18-39)	.068
Weight percentiles at 1 y, median (IQR) ^{†,§}	40 (14-72)	43 (26-51)	.725
Length percentiles at 1 y, median (IQR) ^{†,§}	50 (25-79)	30 (25-52)	.451
Head circumference percentiles at 1 y, median (IQR) ^{†,§}	71 (38-90)	76 (25-86)	.941
GQ at 1 y, median (IQR) ^{†,§}	95 (90-101)	93 (87-100)	.406
Major developmental delay, No. ^{†,§}	1/31	0/12	1.000
Minor developmental delay, No. ^{†,§}	2/31 (6.4%)	4/12 (33%)	.053

*Long segment group includes 3 infants with total colonic aganglionosis.

[†]Number of infants with recorded weights, lengths, and head circumferences at 1 year of age were 34, 32, and 30, respectively.

[‡]Developmental information was available on 43 nonsyndromic infants (see text).

[§]Nonsyndromic infants only.

Discussion

This study shows that although the mean GQ of nonsyndromic infants with Hirschsprung disease at 1 year of age is significantly less than the population mean, the development of most infants with Hirschsprung disease is within the normal range. This study also gives reassuring information on the physical growth of infants with Hirschsprung disease over the first year of life. We found no significant differences in the mean GQ of infants with short-segment vs those with long-segment disease. However, there was a trend toward higher incidence of minor developmental delay in those infants with long-segment disease.

Previous studies have reported that newborn infants undergoing surgery for major birth defects are at high risk of adverse developmental outcomes; these studies have included only small numbers of cases with Hirschsprung disease.^{9,32-34} Ludman et al³² used the Griffiths scales to study 30 infants who had undergone neonatal general surgery at 1 year of age; these infants were performing within the normal range but significantly less well in almost all areas of development compared with a control group. The same children were re-studied at 3 years of age; the children whose condition had resolved early in infancy were developing in a similar way to controls, but those children with ongoing treatments were still functioning below their peers, especially with language development.³⁴ Laing et al⁹ used the Bayley Scales of Infant Development to describe the 2-year outcomes of 97 infants born >32 weeks' gestation who underwent neonatal surgery (cardiac 51, general 46). The developmental outcomes were more concerning as 73% of infants performed below the population average in cognitive and language skills; 41% had developmental delay (24% with mild delay and 17% with significant delay). There was no significant difference in outcome between the cardiac and general surgery groups.

Both our study and that of Ludman et al used the Griffiths Mental Development Scales at 1 year of age. Children in the study by Laing et al had a mean age of 24 months at testing using the Bayley scales. Only 1 study has directly compared the Griffiths and Bayley scales, at ~13 months of age, and found Griffiths scores were consistently higher than Bayley scores.³⁵ Our results are more in keeping with those of Ludman et al; we found noticeably less developmental delay than did Laing et al in an older group of infants with a wider spectrum on neonatal surgical problems. The different developmental scales, the different age at testing, and a more homogeneous condition in our study may help to explain the difference in outcomes between our study and that of Laing et al.

Moore et al,³⁶ in a follow-up study of 178 children >4 years old with Hirschsprung disease, in South Africa, found that the overall growth was within normal limits. They also found that younger children were more likely to be underweight but that growth tended to improve with age. Another follow-up study of children with total colonic aganglionosis found that

25% were underweight at 5 years of age.¹⁴ Growth was one of the key outcomes of our study, and it was reassuring that, in a modern cohort of Hirschsprung disease, optimal early growth can be maintained.

A review of HAEC found a preoperative incidence of 6%-26% and a postoperative incidence of 5%-42%.³⁷ Although the time frames are not exactly the same, our results compare favorably with a perioperative and a post neonatal incidence of 7.5% and 19%, respectively. All infants presenting to our unit with Hirschsprung disease receive routine antibiotics pending diagnosis and septic screen results. Daily rectal washouts of the bowel are performed once a diagnosis of Hirschsprung disease is confirmed. Postoperative washouts were not routinely performed. Parents were counseled about the risks and symptom and signs of HAEC, and oral or intravenous metronidazole is used if there are signs of HAEC.

From our study cohort, we could compute that the incidence rate of Hirschsprung disease was ~1:5000 based on data from Australian Bureau of Statistics. This is in keeping with previously reported incidence of Hirschsprung disease.^{1,38}

Our study cohort has shown similar demographic profile to previous studies.^{4,39} Most were full-term, well-grown infants in good condition at birth. Predominant presenting symptoms were abdominal distention and delayed passage of meconium. Bilious aspirates/vomiting was third most common presenting feature (57%). Hence, it is important to consider Hirschsprung disease in the differential diagnosis of neonates presenting with bilious vomiting.⁴⁰

Some of the important limitations of our study are its retrospective nature with the inherent bias of data extracted from medical records, lack of controls, and the relatively small sample size. Moreover, developmental assessments were not performed with a uniform tool in all patients, thereby limiting generalizability of the results. Hence, although these early results are relatively reassuring, further prospective studies with a large sample size would give invaluable information about the long-term growth and development of children with Hirschsprung disease. ■

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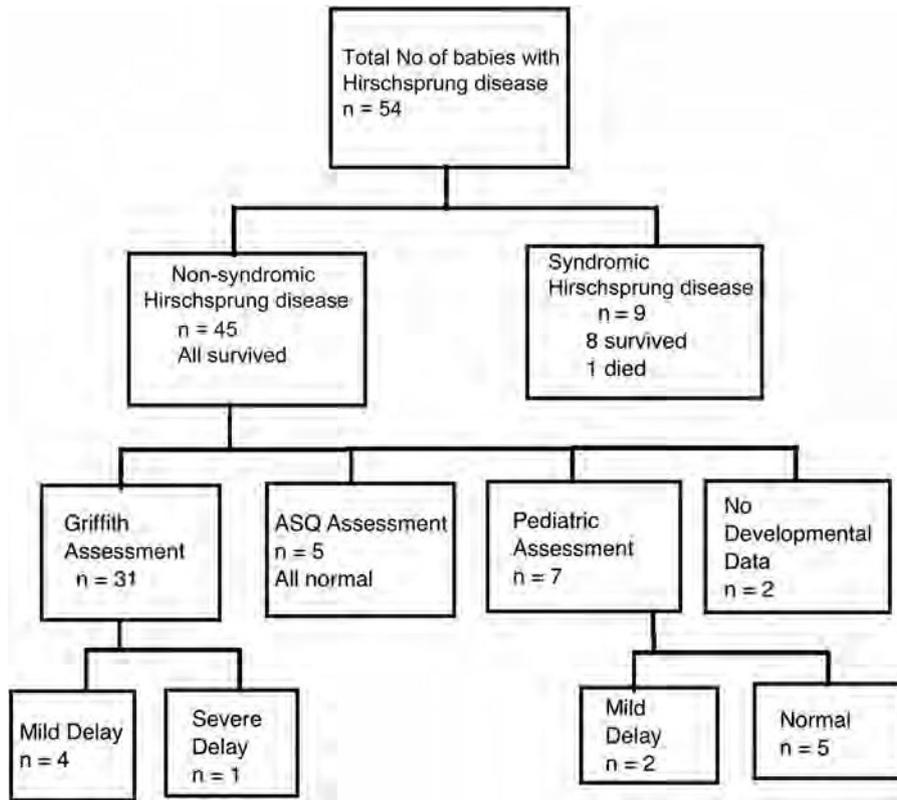


Figure 1. Patient flow diagram with outcomes over 1 year.

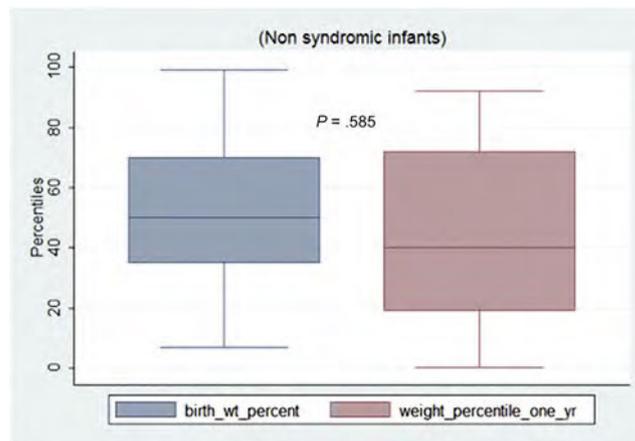


Figure 2. Comparison of weight percentiles between birth vs 1 year.

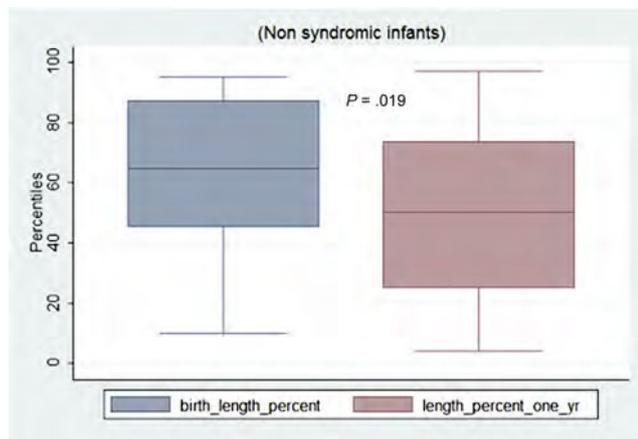


Figure 3. Comparison of length percentiles between birth vs 1 year.

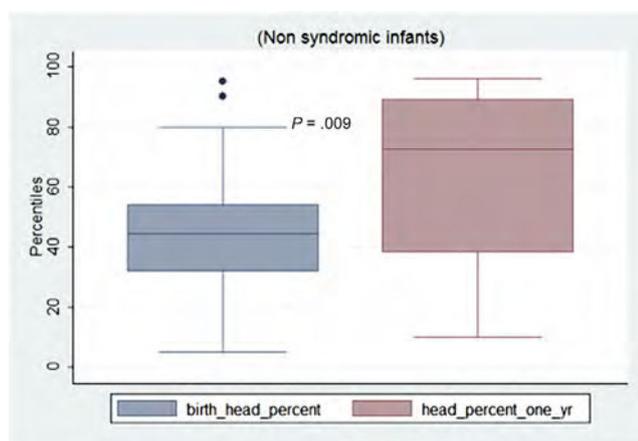


Figure 4. Comparison of head circumference percentiles between birth vs 1 year.



Growth and developmental outcomes of infants with gastroschisis at one year of age: A retrospective study^{☆,☆☆}

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Abstract

Background: The aim of the study was to describe the physical growth and developmental outcomes of babies born with gastroschisis.

Methods: We retrospectively reviewed all cases of gastroschisis in Western Australia born between 1997 and 2010.

Results: In the 128 pregnancies with fetal gastroschisis, 117 babies were live born. 112 (95.7%) survived to one year. 19% had z scores of < -1.28 for weight at birth (<10th centiles) compared with 30% at one year. Neurodevelopmental data were available in 88/112 (79%) of survivors (Griffiths scores in 67; reports of ages and stages questionnaire (ASQ) in 21). The mean GQ at 12 months was 99 (SD 9.8). Suboptimal neurodevelopmental outcomes were noted in eight. Complex gastroschisis (present at birth) and acquired gut related complications were associated with adverse long term outcomes. The incidence of acquired gut complications was least (5%) in those who underwent silo reduction as the primary management. However, on univariate and multivariate analysis, the type of primary reduction did not significantly influence the outcome.

Conclusions: A large proportion of infants with gastroschisis exhibit suboptimal weight gain during the first year. The incidence of adverse developmental outcomes appears to be low.

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Abbreviations: GQ, General Quotient; IQ, Intelligence Quotient; IQR, Inter Quartile Range; CDC, Centers for Disease Control and Prevention; ASQ, Ages and Stages Questionnaire; SD, Standard Deviation; GA, General Anesthesia; TPN, Total Parenteral Nutrition.

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Gastroschisis is a congenital anterior abdominal wall defect characterized by varying degrees of evisceration of the intra-peritoneal contents, which may include small intestine, large intestine, liver, spleen and urinary bladder [1]. The incidence of gastroschisis appears to be increasing worldwide with the current incidence 4–5 per 10,000 live births [2–5]. Postulated reasons for this increase in incidence include young maternal age, maternal substance misuse and other environmental teratogens [6–8]. However, a definitive etiology is yet to be established.

Prenatal recognition of fetal gastroschisis is now almost universal but there remains a substantial variation in the postnatal management. Various surgical approaches have been used in the management of gastroschisis including primary reduction under general anesthesia, primary ward reduction without general anesthesia and staged reduction with the use of preformed silos [9,10]. Overall, neonatal survival has improved over the past two decades, with reported rates of survival to discharge now greater than 90% [11,12]. Many studies have reported on the short term outcomes of babies with gastroschisis, typically survival until discharge and surgical complications (e.g. abdominal compartment syndrome, intestinal atresia, necrotizing enterocolitis) [13–15]. However, there is limited information on neurodevelopmental outcomes and physical growth parameters for children born with gastroschisis [16–18]. Hence, the aim of this study was to determine the physical growth and neurodevelopmental outcomes of infants born with gastroschisis at one year of age. We also aimed to identify modifiable risk factors associated with adverse outcomes to potentially improve postnatal management approaches.

1. Materials and methods

This was a retrospective study of all infants born with gastroschisis in Western Australia between January 1997 and December 2010.

All women with a prenatal diagnosis of fetal gastroschisis in Western Australia are provided with antenatal care and delivery management at King Edward Memorial Hospital, the sole tertiary obstetric unit in the state. A uniform antenatal management strategy is provided for pregnancies complicated by fetal gastroschisis. The timing and mode of delivery for fetal gastroschisis are based on obstetric indications. Elective early delivery is not performed. Immediately after birth, the gastroschisis is managed in a standard way by protecting the herniated viscera using a sterile bowel bag, decompressing the stomach using a large bore nasogastric tube, administering prophylactic antibiotics, fluid resuscitation with normal saline boluses as required and prompt transfer to Princess Margaret Hospital, which has the only level 3 neonatal surgical intensive care unit in Western Australia. Any infant with postnatally diagnosed gastroschisis in the state is transferred directly to Princess Margaret Hospital.

The majority of infants prior to 2008 underwent the procedure of primary reduction in the operating room under general inhalational anesthesia, while a small percentage underwent primary reduction without general anesthesia under the cover of intravenous paracetamol (ward reduction). Since 2008, the unit has moved predominantly to the method of silo reduction followed by final closure under general anesthesia [13]. In total, ten surgeons were part of the surgical team during different stages of the study period and the number of gastroschisis surgeries done by each surgeon was almost equal. The experience of the surgeons ranged from 1 to 25 years post specialist qualification.

Post-reduction, infants in all groups were monitored in a similar way for the development of abdominal compartment syndrome, that is, systemic blood pressure, urine output, metabolic or mixed acidosis, lactate levels, discoloration and mottling of lower limbs and abdomen, excessive pain necessitating the administration of high dose narcotic analgesics, need for excessive ventilator pressures. Formal measurement of intra-abdominal pressure (bladder pressure or intra-gastric pressure) was not performed. In all groups, feeds were started as soon as the clinical condition was considered stable. The unit policy is to begin enteral feeds using expressed breast milk via nasogastric tube as soon as possible, preferably within 48 h after the reduction. This policy is based on the results from a previous study from our unit in 2000 which demonstrated that the early commencement of enteral feeds is associated with a decreased time to full enteral feeds and early discharge [19]. Feeds are commenced at the rate of 1–2 ml every 3 h as boluses and gradually increased at daily increments of 15–20 ml/kg/day as tolerated. Presence of small to moderate amounts of bilious aspirates is not considered a contraindication to enteral feeds; however, feeds are withheld if there are regular nasogastric aspirates >50% of previously administered feeds. Elemental or semi-elemental formulae are used at the clinician's discretion in babies with prolonged intolerance to feeds with expressed breast milk.

Postoperatively, all infants were managed in the neonatal intensive care unit until their discharge. All discharged infants with surgical conditions, including gastroschisis, are routinely followed to assess physical growth and neurodevelopmental outcomes at least until one year of age.

For this study, cases were identified by interrogating the Fetal Medicine Service and Neonatal Databases at King Edward Memorial Hospital for Women and Princess Margaret Hospital for Children, Perth, Western Australia. Relevant clinical details during hospital stay and until one year of age were obtained by reviewing the medical record charts of the cases. All live born neonates with gastroschisis were eligible for inclusion. None were excluded.

Physical growth was assessed using weight, head circumference and length measurements at birth and one year of age. Z scores for the physical growth parameters at birth were calculated using Fenton charts [20], which are the commonly used charts for monitoring the growth of preterm infants until post-conceptual age of 44 weeks. Z scores for

Table 1 Clinical characteristics (simple versus complex gastroschisis).

Simple gastroschisis (n = 102)				Complex gastroschisis (n = 15)			
	Median	IQR	N ^d	Median	IQR	N ^d	P value
Maternal age (years)	22.5	20, 27	96	24	22, 26	13	0.162
Gestational age at delivery (weeks)	36	35, 37	102	35	35, 36	15	0.244
Birth weight (g)	2425	2100, 2735	102	2350	2045, 2660	15	0.91
Age at primary treatment (h)	4	3, 5	70	3.5	3.0, 4.6	11	0.427
Age at commencement of enteral feeds (days)	5	3, 9	100	6.5	3, 9	14	0.396
Age at full enteral feeds (days) ^a	17	14, 23	98	56	22, 90	12	0.0047
Length of hospital stay (days) ^b	23	19, 34	102	90	22, 285	15	0.0026
Days to discharge for survivors ^c			102			15	<0.0001
≤30	69 (68%)			4 (27%)			
31–59	22 (21%)			3 (20%)			
≥60	11 (11%)			8 (53%)			

^a 7 infants did not achieve full enteral feeds (five died).

^b Includes data from five infants who died.

^c One patient was still in hospital at one year of age.

^d Number of patients from which data were available.

physical growth at one year were calculated using the CDC 2000 growth charts [21]. To assess infant neurodevelopment, the scores in the Griffiths Mental Development Scales General Quotient (GQ) at one year of age were used. If the Griffiths scores were not available, data from Ages and Stages Questionnaire (ASQ) were used.

The Griffiths Mental Development Scales assess development in five separate areas: Locomotor, Personal and Social, Hearing and Speech, Eye and Hand Coordination and Performance. The five subscales can be assessed and scored separately and then combined to provide an overall General Quotient reflecting the child's developmental performance level relative to the population. When all five subscales are combined to form the total scale, the mean score is 100.2 and the standard deviation (SD) is 12.8. A GQ more than 87 is considered normal. A GQ of <75 falls >2 SD below the mean and hence denotes severe disability [21]. The Griffiths developmental assessments in our cohort were conducted by a single developmental pediatrician (JM) in nearly 90% of cases; the remaining 10% were performed by qualified pediatricians.

The ASQ is a parent-completed screening tool that uses parent observation to assess child development and behavior. The ASQ questionnaire at each age point contains six questions in each of five domains of development—communication, fine motor, gross motor, problem solving and personal social, for a total of 30 questions. Answer options for each question include 'yes', 'sometimes' or 'not yet'. A 'yes' response receives 10 points, 'sometimes' receives 5 points and 'not yet' receives 0 point. Each of the five domains is scored separately. A score of ≥2 SD below the mean in any one of the domains is considered a 'fail' on the ASQ [22]. It is a validated screening tool for identifying neurosensory disability [23,24]. Parents of all high risk infants discharged from our unit routinely receive the ASQs at 4, 8, 12 and

24 months of age. The coordinator of developmental follow up program enters the results of the ASQ into the departmental computer system, which then identifies a baby as having "passed" or "failed" the ASQ.

For this study there were three main outcomes of interest:

1. Suboptimal long-term neurodevelopmental outcome, defined as a GQ >2 SD below the mean (i.e., GQ <75) or ASQ score >2 SD below the mean in at least one of the domains or one or more of the following: moderate to severe cerebral palsy (CP), blindness (defined as visual acuity of <6/60 in the better eye) and sensorineural deafness requiring hearing aids.
2. Failure to thrive at one year of age: defined as z scores for weight <-1.28 (i.e., less than 10th centile) and
3. Prolonged duration of hospital stay (>60 days).

The conduct of this study was approved by the Institutional Ethics Committee.

1.1. Statistical analysis

Statistical analysis was performed with Stata 11 software (StataCorp LP, 4905 Lakeway Drive, College Station, Texas, USA). Median and interquartile range (IQR) were calculated for the nonparametric data; mean and standard deviation were calculated for normally distributed data. To compare the physical growth z scores at birth compared with one year of age, Wilcoxon matched pairs signed rank test was used. Univariate and multivariate logistic regression analyses were conducted to explore the associations between clinical variables and outcomes of interest. Odds ratios and their 95% confidence intervals were calculated. All P values less than 0.05 were considered as statistically significant.

2. Results

There were 128 pregnancies complicated with fetal gastroschisis identified between January 1997 and December 2010. Six women elected to terminate their pregnancy (all <20 weeks gestation) and a further 5 fetuses were stillborn. The stillbirth rate in this cohort was 4.1% (5/122). 113 (96.6%) of the babies were born at King Edward Memorial Hospital, reflecting a high rate of prenatal diagnosis within the state. All infants were transferred to Princess Margaret Hospital for Children soon after delivery for definitive management.

Baseline characteristics and short-term in-hospital outcomes are shown in Table 1. Of the 117 live born infants, 65 (55%) were male and 52 (45%) female. 102 were simple and 15 complex gastroschisis cases. Gastroschisis was defined as simple if the bowel was intact, continuous and not compromised or breached at delivery. "Complex gastroschisis" was defined as the presence of at least one of the following: intestinal atresia, perforation or intestinal necrosis at delivery or presentation, or missed atresia [25,26]. "Acquired gut complication" was defined as the occurrence of ischemic gut necrosis, enteric fistula, strictures, intraabdominal abscess or abdominal wall infections after the primary reduction of gastroschisis.

Complete closure under general anesthesia (GA) was performed in 68 neonates, preformed silo under GA in 14, preformed silo in the ward without GA in 19 and ward reduction without GA in 16 cases.

There were five deaths in this cohort. The first case was delivered at 35 weeks gestation and underwent a ward reduction which was complicated by abdominal compartment syndrome. The intestines were subsequently exteriorized and placed in a bowel bag, leading to prolonged gut morbidity. Severe nosocomial pneumonia due to Respiratory Syncytial Virus occurred on day 18 of life and the baby died on day 49 secondary to intractable respiratory failure. The second infant had congenital short gut syndrome and died on day 255 due to Total Parenteral Nutrition (TPN) related complications. The third infant had congenital volvulus and gangrene of the intestines at birth and died on day 21. The fourth death was secondary to multiple endocrine disorders and lobar holoprosencephaly. The final death occurred in an extremely preterm infant (26 weeks gestation at birth) who developed necrotizing enterocolitis and adhesions requiring multiple laparotomies and died on day 178.

The remaining 112 infants survived until at least one year of age. One year data were available on weight for 95/112 (85%), length for 82 (73%) and head circumference for 81 infants (72%). One woman moved interstate and her infant was not able to be assessed.

There were no significant differences between z scores for weight or length at birth and one year. The z scores for head circumference were higher at one year of age compared with birth in those with simple gastroschisis ($p < 0.0001$)

(Tables 2A, 2B). There were more infants with z scores for weight less than -1.28 (i.e., <10th percentile) at one year of age compared with birth, but this was not statistically significant (Tables 2C and 2D). The incidence of intestinal failure (time to full enteral feeds ≥ 60 days) was 21% (6/28) in those with failure to thrive (z scores for weight less than -1.28) compared with 6% (4/67) in those who had adequate weight gain (z scores ≥ -1.28); $p = 0.059$. The incidence of prolonged hospitalization (≥ 60 days) was 25% (7/28) in those infants with failure to thrive at one year of age compared with 9% (6/67) in those who had adequate weight gain ($p = 0.051$).

Neurodevelopmental follow up data were available in 79% of survivors (Griffiths scores in 67; reports of ASQ in 21). Twenty nine infants were lost to follow up. There were no significant differences in baseline characteristics and hospital outcomes between those who were assessed at one year of age compared with those who were lost to follow up (details not provided). The mean GQ at 12 months was 99 (SD 9.8). This compares favorably with the healthy population norms wherein the mean score is 100.2 and the SD is 12.8. Only one infant had a GQ < 75. Details of the eight infants who had adverse neurodevelopment outcomes are shown in Table 3.

Lower gestational age, high CRPs within 72 h of life, complex gastroschisis and prolonged duration of hospital stay were associated with increased risk of the composite outcome of "death or severe disability" on univariate analysis (Table 4). However, on multivariate analysis, only complex gastroschisis was associated with increased risk of this outcome (OR: 5.34, 95% CI: 1.05, 27.3; $p = 0.044$).

Low birth weight, small for gestational age at birth, high CRPs within the first 72 h of life, complex gastroschisis, delayed commencement of enteral feeds and prolonged duration of hospital stay were associated with increased risk of failure to thrive at one year of age on univariate analysis (Table 5). However, on multivariate analysis, none of these were statistically significant.

Lower gestational age, lower birth weight, delayed commencement of enteral feeds, complex gastroschisis and acquired gut complications were associated with an increased risk of prolonged hospitalization (> 60 days) (Table 6). However, on multivariate analysis, only complex gastroschisis (OR: 30.6; 95% CI: 4.6, 203.6; $P: 0.0001$) and acquired gut complications (OR: 86.3; 95% CI: 7.2, 1033.7) were associated with a higher risk of prolonged duration of hospital stay. Higher birth weight was associated with a lower risk of this outcome.

3. Discussion

Gastroschisis represents a birth defect which is continuing to evolve in terms of the perinatal management, predominantly due to an increase in both its occurrence and prenatal recognition [27]. The improved prenatal detection facilitates delivery in perinatal centers with the capability of providing

Table 2A z scores for anthropometry at birth versus at one year (simple gastroschisis).

	Birth Median (IQR)	One year Median (IQR)	P value
Weight z scores	0.69 (1.08, 0.05)	0.48 (1.59, 0.02)	0.655
Length z scores	0.5 (1.4, 0.4)	0.37 (1.3, 0.49)	0.333
Head circumference z scores	0.3 (0.9, 0.4)	0.11 (0.51, 0.74)	<0.00001

Table 2B z scores for anthropometry at birth versus at one year (complex gastroschisis).

	Birth Median (IQR)	One year Median (IQR)	P value
Weight z scores	0.95 (1.14, 0.17)	1.26 (1.8, 0.11)	0.859
Length z scores	0.4 (1.1, 0.15)	0.29 (1.27, 0.03)	1.000
Head circumference z scores	0.25 (0.8, 0.7)	0.24 (0.78, 0.55)	0.263

prompt surgical management strategies and the increase in incidence provides a case load of sufficient volume for pediatric surgical units to development expertise in care of the neonate with gastroschisis. These two phenomena are demonstrated in the data from our study. Although Western Australia is a geographically large state with an annual birth rate of approximately 25,000 during the period of review, survival rates, length of hospital stay and the time to full enteral feeds in our cohort all met national as well as international benchmarks [1,11,15,28].

Even though the weight z scores at one year were not significantly different from the weight z scores at birth, it was concerning that nearly 30% of the infants had a weight z score below the -1.28 (<10th percentile) at one year of age. Failure to thrive in infancy can be associated with persisting deficits in IQ later in life[29]. We speculate that the combination of congenital gastroschisis and postnatal growth deficit may adversely impact on infant neurodevelopment. More attention needs to be paid to optimizing the nutrition of these infants both during hospital stay as well as after discharge home. Interestingly, head growth seemed to be spared in those with simple gastroschisis, with better catch up on z scores at one year of age compared to birth measurements. This may be nature's mechanism of selective brain protection at the cost of undernourishment of the rest of the body.

Some studies have reported on the developmental outcomes of congenital abdominal wall defects without differentiating between gastroschisis and omphalocele [30,31] whereas few studies have focused exclusively gastroschisis [14,17,18]. In a prospective study, South and co-workers assessed neurodevelopment at 16 to 24 months in 17 infants born with gastroschisis. Interestingly, similar to the data in our cohort, approximately one-third had weight or length below the 10th percentile at assessment. The small for gestational age fetuses tended to be smaller at assessment and have lower neurodevelopmental scores, although overall there was no neurodevelopmental delay [17]. Henrich et al. followed 40 infants with gastroschisis until ten years of age. They concluded that normal intellectual function can be expected in children with gastroschisis. However it is difficult to interpret their results as details of the parental questionnaire used to assess developmental and other outcomes were not provided. Additionally, the follow up rate in this series was low (22/40) [18]. In a recent publication, Gorra et al. reviewed 46 infants with simple gastroschisis up to two years of age using the reports from the state-sponsored Developmental Tracking Infant Progress Statewide (TIPS) program. They excluded 59 infants with "complex gastroschisis". Poor outcomes were defined as scores of "failure" or "moderate/high risk" on the screening

Table 2C Number of infants with z scores less than 1.28 (<10 centiles) (simple gastroschisis).

	Birth	One year	P value
Weight z scores less than 1.28	19/102 (19%)	24/85 (28%)	0.120
Length z scores less than 1.28	25/88 (28%)	19/74 (26%)	0.696
Head circumference z scores less than 1.28	16/94 (17%)	4/72 (6%)	0.024

Data presented as number (%).

Table 2D Number of infants with z scores less than 1.28 (<10 centiles) (complex gastroschisis).

	Birth	One year	P value
Weight z scores less than 1.28	3/15 (20%)	4/9 (44%)	0.20
Length z scores less than 1.28	3/12 (25%)	1/7 (14%)	0.580
Head circumference z scores less than 1.28	0/14 (0%)	1/8 (12.5%)	0.175

Data presented as number (%).

Table 3 Clinical characteristics of infants who had suboptimal neurodevelopmental outcomes.

Case	1	2	3	4	5	6	7	8
Gestational age (weeks)	36	37	36	34	33	36	35	34
Gender	Female	Female	Female	Female	Female	Male	Female	Female
Birth weight (g)	2185	2645	1980	1970	1640	2365	1950	1650
Birth weight z scores	1.35	0.74	1.8	0.79	1.05	0.95	1.36	1.53
Birth head circumference z scores	0.8	0.3	1.4	0.7	2.2	0.7	0.7	.5
Primary treatment ^a	WR	TR	TR	TR	SR, no GA	TR	TR	SR with GA
One year weight z scores	1.46	1.11	2.2	1.61	NA	1.54	0.11	2.57
One year head circumference z scores	1.03	0.5	1.24	2.54	NA	NA	1.58	0.88
Associated anomalies	Nil, simple gastroschisis	Ileal atresia, complex gastroschisis	infarcted bowel at birth; Complex, gastroschisis	Duplication cyst, complex gastroschisis				
Time to full enteral feeds (d)	11	14	19	13	24	270	475	440
Duration of hospital stay(d)	13	16	27	18	30	285	299	472
Co morbidities		Nil	Nil	Nil	Microcephaly at birth	Nil	Nil	
Type of adverse outcome	Failed ASQ	Amblyopia in one eye	Cerebral palsy	Cerebral palsy	Failed ASQ	Delayed motor milestones	Failed ASQ	GQ 65
Number of surgical procedures under GA within first year of life	0	0	0	0	NA	8	4	8

NA: Not available.

^a WR: Ward reduction without GA; SR, no GA: Silo reduction without GA; TR: Reduction in operating room under GA; SR with GA: Silo application in operating room under GA.

assessment or enrolment in early intervention services by 2 years. Children with gastroschisis were compared with case-matched nonsurgical, non-syndromic children of similar gestational age and birth weight [14]. They found a high number of children who either failed the screening test (23.9%) or who had enrolled in early intervention services (15.2%). However, they attributed the developmental delays to associated prematurity rather than gastroschisis as the gestational matched controls had similar neurodevelopmental outcomes. They concluded that children born with gastroschisis had similar 2-year neurodevelopmental outcomes as nonsurgical, non-syndromic neonatal intensive care unit children of similar gestational age and birth weight.

The data from our state-wide cohort are consistent with these prior reports, with the majority of children having no adverse neurodevelopmental outcomes at one year of age. Clearly, ongoing assessments of the children are required beyond this early age and we are currently completing a

prospective study to provide further long-term outcome data [32].

It is recognized that neonates undergoing cardiac surgery have an increased incidence of subsequent adverse neurodevelopmental outcomes which may be related to the intrinsic cardiac defect in addition to the surgical procedures conducted [33]. Evidence is accumulating that those neonates with congenital anomalies undergoing non-cardiac surgeries are also at increased risk of adverse neurodevelopmental outcomes [34–37]. Hence, further studies focusing on long term outcomes (at least until primary school age) in children born with gastroschisis are clearly required and are currently in progress in our state and other centers.

This series is one of the largest published to date on short-term neurodevelopmental outcomes in children born with gastroschisis. The main strength of our study is its population rather than institution basis with near complete ascertainment of data, commencing from the antenatal period until

Table 4 Death or disability (Univariate analysis).

	Odds Ratio	95% CI	P value
Gestational age	0.69	0.50, 0.95	0.025
Gender (male compared to female)	0.38	0.11, 1.36	0.138
Birth weight	0.998	0.996, 1.00	0.052
Birth weight z scores	1.02	0.51, 2.05	0.950
Maximum CRP within 72 h	1.01	1.001, 1.02	0.019
Age at commencing enteral feeds	0.99	0.87, 1.14	0.980
Complex gastroschisis	6.43	1.68, 24.51	0.006
Acquired gut complications	3.6	0.92, 13.9	0.065
Cesarean section	0.38	0.08, 1.88	0.240
Primary treatment (when compared to reduction under GA)			
Silo reduction without GA	0.40	0.05, 3.5	0.412
Ward reduction without GA	1.6	0.29, 9.07	0.584
Silo reduction in operating room after attempted complete closure	0.61	0.07, 5.41	0.655
Silo versus complete primary reduction	0.44	0.09, 2.18	0.319
Hospital stay more than 60 days	5.70	1.52, 21.44	0.010

Table 5 Failure to thrive at one year of age (Univariate analysis).

	Odds Ratio	95% CI	P value
Gestational age	0.93	0.71, 1.22	0.063
Gender (male compared to female)	0.88	0.36, 2.13	0.778
Birth weight	0.99	0.99, 1.00	0.002
Birth weight z scores	0.37	0.20, 0.70	0.002
Maximum CRP within 72 h	1.01	1.003, 1.02	0.007
Age at commencing enteral feeds	1.12	1.01, 1.23	0.021
Complex gastroschisis	2.06	0.51, 8.35	0.308
Acquired gut complications	3.38	0.93, 12.19	0.063
Cesarean section	0.75	0.29, 1.90	0.540
Primary treatment (when compared to reduction under GA)			
Silo reduction without GA	0.68	0.19, 2.38	0.845
Ward reduction without GA	0.67	0.13, 3.63	0.650
Silo reduction in operating room after attempted complete closure	1.78	0.53, 5.96	0.349
Silo versus complete primary reduction	1.14	0.45, 2.56	0.787
Hospital stay more than 60 days	4.1	1.18, 14.4	0.026

Table 6 Prolonged duration of hospitalization (>60 days) (Univariate analysis).

	Odds Ratio	95% CI	P value
Gestational age	0.66	0.50, 0.87	0.003
Gender (male compared to female)	0.17	0.60, 4.99	0.306
Birth weight	0.997	0.996, 0.998	<0.0001
Birth weight z scores	0.70	0.38, 1.28	0.246
Maximum CRP within 72 h	1.007	0.99, 1.01	0.055
Age at commencing enteral feeds	1.11	1.02, 1.22	0.020
Complex gastroschisis	10.5	3.15, 35.12	0.0001
Acquired gut complications	29.5	7.99, 109.11	0.0001
Cesarean section	0.39	0.10, 1.46	0.160
Primary treatment (when compared to reduction under GA)			
Silo reduction without GA	0.28	0.03, 2.34	0.242
Ward reduction without GA	1.17	0.28, 4.82	0.823
Silo reduction in operating room after attempted complete closure	0.49	0.17, 4.33	0.843
Silo versus complete primary reduction	0.49	0.13, 1.84	0.293

discharge from the hospital. More than 95% of children with gastroschisis received antenatal care and delivered at the tertiary perinatal center providing a uniform care strategy. All live born children were managed in the sole tertiary neonatal surgical center in Western Australia and although surgical strategies have altered over time these changes have been uniform throughout the population studied. The limitations of the study are its retrospective nature, short duration of follow up (one year) and relatively low follow ups and low percentage of Griffiths assessments at one year of age. Another limitation may be the use of ASQ where Griffiths assessment reports were not available. Some studies have suggested that the ASQs provide a simple, valid, and cost-effective method to identify developmentally delayed infants [20,22,23,31,38] whereas the others have not [15,21,24].

In our study cohort, the two main variables associated with adverse outcomes were complex gastroschisis (present at birth) and acquired gut complications following primary reduction. Of these, complex gastroschisis may not be preventable as the intestinal complications are already present at birth whereas acquired gut related complications are potentially preventable. The incidence of acquired gut complications was least (5%) in those who underwent silo reduction as the primary management when compared with 12% for primary reduction under GA, 25% for ward reductions and 14% for those who underwent silo reduction after a failed attempt at complete reduction under GA. However, on univariate as well as multivariate analyses, the type of primary reduction did not significantly influence the outcomes. Hence, "the jury is still out" regarding the best modality of primary reduction [39]. Randomized trials or prospective studies with large sample size are needed to answer this question definitively.

4. Conclusions

Infants born with gastroschisis have high survival rates. A large proportion of infants with gastroschisis have sub-optimal weight gain by one year of age. The outcomes for the West Australian cohort compare favorably with international benchmarks. The incidence of adverse neurodevelopmental outcomes at one year of age appears to be low.

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Dr Shripada Rao | Neonatologist | Neonatal Intensive Care Unit
15 Hospital Avenue, Nedlands | Locked Bag 2010, Nedlands WA 6909
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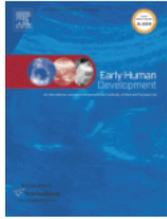
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Publication: Early Human Development

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A modern era comparison of right versus left sided congenital diaphragmatic hernia outcomes

Author:

Michael Collin, Sarah Trinder, Corrado Minutillo, Shripada Rao, Jan Dickinson, Naeem Samnakay

Publication: Journal of Pediatric Surgery

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Author: Kiran More, Shripada Rao, Judy McMichael, Corrado Minutillo

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