Use of enzymes to improve feed conversion efficiency in Japanese quail fed a lupin-based diet

by

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Declaration

The work presented in this thesis is the original work of the author, and none of the material in this thesis has been submitted either in full, or part, for a degree at this or any other university or institution. The design of the study and the writing of the manuscript were carried out by myself after discussion with my supervisors, Professor Graeme B. Martin and Associate Professor Irek Malecki.

Mahmoud Mohamed Khalil

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Table of Contents

Summary

There is growing interest in quail production worldwide because, compared to broiler chickens, they are fast-growing, healthy, easy to handle, and have a high feed conversion ratio (FCR). Australian quail have a large body mass and therefore the potential to be some of the best meat-producing quail in the world, but Australian quail producers have been experiencing unprecedented increases in feed costs, mostly driven by the price of imported soybean meal. Feed is the biggest cost (70%) of total quail production, so there is great interest in replacing soybean meal.

One possibility is to replace soybean meal with Australian sweet lupin meal because they have similar contents of protein and energy. However, lupin meal rarely comprises more than 5% of commercial poultry diets. This is mainly because 35% of the lupin kernel is composed of complex non-starch polysaccharides (NSPs). The main NSP in lupin is pectin with branched side-chains of xylan.

Non-starch polysaccharides are indigestible in monogastric animals because they do not secrete the required enzymes to break them down. The digestion of lupin meal is thus very limited with several adverse consequences: i) accumulation of undigested NSP or pectin increases the viscosity of the gut, reducing digestibility of dry matter and growth performance; ii) undigested pectin in the gut increases the water intake, resulting in wet droppings (wet litter), causing odours, coccidiosis outbreaks, soiled eggs; iii) undigested nutrients are excreted into the environment.

The work in this thesis aims to eliminate the negative effects of feeding lupin-based diets to Japanese quail by supplementing lupin meal with exogenous enzymes that break down NSPs. To achieve this goal, three hypotheses were tested:

a) A synergistic interaction between pectinase and xylanase will improve the physico-chemical properties of lupin more than pectinase or xylanase alone;
b) For Japanese quail, a combination of pectinase and xylanase will improve feed conversion ratio (FCR) more than the pectinase or xylanase alone;
c) There will be an optimal dose of the combination of pectinase and xylanase that will offer the best improvement in the nutritive value of lupin meal.

To test my first hypothesis, ground lupin kernels were incubated *in vitro* with pectinase and xylanase, alone and in combination, and the effects on the physical and chemical properties were measured (water holding capacity, viscosity, filtration rate, pectin content). The results revealed that the combination of pectinase and xylanase significantly improved the physico-chemical properties of lupin more than the individual enzymes. This was achieved by a reduction in water-holding capacity by (3%), viscosity (11%) and pectin content (24%) compared to the individual enzymes.

The concept was transferred to an *in vivo* experiment with Japanese quail, using diets containing 10% or 20% lupin meal, with each level of lupin content supplemented with no enzyme (control), pectinase, xylanase, or pectinase + xylanase. The combination of pectinase and xylanase improved growth performance and the FCR in quail consuming a diet containing 10% and 20% lupin kernel compared to the individual enzymes. On the other hand, at this stage, the best diet is one based on 10% lupin meal supplemented with the enzyme combination.

Finally, in the third experiment, I returned to *in vitro* methodology to attempt to identify the optimal doses for the enzyme combination. Ground lupin kernels were incubated with combinations of 3 doses of polygalacturonase (0.35, 0.7, 1.4 U/g), 3 doses of pectinesterase (0.1, 0.2, 0.4 U/g), and 3 doses of xylanase (0.095, 0.19, 0.38 U/g). In the physico-chemical properties, there were some significant differences among the enzyme-dose combinations, but clear overall dose-responses were rarely evident. It seems that all of treatments led to major improvements in the physico-chemical properties of lupin meal, but all the doses were exerting maximum effects, making it difficult to detect a dose-response and thus identify clear optimal doses.

In conclusion, quail producers can include up to 10% of Australian sweet lupin meal in the diet if they supplement with a combination of pectinase and xylanase. This will bring important benefits to the industry by reducing the total production cost without compromising growth performance, FCR or bird welfare.

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Thank you all,
Mahmoud Khalil

List of Abbreviations

ANOVA	Analysis of variance
DM	Dry matter
FCR	Feed conversion ratio
G	Gram
ICU	*Intensive care unit*
IU	International Unit
Kcal	Kilocalorie
Kg	Kilogram
M	Molarity
mg	Milligram
MJ	Megajoule
µl	Microliter
ml	Milliliter
mm	Millimeter
mPas	Millipascal
NSP	Non-starch polysaccharides
P	Probability
pa	Per annual
rpm	Rotation per minute
SBM	Soybean meal
sec	Second
sem	Standard error of mean
t.	Tonne
U/g	Unit/gram
vs.	Versus

Chapter 1

General Introduction

There is growing interest in quail production worldwide because, compared to broiler chickens, they are fast-growing, healthy and easy to handle. Australia has some of the best meat-producing quail in the world, with a greater average live weight (240 g vs. 160 g), but their feed conversion ratio (FCR) is much higher than that of broilers (3.3 vs. 1.7; Arslan & Seker, 2002; Selle *et al,* 2003; Wu *et al*, 2004; Namra, 2006; Olkowski *et al*, 2010; Nalle *et al*, 2010; Chantiratiku *et al*, 2010).

Exacerbating this problem, Australian quail producers have been experiencing unprecedented increases in costs due to the global increase in prices for major dietary components, such as soybean meal (SBM). Feed is the biggest cost (70%) of total quail production (Bhuiyan, 1989; Perez-Maldonado *et al*, 1999; Louw *et al*, 2011; Glatz, 2012), so there is great interest in replacing expensive and imported SBM ($590/tonne) with cheaper, local feedstuffs such as lupin meal ($320/tonne).

However, Australian feed manufacturers and poultry producers still only use 5% lupin in poultry diets because it contains large amounts of indigestible carbohydrates (35%), mainly non-starch polysaccharides (NSPs, Evans *et al,* 1993; Chesson, 1993; Annison & Choct, 1993; Perez-Maldonado *et al*, 1999; Kocher *et al*, 2000). The main NSP content of lupin, in particular in the kernel, is pectins (polygalacturonans), galactans, rhamno-galacturonans, arabinans and xylans (Bailey *et al*, 1974; Brillouet & Roichet, 1983; Evans *et al*, 1993; Hughes *et al*, 1997). Pectin increases the viscosity of digesta in the intestinal tract and inhibits the digestion of nutrients. In addition, pectin is a gel-like material that entraps water within cell-wall lattices, thus increasing the water-holding

capacity of the digesta and, in turn, the incidence of wet droppings (wet litter) in the poultry shed. The poor growth rates and feed conversion efficiencies of lupin (Choct & Annison, 1992; Annison *et al*, 1996; King *et al*, 1997; Hughes *et al*, 2000; Kocher *et al*, 2000; Ali *et al*, 2005; 2006ab), are therefore associated with management problems such as odour, coccidiosis outbreaks, soiled eggs and excretion of digestible nutrients to the environment (Annison *et al*, 1996; Ali, 1997; Hughes *et al,* 2000; Kocher *et al,* 2000).

To overcome these issues, the main pectin chains in lupin, and the xylan chains attached to them, must be broken down. However, poultry do not produce the necessary enzymes so we need to treat lupin-based poultry diets with exogenous pectinases. Studies to date have shown that this leads to a 5-fold breakdown of the complex cell-wall network, reduces viscosity by 18%, releases digestible nutrients, and increases nutrient absorption by 17% (Annison and Choct 1993; Schutte *et al*. 1995). The outcome is a 6% increase in weight gain, 6% reductions in FCR and wet droppings, and an 8–20% reduction in excretion of digestible nutrients into the environment (Gracia, *et al*, 2003; Saleh, *et al*, 2003; Odetallah, *et al*, 2005; Wang *et al*, 2005; Hua & Kang-ning, 2008; Ali *et al,* 2009).

Importantly, the various available enzymes that can act on cell-wall polysaccharides can also work synergistically to improve productivity. For example, for broilers fed wheat-based diets, a combination of xylanase and phytase can improve both weight gain and FCR by 8-9% above the levels achieved by using phytase or xylanase on their own (Ravindran *et al*, 1999; Zyla *et al*, 1999; Wu *et al*, 2004).

In this thesis, I tested the general hypothesis that breakdown of cell-wall materials, in particular pectins and xylans, would improve the nutritive value of lupin for quail. I expect that successful breakdown of pectins and xylans would reduce viscosity and water-holding capacity, and therefore the incidence of wet droppings. The breakdown of pectins would improve dietary metabolisable energy, growth and feed conversion efficiency with considerable financial benefits for quail producers.

Chapter 2

Literature Review

2.1. Introduction

Soybean meal (SBM) is commonly used as a source of protein in poultry feed, all around the world. However, the increasing cost of SBM, the most expensive ingredient in poultry feed, is becoming a serious threat for poultry producers who wish to expand their production. It is therefore necessary to evaluate alternative protein sources. One alternative is lupin, a legume crop grown worldwide. There are over 200 lupin species, one of which is the Australian sweet lupin (*Lupinus angustifolius*). Lupin is commonly used as a protein source for ruminant livestock, as well as for human consumption, but their use for monogastric livestock is still very limited, primarily because they contain anti-nutritional factors that depress digestibility and utilization, resulting in poor animal performance. In this review, I will describe some of these issues with a focus on poultry species, especially the Japanese quail.

2.2. Japanese quail (*Coturnix japonica*)

Japanese quail (*Coturnix coturnix japonica*) is a species that belongs to the same biological family as chickens and pheasants, the *Phasianidae*, so they have many similar physiological and behavioral characteristics. The Japanese quail has a small to medium body size and plays major roles in industry and research, and it is the most common species bred for human consumption (Mizutani, 2003; Ikhlas *et al*, 2010). The species was first domesticated in Japan, from where it spread all over the world, and large numbers can now be found in Asia, Europe and Africa. Nowadays, the main

producer of quail meat is China (over 160,000 t), followed by Spain (9,000 t), France (8,000 t), and Italy and the USA (3,000 t each; Ioniță *et al*, 2011).

The breeding of quail for meat and egg production has grown rapidly, aided by early sexual maturity (4-6 weeks of age), short generation interval, inexpensive maintenance and good egg-laying ability (Baumgartner, 1994; Cain and Cawley, 2000). Quail meat is considered superior to red meat as it has lower levels of calories, and considered superior to chicken and duck meat because it has higher levels of protein, omega-3 fats, iron and vitamins A, C, B_2, B_6, and B_{12} (Ioniță *et al*, 2011). Quail eggs are rich in vitamins, minerals and antioxidants, and have a much higher nutritive value than chicken eggs. Quail eggs are also thought to confer medical benefits by strengthening the immune system, increasing brain activity, increasing haemoglobin levels, and removing heavy metals and toxins from the blood (Troutman, 1999-2012; Lalwani, 2011; Tunsaringkarn *et al,* 2013).

Nutritional requirements for Japanese quail

The requirements for Japanese quail differ from those for the chicken, as can be seen in the 1994 recommendations of the NRC (Table 2.1). In addition, feed conversion ratio (FCR) for quail is higher than for chickens (3.3-4.9 vs. 1.3-2.2), and this increases the cost of feeding quail compared to chicken (Scott, 2005; Ali *et al,* 2009; Chantiratikul *et al*, 2010; Hascik *et al*, 2010; Hazim *et al*, 2010; Prayogi, 2011).

2.3. Australian Sweet Lupin

There are 12 native lupin species under the *Lupinus* genus in Europe and the Mediterranean regions, only three of which have been domesticated for agriculture, including the narrow-leafed lupin or Australian sweet lupin (*Lupinus angustifolius*). The domesticated Australian sweet lupin species has been generated by breeding the wild

type for sweetness, early-flowering, non-shattering pods and permeable seed coats (Gladstones, 1972). Australian production of lupin grain has been increasing over the past 5 years and, according to FAO (2012), Australia is now the dominant international producer (about 1 million t pa).

Table 2.1. Comparison between the nutritional requirements for chicken and Japanese quail at starter stage (NRC, 1994).

Nutrient	Unit	Starter		Grower	
		Japanese quail	*Meat-type chicken*	*Japanese quail*	*Meat-type chicken*
Energy (kcal/kg)	%	2900	3200	2900	3200
Protein	%	24	23	24	20
Lysine	%	1.3	1.1	1.3	1
Methionine	%	0.5	0.5	0.5	0.38
Methionine+Cystine	%	0.75	0.9	0.75	0.72
Threonine	%	1.02	0.8	1.02	0.74
Tryptophan	%	0.22	0.2	0.22	0.18
Arginine	%	1.25	1.25	1.25	1.1
Isoleucine	%	0.98	0.8	0.98	0.73
Valine	%	0.95	0.9	0.95	0.82
Calcium	%	0.14	1	0.14	0.9
Total phosphorus	%	0.3	0.45	0.3	0.35
Sodium	%	0.15	0.2	0.15	0.15
Potassium	%	0.4	0.3	0.4	0.3
Chlorine	%	0.14	0.2	0.14	0.15
Magnesium	mg	300	600	300	600
Fat soluble vitamins					
A	IU	1650	1500	1650	1500
D3	ICU	750	200	750	200
E	IU	12	10	12	10
K	mg	1	0.5	1	0.5
Water soluble vitamins					
B12	mg	0.003	0.01	0.003	0.01
Biotin	mg	0.3	0.15	0.3	0.15
Choline	mg	2000	1300	2000	1000
Folacin	mg	1	0.55	1	0.55
Niacin	mg	40	35	40	30
Thiamin	mg	2	1.8	2	1.8

2.4. Nutritional value of lupin

Nutrient composition

Australian Sweet Lupin (ASL) seed has a typical dicotyledonous structure: the hull represents about 25% of the total seed weight and is comprised mostly of cellulose fiber; the kernel mainly consists of protein, cell wall materials and oligosaccharides. The cells of the seed contain protein bodies (400 g/kg), fat bodies (70 g/kg),

oligosaccharides (60 g/kg), starch (20 g/kg), phytic acid (10 g/kg) and water (100 g/kg). (Table 2.2; Petterson, 1998; Kingwell, 2005; Sipsas and Glencross, 2005).

Table 2.2. Nutrient composition of Australian sweet lupin (*L. angustifolius*).

	Seed	*Kernel*
Dry matter	911.1	911.5
Crude fiber	131	21
Crude protein	290	372
Fat	53	67
Ash	25	25
Lysine	13.0	16.5
Methionine	1.7	2.2
Methionine and cystine	4.9	6.6
Threonine	9.4	12.3
Tryptophan	2.2	3.0
Isoleucine	11.7	15.1
Leucine	19.2	24.8
Valine	10.6	13.6
Histidine	7.9	10.1
Gross energy (MJ/kg)	18.06	18.92
Digestible energy	15.81	16.85
Neutral-detergent fiber	260	58
Acid-detergent fiber	172	32
Composition of non-starch polysaccharide fraction		
Rhamnose	2.8	1.2
Fructose	1.7	0
Ribose	0.2	0.1
Arabinose	45.7	43.9
Xylose	27.9	22.3
Mannose	16.0	12.5
Galactose	190.1	191.8
Glucose	115.8	47.4

Evans *et al*, 1993; Petterson & Mackintosh,1994; King *et al*, 2000; Hanbury *et al*, 2000.

Lupin has great potential as a protein and energy source for broiler diets. Australian poultry breeders and feed manufacturers are keen to replace soybean meal with ASL in poultry diets because the nutritive value of lupin is similar to SBM, while the price is about half (Table 2.3). However, the inclusion of sweet lupin in broiler diets is restricted due to the presence of high levels of anti-nutritional factors (ANFs), such as non-starch polysaccharides and oligosaccharides, and the absence of endogenous enzymes that can hydrolyze these components (Carre *et al*, 1985; Petterson *et al,* 1997; Naveed *et al*,

1999). Nevertheless, Bekrić *et al* (1990) reported that SBM could be replaced by ASL in broiler diets up to 250 g/kg with no negative effects on bird's performance compared to soybean meal diets. In addition, other papers reported that broilers can consume diets containing more than 200 g/kg ASL in their diets without any detrimental effects on their performance (Brenes *et al*, 1989; Bekrić *et al*, 1990; Castell *et al*, 1996; Olver & Jonker, 1997; Perez-Maldonado, 1997; Bennet, 2002; Nalle, 2009).

Table 2.3. The nutritive value of common ingredients fed to poultry.

Nutrients (%DM)	Wheat[1]	Soybean meal[1]	Lupin[1]
Moisture	8.9	8.5	9.1
Protein	11.8	46.3	34.0
Fat	2.1	7.0	5.9
Ash	2.0	3.0	2.9
Fiber	4.6	6.5	13.7
Nitrogen-free extract	70.9	28.7	35.9
Gross energy (MJ/kg)[2]	19.1	16.1	17.8
Metabolisable energy (MJ/kg diet)[3]	12.8	13.1	9.7
Price ($/tonne)[5]	380	591	317

[1]Choct *et al,* 1995; Choct, 2006; Petterson & Mackintosh, 1994; Ali, 1997; [2]Kim *et al,* 2003; [3]Graham & Balnave, 1995; Leeson & Atteh, 1995; Annison *et al, 1*996; FAO, 2011.

In contrast, Olkowski *et al* (2001) stated that broilers fed 350 g/kg dehulled lupin-based diets showed poor growth and feed consumption compared to birds fed an SBM-based diet. Moreover, there are several reports indicating that inclusion of 200 g/kg of ASL or white lupin reduced growth rate and feed efficiency in broilers (Farrell *et al*, 1999; Steenfeldt *et al*, 2003; Viveros *et al*, 2007). Finally, Hughes *et al* (1998) showed that the high rates of inclusion of ASL (400 g/kg) in broilers diet resulted in sticky wet droppings and therefore high environmental contamination.

Antinutritional Factors (ANFs) in Lupin

Antinutritional factors can be defined as naturally occurring substances generated in plants, as a normal outcome of their metabolic processes, which interfere with nutrient

intake or availability when ingested by an animal and have adverse physiological effects, such as poor nutrient utilisation and animal performance (Saini, 1989). Antinutritional factors in grain legumes can be divided into 10 groups: alkaloids, glycosides, isoflavones, tannins, oligosaccharides, saponins, phytate, lectins, protease inhibitors and non-starch polysaccharides (Petterson *et al,* 1997). For the present study, the last of these is the most important.

2.5. Non-starch polysaccharides (NSPs)

Polysaccharides are polymers of monosaccharide units joined by glycosidic linkages (Choct, 1997). Polysaccharides can be classified into cellulose and non-cellulosic polysaccharides, hemicelluloses, β-glucans and pectic substances (Englyst and Hudson, 1996). Non-starch polysaccharides contain soluble components such as hemicellulose, pectins, β-glucans, galactomannan gums, and insoluble components such as cellulose, lignin and some hemicelluloses (Cho *et al*, 1997). The lupin kernel contains pectic-like substances (Fig. 2.1), consisting of a long linear chain of galacturonic acid units that are usually linked to each other by beta type 1→4 glycosidic bonds (Kertesz, 1951; Worth, 1967; Albersheim, 1974; BeMiller, 1986; Rolin & de Vries, 1990; Choct, 2006). Pectic substances are mainly found in the lupin kernel, whilst cellulose and xylans, are mainly found in the hull.

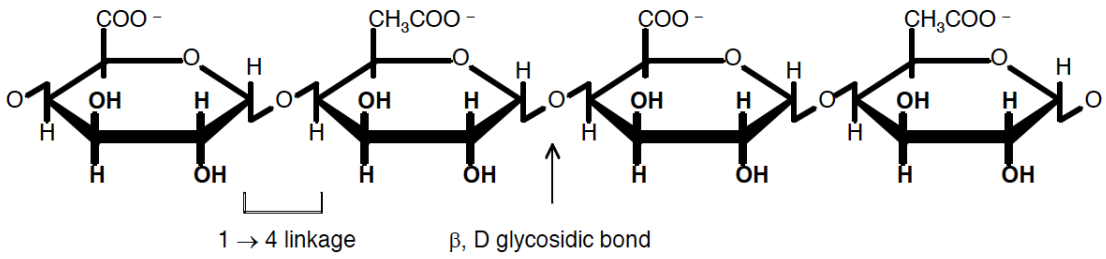

Figure 2.1 The molecular structure of pectin (the linear galacturonic acid units, glycosidic bonds, and the carboxyl (COO^-) or methyl ester (CH_3COO^-) groups).

The level of soluble, insoluble and total NSPs in grain legumes are summarised in Table 2.4. The highest total NSP content was found in Australian sweet lupin, followed by the white lupin. Lupin kernel also contains 50-80 g/kg of oligosaccharides of the rhamnose family (Saini, 1989). Gdala *et al*, 1997 showed that the NSP of Australian sweet lupin contains galactose (349 g/kg) and glucose (315 g/kg). Uronic acid, arabinose and xylose were found at intermediate levels (120, 107 and 83 g/kg, respectively).

Table 2.4. Soluble, insoluble and total NSP contents (g/kg DM) of some grain legumes

Legume	*Soluble NSP*	*Insoluble NSP*	*Total NSP*	*References1*
Australian sweet lupin	22- 40	229-340	251-392	1,2,8
White lupin (cotyledon)	14-134	170-244	244-280	1,2,4
Soybean meal	63-139	154-164	217-303	1,4
Peas	25-59	129-322	173-347	1,3,4,6,7,8
Faba beans	50	140	190-209	3,8
Chickpeas	20-33	74-76	96-107	1,5

1. Smits and Annison (1996); 2. Van Barneveld (1999); 3. Knudsen (1997); 4. Knudsen (2001); 5. Periago *et al* (1997); 6. Anguita *et al,* (2006); 7. Englyst and Hudson (1996); 8.Gdala *et al (*1997).

For monogastrics, the soluble NSP content of lupin grain is the main cause of low growth rate, high feed conversion ratio, low metabolisable energy content, and the wet droppings. These problems arise because of several reasons:

a) Increase the viscosity of the gut:

Soluble NSP content in particular pectin has high water-holding capacity, this is because the molecular structure of pectin which gives pectin the ability to absorb large amount of water, which leads to increase in the viscosity of the gut contents (Smits and Annison, 1996). This leads to negative effects on gastrointestinal physiology and morphology, and on the interaction with gut microflora. It also alters intestinal transit time and changes the hormonal regulation of metabolism because of variations in the nutrient absorption rate (Carre *et al*, 1995ab; Langhout & Schutte, 1996; Ali, 1997; Choct, 2001).

b) Block the enzymatic activity:

Non-starch polysaccharides in cell walls block the access of enzymes to nutrients that are encapsulated within cell walls, leading to reductions in nutrient digestion and utilisation (Bedford & Classen, 1991, 1992; Choct, 1997). Moreover, soluble NSP could increase the microbial contents at the ileum by stimulating microbial growth (Ikeda & Kusano, 1983; Erdman *et al*, 1986; Fengler & Marquardt, 1988).

c) Increase water intake:

Pectin is a gel-like material that entraps water within cell-wall lattices, this leads to increase of water intake for birds fed diet containing large amounts of pectin, resulting in wet dropping (wet litter), odour, coccidiosis outbreak, soiled eggs and excretion of digestible nutrients to the environment (Annison *et al*, 1996; Ali, 1997; Hughes *et al,* 2000; Kocher *et al,* 2000).

2.6. Using enzymes to break down NSPs

Monogastrics cannot break down the complex lupin NSPs or pectin because they do not produce the appropriate digestive enzymes. For this reason, NSP-degrading enzymes have become widely used in commercial poultry production: more than 95% of poultry diets are supplemented with exogenous enzymes which have been reported to have significant effects in alleviating the anti-nutritive effects of NSPs in many feedstuffs such as wheat, barley, sorghum, lupin and peas. Thus, glucanases and arabinoxylanases have been used to reduce the negative effect of beta glucans and arabinoxylans (Bedford, 2000). The outcome is better nutrient digestibility and availability (Choct and Annison, 1992; Choct *et al*, 1995; Hughes *et al,* 2000; Meng *et al*, 2005; Saleh *et al*, 2005; Choct, 2006; Wang *et al*, 2008; Ali *et al,* 2009; Selle *et al*, 2010).

On the other hand, the benefits of supplementing lupin-based poultry diets with enzymes have been variable with respect to growth performance, nutrient utilisation and feed conversion ratio. A major factor seems to be the quantity of lupin meal as well as the type and quantity of enzymes used (Brenes *et al*, 1993, 2002; Annison *et al*, 1996; Ferraz de Oliveira, 1998; Naveed *et al*, 1999; Bedford and Partridge, 2001; Rubio *et al,* 2003; Mieczkowska and Smulikowska, 2005).

Pectin breakdown

Pectin can be broken down using specific enzyme such as polygalacturonase (PG) that attacks the glycosidic units between the basic units (galacturonic acid) of the pectin chains (Fig 2.2; Ali, 1997; Ali *et al*, 2001, 2006ab). Using pectinases in poultry diets results in 5-fold breakdown of complex cell-wall network, reduced viscosity by 18% and hence release of digestible nutrients ready for digestion, and increased nutrient absorption by 17% (Annison and Choct, 1993; Schutte *et al*, 1995). This results in increased weight gain by 6%, reduced FCR by 6%, reduced wet droppings by 6% and reduced the excretion of digestible nutrients to the environment by 8 – 20% (Gracia, *et al*, 2003; Saleh, *et al*, 2003; Odetallah, *et al*, 2005; Wang *et al*, 2005; Hua & Kang-ning, 2008; Ali *et al,* 2009).

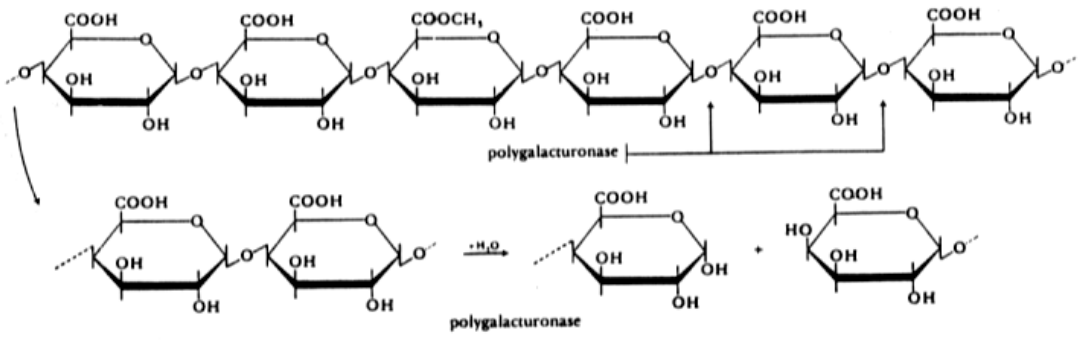

Figure 2.2. Enzymatic degradation of beta-glycosidic bonds of pectin by polygalacturonase.

However, the ability of PG is limited – complete breakdown of the pectin structure is not possible because methyl ester radicals attached to the galacturonic acid units in the pectin main chain block access of the enzyme to the glycosidic bonds (Rexova-Benkova *et al*, 1977; Rombouts & Thibault, 1986; Ali *et al*, 2001). Another enzyme is required to overcome this complexity in the structure of pectins, and attention turned to pectinesterase (pectin methyl esterase, PME) with its ability to de-esterify an esterified galacturonate unit next to a non-esterified galacturonate unit. The result is a linear chain of galacturonic acid units that are susceptible to attack by PG (Fig. 2.3; Cooke *et al,* 1976; Coleman *et al,* 1980; Bonhomme, 1990).

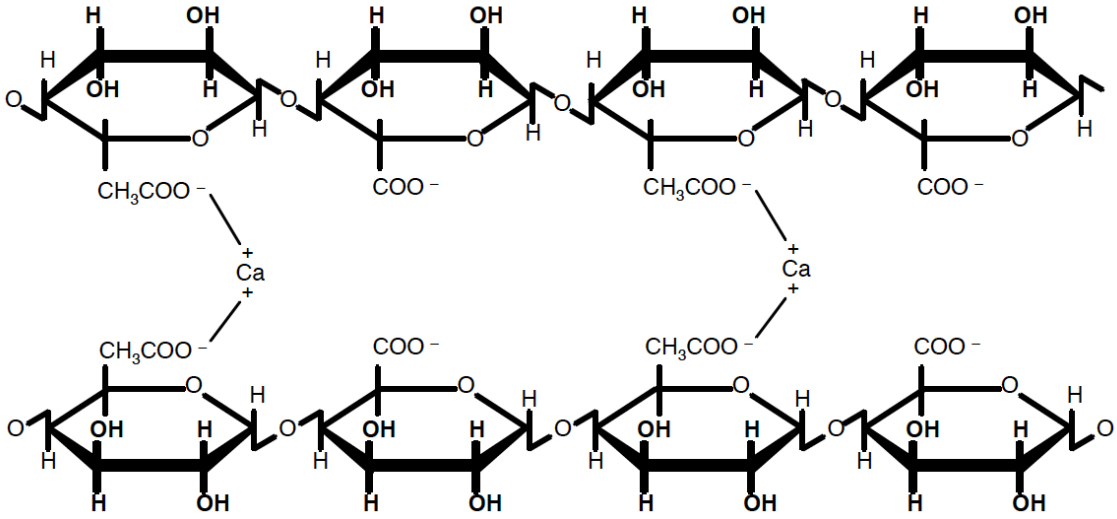

Figure 2.3. Cross-linking of pectic polymers via methyl ester and Ca^{++} ions.

With a combination of PG and PME, the breakdown of the glycosidic bonds was 4-10-fold more than with PG alone. The outcome was further improvements in water holding capacity, viscosity, pectin chain length (Rexova-Benkova & Markovic, 1976; Chesson, 1987; Christgau *et al,* 1996; Kollar, 1998; Ali *et al,* 2009).

Xylan breakdown

Lupin also contain xylans that are attached to the main chain of pectin (Fig. 2.4) so, when pectinase breaks down the main chain, it gives xylanase access to the xylan side branches attached to main backbone (Wood & McCrea, 1979; Greve *et al,* 1984; Biely

et al, 1986; Lee & Forsberg, 1987; Joseleau *et al,* 1992; Cowan *et al*, 1999; de Vries *et al,* 2000). This offers further possibilities for synergistic interactions among enzymes and thus further improvements in the value of diets based on lupin.

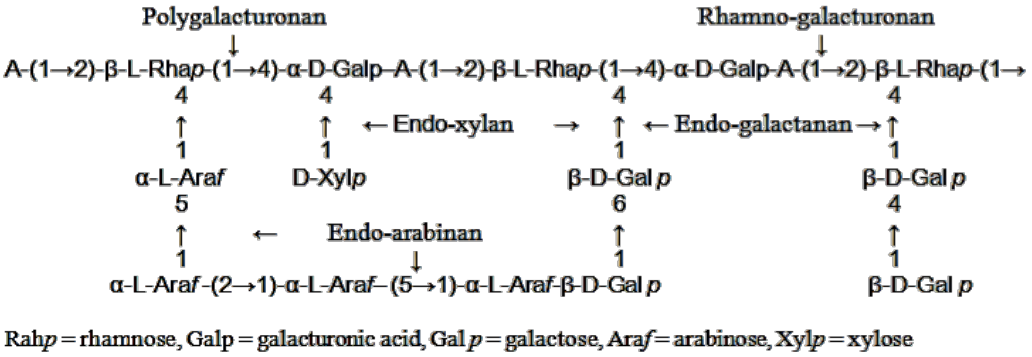

Figure 2.4. Endo-xylan attached to the main chain of pectin.

2.7. Synergistic interactions between enzymes

Many enzymes used in diet formulation can interact synergistically. For example, for broilers fed wheat-based diets, a combination of xylanase and phytase has been shown to improve weight gain by 8% and FCR by 9%, above the outcomes with phytase or xylanase alone (Ravindran *et al*, 1999; Zyla *et al*, 1999; Wu *et al*, 2004). There were also benefits of the combination of pectinase and phytase for feed efficiency (Zyla *et al*, 2000). Similarly, for broilers fed sorghum-based diets, the combination of pectinase and xylanase improved weight gain by 3% and reduced FCR by 2%, compared to single enzyme diets (Cowan *et al*, 1999).

Extrapolating this concept, the breakdown of complex carbohydrates is likely to be greatly improved by using a mixture of enzymes with diverse activities that can target a variety of components in the diet. Indeed, it has been reported that the use of enzyme mixtures (such as protease plus amylase) in broiler diets is more likely to lead to significant improvements in growth performance than supplementing each enzyme

separately (Gracia *et al*, 2003; Odetellah *et al*, 2003). In the quest for more predictable improvements in growth performance, nutrient digestibility and FCR, and thus greater economic returns for producers, the correct enzyme mixture should be determined experimentally rather simply relying on an untested mixture.

2.8. Aims and hypothesis

Aims:

1- Improve the nutritive value of lupin meal using a combination pectinase and xylanase.
2- For Japanese quail, improve feed conversion ratio as well as the metabolisable energy content of the diet.

Hypotheses:

1- The synergistic interaction between pectinase and xylanase will improve the physico-chemical properties of lupin more than pectinase or xylanase alone.
2- For Japanese quail, a combination of pectinase and xylanase will improve FCR more than the pectinase or xylanase alone.
3- The optimal dose of the combination of pectinase and xylanase will further improve lupin nutritive value

Chapter 3*

Improving physico-chemical properties of lupin using a combination of pectinase and xylanase

Khalil, M., Ali, A., Malecki, I. A. and Martin, G. (2014). Improving the nutritive value of lupin using a combination of pectinase and xylanase. *Proceedings of the Australian Poultry Science Symposium* **25**: 50-53.

3.1. Introduction

Australian varieties of lupin have been bred to have a very low alkaloid content (0.01%) and these 'sweet lupin' are currently used as a source of protein and energy in a range of monogastric diets, for poultry, pigs, fish and rabbits (Inborr, 1990; Glencross *et al*, 2004). However, the use of lupin in poultry diets by feed manufacturers and poultry producers is still limited because the lupin contains high concentrations (35%) of non-starch polysaccharides (NSPs). The main NSPs content in lupin are pectins, galactans, rhamno-galacturonans, arabinans and xylans (Cheetham *et al*, 1993; Choct, 2006). Monogastrics do not have endogenous enzymes that can digest these NSPs into simple sugars, so NSPs in their diets increase the viscosity of digesta in the intestinal tract, inhibit the digestion of nutrients, and reduce growth and feed conversion efficiency (Kocher *et al*, 2000; Steenfeldt *et al*, 2003).

The detrimental effects of lupin NSP can be alleviated by supplementing lupin with NSP degrading enzymes, one possible enzyme is pectinase. Pectinase will break down pectin main chain, hence improve lupin physical and chemical properties such as: water holding capacity, viscosity, and pectin content (Annison & Choct, 1993; Schutte *et al,* 1995; Ali *et al,* 2009).

Since lupin contain xylans that are attached to the main pectin chain, it seems likely that a combination of enzymes, pectinase and xylanase, will work synergistically to break down pectins and xylans in lupin (Ravindran *et al*, 1999; Wu *et al*, 2004). The synergism would result from pectinase breaking down the main pectin chain and thus giving xylanase access to the xylan side branches attached to main pectin backbone. In the present study, we tested this hypothesis by incubating lupin kernel with pectinase and xylanase, alone and in combination.

3.2. Materials and Methods

3.2.1. Experimental design

Lupin kernels were ground to pass through a 1 mm sieve and 12 replicate samples were then incubated with each of four treatments: i) no enzyme (control); ii) pectinase (1.4 U/g polygalacturonase and 0.2 U/g pectinesterase, Novozymes Australia) alone; iii) xylanase (0.38 U/g, Rovabio) alone; iv) the combination of these two pectinase and xylanase treatments. Lupin samples were suspended in 25 ml deionized water and incubated without enzymes (control) or with enzymes in an incubator-shaker at 38°C for 1 hour and 150 rpm. The physico-chemical measurements were then made, as described below.

3.2.2. Water holding capacity (WHC)

After incubation, samples were centrifuged at 15000 g for 15 min at 20°C. The residues were collected, weighed and freeze-dried. Water holding capacity was calculated as grams of water per gram of organic matter (Armstrong *et al*, 1993).

3.2.3. Filtration rate

The filtration rate was calculated by measuring the volume of supernatant filtered through filter paper (Whatman, no. 41) divided by the filtration time (μl/sec). After filtration, the viscosity of the solutions was measured.

3.2.4. Viscosity

Viscosity was measured using a Viscotester (HAAKE, PK 100, VT 550). Supernatant was filtered through cheese cloth to remove any floating particles, then a sample (0.50 ml) was placed on a cone plate PK5 at a shear rate of 3000/sec and speed rate of 500/min at a temperature of 23°C.

3.2.5. Neutral detergent fiber (NDF) and Acid detergent fiber (ADF)

Neutral detergent fiber and ADF were measured following the method described by Van Soest *et al,* (1991). Samples (0.5 g) of the dried residue were placed in a filter bag in duplicate and digested in an ANKOM200 fiber analyzer (ANKOM Corporation Technology Fairport, NY).

3.2.6.Pectin content

Pectin content was measured in the dried residue using the method described by McGready (1996). Samples were transferred into a beaker with 500 ml water, 2.5 ml HCl to give a pH of 2.2, and then boiled for 45 min. Samples were filtered using (Whatman No. 1) filter paper and the residue was washed with 250 ml boiling water and left to cool to 25°C. Pectin was precipitated by adding 200 ml of 95% acetone for 30 min. Floating pectin was then purified by centrifugation, washed in acetone, and dried.

3.2.7. Galacturonic acid concentration

Galacturonic acid molecules are the main sub-units of pectin molecules, so a quantitative measurement of this acid is used to determine the concentration of pectic substances. A sample (5 mg) was weighed into a 20 ml beaker and treated with 2 ml chilled, concentrated sulfuric acid. The mix was stirred with magnetic stirrer and 0.5 ml deionized water was added drop-wise to the beaker. After 5 min, another 0.5 ml water was added drop-wise and stirred until the sample was completely dissolved.

One ml of standard or sample solution was placed into test tubes then placed in an ice bath for a few minutes to cool, then 6.0 ml of 0.0125M sodium tetraborate solution (prepared using concentrated sulfuric acid) were added to the test tube, mixed properly, then kept in an ice bath until all the samples had been prepared.

Tubes were placed in a boiling water bath for about 5 min then cooled immediately in an ice bath. A 0.15% solution of *m*-hydroxydiphenyl was prepared in 0.5% sodium hydroxide then covered with aluminum foil to protect it from light and kept in refrigerator; 0.1 ml of this solution was added to two tubes for color reaction and 0.1 ml sodium hydroxide (0.5%) was added for the blank tube. All tubes were mixed properly and left for 15-20 min at room temperature. Galacturonic acid concentration was then measured using spectrophotometer at 520 nm by reading samples against corresponding blank tube.

A standard solution of galacturonic acid (1 mg/ml) was prepared and stored at 4°C. It was used to prepare a range of concentrations (20, 40, 60, 80, 100 µg /ml). The absorbance due to *m*-Hydroxydiphenyl was corrected by subtracting the absorbance for sample blank from the absorbance for the samples (El-Rayah & Labavitch, 1977).

3.2.8. Pectin chain length

Pectin chain length was calculated based on the viscosity and galacturonic acid concentration using the equation of Barash & Eyal (1970):

$$DP = \frac{\left(\frac{\eta}{0.49 \times 10^{-4}}\right)^{0.82} \times C}{100}$$

where DP is degree of polymerization

η is intrinsic viscosity (m.Pas/sec measured at 22°C in 250 μl), divided by the viscosity coefficient, 0.49×10^{-4}

C is concentration of galacturonic acid (μg/250 μl).

3.2.9 Statistical analysis

The data were subjected to ANOVA in a complete randomized design using statistical package SAS 9.3. If a treatment effect was statistically significant ($P < 0.05$), Duncan's multiple range test (Duncan, 1955) was used to compare the differences between means.

3.3. Results

The changes in physico-chemical properties of lupin kernel were less with pectinase and xylanase used alone than with the combination of enzymes (Table 3.1). Compared to control values, the combination of pectinase and xylanase reduced viscosity by 21%, WHC by 4% and the filtration rate by 50% ($P < 0.05$). This breakdown was manifested by reductions in the contents of cell wall (13%) and pectin (35%). In addition, the combination of enzymes reduced the pectin chain length by 51% and galacturonic acid content by 40%.

Table 3.1. Effect of enzymes on physico-chemical properties of lupin kernel *in vitro* (mean ± sem, n = 12).

Parameters	*Control*	*Pectinase*	*Xylanase*	*Combination*
WHC (g:g)	3.6 ± 0.01^{a}	3.5 ± 0.02^{a}	3.5 ± 0.09^{a}	3.4 ± 0.03^{b}
Viscosity (mPas/sec)	1.5 ± 0.01^{a}	1.4 ± 0.01^{b}	1.3 ± 0.01^{c}	1.2 ± 0.01^{d}
Filtration rate (µl/sec)	31.2 ± 1.5^{a}	26.7 ± 1.8^{b}	20.6 ± 1.2^{c}	15.4 ± 0.9^{d}
NDF (%)	11.5 ± 0.1^{a}	11.1 ± 0.1^{b}	10.9 ± 0.1^{b}	10.5 ± 0.1^{c}
ADF (%)	5.3 ± 0.1^{a}	5.1 ± 0.1^{b}	4.7 ± 0.1^{c}	4.5 ± 0.1^{d}
Pectin (%)	10.8 ± 0.3^{a}	9.0 ± 0.2^{b}	9.3 ± 0.1^{b}	6.9 ± 0.1^{c}
GA concentration (µg/g)	32.8 ± 1.4^{a}	26.9 ± 0.4^{b}	29.4 ± 1.5^{ab}	19.5 ± 0.7^{c}
Pectin chain length (%)	76.5 ± 3.5^{a}	58.9 ± 1.2^{b}	60.2 ± 2.9^{b}	37.3 ± 1.4^{c}

^{abcd} Means within rows with different superscripts differ ($P < 0.05$).

3.4. Discussion

These results support the hypothesis that the combination of pectinase and xylanase will improve the physico-chemical properties of lupin kernel more than the individual enzymes. The synergistic interaction between pectinase and xylanase was clearly evident in the superior effect of the enzyme combination on the content of pectin and galacturonic acid. The basis of the synergism is that, acting independently, xylanase can do little to breakdown xylans in the lupin meal unless the substrate is first exposed by pectinase.

Reductions in the content of NSPs by application of the enzyme mix should improve nutritive value and digestibility of lupin kernel for monogastrics. Indeed, in chickens, treating lupin with other multi-enzyme combinations has been shown to improve production performance (Brenes *et al*, 1993; Naveed *et al*, 1999; Ali *et al*, 2009; Olkowski *et al*, 2010).

3.5. Conclusion

The combination of pectinase and xylanase is more effective than the individual enzymes *in vitro.* We now need to test whether this outcome can be reproduced *in vivo*, with a focus on potential benefits in feed conversion efficiency and growth rate in quail. If experiments with growing birds support the *in vitro* study, then industry could make use of the synergistic interaction between the two enzymes to make progress towards greater inclusion of lupin kernel in diets for poultry, reducing the cost of production without compromising production performance or compromising welfare by increasing wet droppings.

Chapter 4

The effect of enzyme supplementation of lupin-based diets on Japanese quail performance

4.1. Introduction

To bridge the gap between supply and demand for animal protein, there is growing interest in quail production worldwide because they are fast-growing birds that provide healthy food, and easier to handle than broiler chickens. They have a short incubation period of 18 days and reach sexual maturity in 5-6 weeks, much faster than other poultry species (Padgett & Ivey, 1959; Wilson *et al*, 1961; Reese & Reese, 1962; Cooper, 1976; Mady, 1976 & 1981; Smith, 2001). Australian quail are among the best meat-producing quail in the world, with a higher average live weight (240 g) than seen in other countries (160 g). Quail meat is lean and the egg has low cholesterol content, with benefits for public health in terms of conditions like atherosclerosis (Agwuonobi & Ekpenyong, 1990; Okon *et al*, 2007).

Feed conversion efficiency plays a major role in the economy of poultry industries, and it is much higher for quail (3.3) than for broiler chickens (1.7), but it is nevertheless essential to retain a focus on this measure of productivity (Arslan & Seker, 2002; Selle *et al,* 2003; Wu *et al*, 2004; Namra, 2006; Olkowski *et al*, 2010; Nalle *et al*, 2010; Chantiratiku *et al*, 2010). One way to do that is by supplementing feed with additives such as probiotics or enzymes to improve digestibility, nutrient availability and absorption (Mujeeb Ather, 2001). This issue is now becoming critical because quail producers have been experiencing an unprecedented rise in feed costs due to the global increase in the price of the most common protein concentrates, such as SBM (Teguia &

Fon Fru, 2007). Since feed is the single major cost (70%) of total quail production (Bhuiyan, 1989; Perez-Maldonado *et al*, 1999; Louw *et al*, 2011), it is essential to find a cheap and local protein source to replace SBM.

One realistic alternative is lupin, a legume grain that is far less expensive than SBM ($650 vs $270/t) and has previously been considered as the main protein source in poultry diets (Petterson & Mackintosh, 1994). Moreover, Australia is the world-leading producer of lupin, accounting for around 85% of world total production, so importation can be avoided. Unfortunately, the usage of lupin meal in commercial poultry diets is limited by the presence of large amounts of soluble non-starch polysaccharides (NSP; Evans *et al,* 1993; Chesson, 1993; Annison & Choct, 1993; Perez-Maldonado *et al*, 1999; Kocher *et al*, 2000) that have negative effects on growth, nutrient digestion and FCR (Choct & Annison, 1992; Annison *et al*, 1996; King *et al*, 1997; Hughes *et al*, 2000; Kocher *et al*, 2000; Ali *et al*, 2005 & 2006ab).

In the previous chapter, incubating lupin meal with exogenous enzymes (pectinase, xylanase and pectinase + xylanase) showed significant improvement in physical and chemical properties of lupin such as viscosity, WHC, pectin content – these outcomes would likely improve nutritive value of lupin meal (Khalil *et al,* 2014). The improvement in lupin nutritive value associated with enzymes supplementation should eliminate the negative effects of feeding lupin to quail chicks. In this experiment, we examined the effect of supplementing lupin-based diets with NSP-degrading enzymes: pectinase, xylanase and the pectinase + xylanase, on growth and FCR in Japanese quail.

4.2. Materials and Methods

4.2.1. Experimental design

This experiment consisted of a 2 x 4 factorial design with 2 levels of lupin (10 and 20%) and 4 enzymes (no enzyme, pectinase, xylanase, combination of pectinase and xylanase) with 3 replicates/treatment. Two hundred and eighty eight quail chicks (18 days old) were allocated among the 8 treatments. Chicks were housed into rearing cages with wire floors, with feed and water offered *ad-libitum*. They were fed experimental diets for three days as adaptation to the experimental diet as shown in (Table 4.1).

4.2.2. Experimental diets

Diets were in crumble form and formulated to meet the nutritional requirements for Japanese quail (NRC, 1994). Experimental diets were formulated to contain 2 levels of lupin kernel (10 and 20%) replacing SBM (Table 4.1).

Table 4.1. Ingredients and nutrient composition of the experimental diets in this study.

Ingredients	**10% lupin**	**20% lupin**
Wheat	49.770	44.270
Lupin	10.000	20.000
Crude canola oil	1.629	1.629
Soya bean meal	30.656	26.156
Wheat gluten	4.588	4.588
Di calcium phosphate	1.631	1.631
DL-methionine	0.010	0.010
Limestone fine	0.999	0.999
Salt (sodium chloride)	0.448	0.448
Quail premix*	0.183	0.183
Choline chloride	0.086	0.086
Calculated analysis of dietary nutrients and apparent metabolizable energy (AME)		
AME (Kcal/kg)	2890	2880
Crude protein	24.9	24.5
Crude fiber	4.61	4.75
Calcium	0.80	0.80
Available phosphorus	0.30	0.30

*Quail premix provided per kg diet: Vitamin A, 8,980 IU; Vitamin D_3, 2,030 IU; Vitamin E, 35 IU; Vitamin K, 1 mg; Vitamin B_1, 0.7 mg; Vitamin B_2, 5.2 mg; Vitamin B_{12}, 10.7 ug; niacin, 20 mg; choline chloride, 695 mg; calcium pantothenate, 7.5 mg; biotin, 130 ug; folic acid, 1.2 mg; manganese oxide, 145 mg; zinc, 78 mg; copper, 10 mg; magnesium, 48 mg iron, 36 mg; selenium, 0.4 mg.

4.2.3 Measurements

Body weight (g)

Chicks were individually weighed every week using a 20 x 20 x 30 cm cardboard box (with small holes for the aeration and light, and a lid) that was placed on a digital balance. Weight gain was calculated as the difference between two successive weeks.

Feed consumption (g)

Chicks in each replicate were provided with a certain amount of feed weekly, the residuals were obtained at the end of the week, and the amount of feed consumed was calculated by the difference. Average feed consumption was calculated:

$$\text{Feed consumption} = \frac{\text{Feed intake/one week/replicate}}{\text{Number of chicks consuming feed}}$$

Feed conversion ratio (FCR)

Feed conversion ratio was calculated by:

$$\text{FCR} = \frac{\text{Feed consumed/chick/week}}{\text{Body weight gain/chick/week}}$$

Digestibility of dry matter

The percentage digestibility of dry matter of diet (DDM) was determined by:

$$\text{DDM} = \frac{\text{Feed DM intake} - \text{Excreta DM output}}{\text{Feed intake}} \times 100$$

Apparent metabolisable energy (AME)

The freeze-dried samples of excreta and diets were ground to pass through 0.7 mm screen and then compressed into pellet form. The pellet was ignited using Parr 1261Isoperibol Bomb Calorimeter to measure the amount of energy released (kilocalorie/g sample) for calculating the AME:

$$AME = \frac{(\text{Feed intake} \times \text{Diet GE}) - (\text{Excreta output} \times \text{Excreta GE})}{\text{Feed intake}}$$

where GE = gross energy

4.2.4. Animal ethics approval

The Animal Ethic Committees (AEC) of the University of Western Australia approved this experiment (RA/3/100/1335). In addition, the protocol has been approved by the animal & poultry production department committee (approval no. 4/6/14) and approved by the farm committee (approval no. 20/7/14), where the trial has been conducted at the Poultry Research Facility, Faculty of Agriculture, Benha University, Egypt.

4.2.5 Statistical analysis

Data were analysed by analysis of variance (ANOVA) using the general linear model procedure (SAS V9.3). Differences were considered to be significant at $P < 0.05$ and significant differences between means were separated by Duncan's multiple range test, (Duncan, 1955).

4.3. Results

4.3.1. *Body weight*

The effect of treatments on body weight is summarized in (Table 4.2). There was no significant difference ($P > 0.05$) between any of the treatments at 28 and 35 days of age. However, at age of 42 days, supplementing 10% lupin with the enzyme combination improved body weight by 8% compared to the control group (no enzyme) and by 2.5% compared to 10% lupin + pectinase and by 4.5% compared to 10% lupin + xylanase.

In contrast, for 20% lupin diet the enzyme combination did not result in any significant improvement in body weight compared to other groups.

Table 4.2. Effect of experimental diets on body weight (g) of Japanese quail at ages 28 (BW1), 35 (BW2) and 42 (BW3) days (mean ± sem, n = 288).

Lupin	Treatment	BW1	BW2	BW3
10%	No enzyme	145.5± 2.5[a]	195.5 ± 2.8[a]	221.2 ± 4.1[b]
	Pectinase	142.4 ± 2.5[a]	197.6 ± 2.8[a]	233.6 ± 4.1[ab]
	Xylanase	145.8 ± 2.5[a]	194.9 ± 2.8[a]	228.1 ± 4.1[ab]
	Combination	147.6 ± 2.5[a]	201.1 ± 2.8[a]	238.7 ± 4.1[a]
20%	No enzyme	141.7 ± 2.5[a]	198.1 ± 2.8[a]	225.9 ± 4.1[ab]
	Pectinase	147.4 ± 2.5[a]	199.6 ± 2.8[a]	223.5 ± 4.1[b]
	Xylanase	139.6 ± 2.5[a]	196.0 ± 2.8[a]	226.7 ± 4.1[ab]
	Combination	145.0 ± 2.5[a]	199.6 ± 2.8[a]	230.9 ± 4.1[b]

[ab] Means within columns with different superscripts differ (P < 0.05).

4.3.2. *Weight gain*

Addition of enzymes to lupin diets did not result in any significant difference in weight gain at the age 28 days (Table 4.3), but there were significant effects at ages 35 and 42 days. At 35 days of age, the combination of pectinase and xylanase improved weight gain by 7% for 10% lupin and by 10% for 20% lupin compared to the control groups. The effect of the enzyme combination extended until the age of 42 days as it improved weight gain by 50% for 10% lupin diet and by 30% for the 20% lupin diet compared to the control groups.

Table 4.3. Effect of experimental diets on weight gain (g) for Japanese quail at age of 28 (WG1), 35 (WG2) and 42 (WG3) days (mean ± sem, n = 288).

Lupin	Enzyme	WG1	WG2	WG3
10%	No-enzyme	53.0 ± 1.7[a]	50.1 ± 1.5[c]	25.3 ± 2.1[c]
	Pectinase	51.6 ± 1.7[a]	55.2 ± 1.5[ab]	35.9 ± 2.1[ab]
	Xylanase	51.9 ± 1.7[a]	49.0 ± 1.5[c]	33.2 ± 2.1[ab]
	Combination	55.6 ± 1.7[a]	53.7 ± 1.5[bc]	38.2 ± 2.1[a]
20%	No-enzyme	55.6 ± 1.7[a]	53.1 ± 1.5[bc]	25.3 ± 2.1[c]
	Pectinase	52.1 ± 1.7[a]	52.7 ± 1.5[c]	30.8 ± 2.1[bc]
	Xylanase	51.8 ± 1.7[a]	56.7 ± 1.5[ab]	24.3 ± 2.1[c]
	Combination	53.4 ± 1.7[a]	58.4 ± 1.5[a]	33.2 ± 2.1[b]

[abc] Means within columns with different superscripts differ (P < 0.05).

4.3.3. *Feed intake*

Pectinase and xylanase, when used alone to 35 and 42 days of age, reduced feed intake compared to groups fed diets without enzymes (Table 4.4). Moreover, the combination of pectinase and xylanase showed superior effect in reducing feed intake for groups fed diet containing 10% and 20% lupin compared to the individual enzymes.

Table 4.4. Effect of experimental diets on feed intake (g) for Japanese quail at age of 28 (W1), 35 (W2) and 42 (W3) days (mean ± sem, n = 288).

Lupin	Enzyme	W1	W2	W3
10%	No-enzyme	145.1 ± 2.5^{c}	181.9 ± 2.5^{c}	318.0 ± 3.2^{b}
	Pectinase	138.9 ± 2.5^{c}	160.9 ± 2.5^{e}	303.9 ± 3.2^{c}
	Xylanase	140.5 ± 2.5^{c}	168.9 ± 2.5^{d}	307.2 ± 3.2^{c}
	Combination	131.3 ± 2.5^{d}	140.8 ± 2.5^{f}	258.7 ± 3.2^{d}
20%	No-enzyme	175.4 ± 2.5^{a}	211.4 ± 2.5^{a}	337.0 ± 3.2^{a}
	Pectinase	142.4 ± 2.5^{c}	174.9 ± 2.5^{d}	322.3 ± 3.2^{b}
	Xylanase	159.9 ± 2.5^{b}	188.0 ± 2.5^{b}	317.4 ± 3.2^{b}
	Combination	142.1 ± 2.5^{c}	173.9 ± 2.5^{d}	307.2 ± 3.2^{c}

^{abcdef} Means within columns with different superscripts differ ($P < 0.05$).

4.3.4. *Feed conversion ratio (FCR)*

The enzymes supplementation did not show any significant differences compared to the control group at 28 days of age. However, at the age of 35 days, supplementing 10% and 20% lupin diets with the enzyme combination had significant reduction in FCR compared to other groups. At the age of 42 days, the enzyme combination improved FCR by 45% for 10% lupin and by 34% for 20% lupin compared to the control group.

Table 4.5. Effect of experimental diets on feed conversion ratio (FCR) for Japanese quail at age of 28 (W1), 35 (W2) and 42 (W3) days (mean ± sem, n = 288).

Lupin	Enzyme	W1	W2	W3
10%	No-enzyme	2.7 ± 0.1^{bc}	3.6 ± 0.1^{b}	12.6 ± 1.0^{ab}
	Pectinase	2.7 ± 0.1^{c}	2.9 ± 0.1^{d}	8.5 ± 1.0^{de}
	Xylanase	2.7 ± 0.1^{bc}	3.4 ± 0.1^{bc}	9.3 ± 1.0^{cd}
	Combination	2.4 ± 0.1^{c}	2.6 ± 0.1^{e}	6.9 ± 1.0^{e}
20%	No-enzyme	3.3 ± 0.1^{a}	4.0 ± 0.1^{a}	14.5 ± 1.0^{a}
	Pectinase	2.7 ± 0.1^{bc}	3.3 ± 0.1^{c}	10.6 ± 1.0^{bcd}
	Xylanase	3.1 ± 0.1^{ab}	3.3 ± 0.1^{c}	12.0 ± 1.0^{bc}
	Combination	2.5 ± 0.1^{c}	3.0 ± 0.1^{d}	9.6 ± 1.0^{cde}

^{abcde} Means within columns with different superscripts differ ($P < 0.05$).

4.3.5 *Digestibility of dry matter and apparent metabolisable energy (AME)*

Increasing lupin level (20%) resulted in reduction in the digestibility of dry matter (DDM) and AME. Supplementing lupin levels (10 and 20%) with enzymes significantly improved DDM and AME. The combination of pectinase and xylanase showed superior effect in improving DDM and AME for the two level of lupin as presented in (Table 4.6).

Table 4.6. Effect of experimental diets on digestibility of dry matter (DDM) and apparent metabolisable energy (AME) for Japanese quail (mean ± sem, n = 96).

Lupin	Enzyme	AME	DDM
10%	No-enzyme	2.8 ± 0.1^{c}	63.9 ± 1.3^{c}
	Pectinase	3.0 ± 0.1^{b}	70.3 ± 1.3^{b}
	Xylanase	3.0 ± 0.1^{b}	70.0 ± 1.3^{b}
	Combination	3.2 ± 0.1^{a}	75.9 ± 1.3^{a}
20%	No-enzyme	2.1 ± 0.1^{f}	43.9 ± 1.3^{f}
	Pectinase	2.6 ± 0.1^{d}	57.6 ± 1.3^{d}
	Xylanase	2.4 ± 0.1^{e}	51.2 ± 1.3^{e}
	Combination	2.8 ± 0.1^{c}	61.9 ± 1.3^{c}

^{abcdef} Means within columns with different superscripts differ ($P < 0.05$).

4.4. Discussion

The results supported the hypothesis that the combination of pectinase and xylanase improves the FCR of lupin-based diets fed to Japanese quail, and is more effective than the individual enzymes, and is in agreement with the observations from the *in vitro* study (Chapter 3) showing that the enzyme combination results in better breakdown of lupin NSP than the individual enzymes. Improved breakdown would result in the release of more nutrients that would otherwise have been trapped within cell wall lattices, leading to improved digestion and absorption of those nutrients in the quail digestive tract. *In vitro*, the enzyme combination also improved the viscosity and water-holding capacity of lupin meal, and this would also explain the improvements in quail in the digestibility of dry matter, metabolisable energy and feed conversion ratio (Ali *et al,* 2009; Khalil *et al,* 2014).

The observation also support the theoretical explanation of the superior effect of the enzyme combination: the main NSP structure in lupin kernel is pectin that comprises galacturonic acid units connected by side-chains including D-galactose, L-arabinose, D-xylose and D-glucuronic acid (Cheetham *et al*, 1993), so enzymes with different activities are required to break down this complex structure. Pectinase solubilises pectins by cleaving the glycosidic bonds between the main pectin chains, leaving smaller highly branched fragments with long side-chains (Annison *et al,* 1996) exposed to xylanase, with the overall result being short-chain NSPs.

Other studies have shown that supplementing lupin-based diets with enzymes has beneficial effects on the performance of chickens (Brenes *et al,* 1993; Bryden *et al,* 1994; Roth Maier & Kirchgessner, 1994ab, 1995; Annison *et al,* 1996; Ferraz de Oliveira, 1998; Naveed *et al,* 1999). As shown by Ali *et al* (2009), the outcome is a significant improvement in the nutritive value of lupin kernel for broilers. A combination of pectinase and xylanase has been shown to improve FCR for broilers fed a sorghum based-diet (Cowan *et al,* 1999). Finally, broiler performance fed sorghum based-diets is also improved by a combination of xylanase and amylase (Annison *et al,* 1996; Pack *et al,* 1996; Creswell *et al,* 1998).

In the present study, increasing lupin content of the diet to 20% led to lower values for DDM, AME and FCR, compared to 10% lupin, for all enzyme treatments. Clearly, for quail to handle diets with 20% lupin meal, further research is needed if we are to avoid anti-nutritive effects of soluble NSP on nutrient digestion, absorption and amino acid digestibility of in poultry diets (Choct and Annison, 1992). The focus should be on optimizing the concentrations of pectinase and xylanase, and on searching for other enzymes that can target other components of the NSPs.

4.5. Conclusion

The combination of pectinase and xylanase improved growth performance and the FCR in quail consuming a diet containing 10% and 20% lupin kernel compared to the individual enzymes. On the other hand, at this stage, the best diet is one based on 10% lupin meal supplemented with the enzyme combination.

Chapter 5

The 'optimal dose' of pectinase and xylanase for breaking down non-starch polysaccharides in lupin kernel

5.1. Introduction

Due to the complex structure of the NSPs in lupin kernel, a combination of pectinase and xylanase is needed to break them down and thus reduce their negative effects in avian nutrition. Pectinase breaks down the galacturonic acid chains in the main pectin chain, thus allowing xylanase to gain access to the xylans attached to the main pectin chain. As shown in Chapter 3, the combination of the two enzymes improved the physico-chemical properties of lupin kernel *in vitro* more effectively than the individual enzymes. As shown in Chapter 4, the same combination of pectinase and xylanase added to lupin-based diets improved growth performance for Japanese quail *in vivo*, again more effectively than the individual enzymes. In this chapter, we have returned to *in vitro* analysis to determine the 'optimal dose' for the combination pectinase and xylanase.

The optimal enzyme dose will ensure sufficient breakdown of pectin and xylan to maximize the release of nutrients entrapped within the cell wall lattices. An inappropriately low dose will result in a feed with low nutritional value but substantial anti-nutritional effects, whereas an inappropriately high dose will be costly and might even result in excessive breakdown of the targeted substrate, causing adverse effects on the physico-chemical properties of the diet.

However, any definition of 'optimal' in this context is bound to be multifaceted, because not all physico-chemical properties will be affected to the same degree and, at industry practice, a wide variety of costs may prevent adoption of a treatment that has small benefits. So, in this study, the aim was to find a combination that satisfied several of the physico-chemical criteria. An extra layer of complexity is added when there are clear synergistic interactions between the various enzymes under consideration. It is nevertheless worth seeking the optimal dose so, we have compared 27 combinations of polygalacturonase, pectinesterase and xylanase.

5.2. Materials and Methods

5.2.1. Experimental design

The results presented in Chapter 3 clearly demonstrate the superior effect of the combination of pectinase and xylanase compared to control (no enzyme) or single-enzyme treatments. Therefore, in the study described in this chapter, we decided to maximize the use of resources and thus not include a zero-enzyme control treatment. Instead, we used a simple design with 3 enzymes, each at 3 doses, with 3 replicates per treatment: two pectinases, polygalacturonase (0.35, 0.7, 1.4 U/g and pectinesterase (0.1, 0.2, 0.4 U/g), and xylanase (0.095, 0.19, 0.38 U/g (Novozymes, Australia). The choice of doses was based on the recommendation of the manufacturer or on a review of the literature, with the lower dose at 50% of the recommended dose and the higher dose being double the recommended dose.

Lupin kernels were ground to pass through a 1 mm sieve then suspended in 25 ml deionized water. The enzymes, all provided by the manufacturer in liquid form, were added and the mixtures were placed in an incubator-shaker (38 ºC, 150 rpm) for 1 h. The physico-chemical properties (WHC, NDF, ADF, pectin content) were measured as

described in Chapter 3. In contrast with the study in Chapter 3, viscosity was measured using a viscometer (HAAKE Mars III Modular Advanced Rheometer System). Supernatant was filtered through cheesecloth to remove any floating particles and viscosity was then determined in 0.10 ml of the supernatant at a shear rate of 3000/sec and speed rate of 300/min at a temperature of 24 °C, as described in the manufacturer's handbook.

5.2.2 *Statistical analysis*

Data were subjected to one-way ANOVA, using the statistical package SAS 9.3. If ANOVA revealed any treatment to be statistically significant ($P < 0.05$), Duncan's multiple range test (Duncan, 1955) was used to compare the differences among means.

5.3. Results

As can be seen in Table 5.1 (treatments pooled across enzymes), there was very little evidence of dose-responses for any of the three individual enzymes, for any of the physico-chemical properties measured.

Table 5.1. Effect of doses of separate enzyme on physico-chemical properties of lupin kernel *in vitro* (mean ± sem; n = 3). PG = polygalacturonase; PME = pectinesterase; Xyl = xylanase. Values in bold text indicate a significant reduction with an increase in dose. WHC = water holding capacity, NDF = neutral detergent fiber, ADF = acid detergent fiber.

Enzyme	Doses (U/g)	WHC (g/g)	Viscosity (mPas/sec)	NDF (%)	ADF (%)	Pectin (%)
PG	0.35	2.8 ± 0.04	3.2 ± 0.2	14.1 ± 0.3	6.8 ± 0.2	4.9 ± 0.1[a]
	0.70	2.8 ± 0.04	3.1 ± 0.2	13.4 ± 0.3	6.5 ± 0.2	**4.5 ± 0.1[b]**
	1.40	2.7 ± 0.04	3.1 ± 0.2	13.4 ± 0.3	6.5 ± 0.2	**4.5 ± 0.1[b]**
PME	0.10	2.9 ± 0.04[a]	3.3 ± 0.2[a]	13.8 ± 0.3	6.7 ± 0.2	4.7 ± 0.1
	0.20	2.9 ± 0.04[a]	3.4 ± 0.2[a]	13.8 ± 0.3	6.6 ± 0.2	4.7 ± 0.1
	0.40	**2.7 ± 0.04[b]**	**2.8 ± 0.2[b]**	13.3 ± 0.3	6.5 ± 0.2	4.5 ± 0.1
Xyl	0.095	2.8 ± 0.05	3.3 ± 0.2	13.7 ± 0.3	6.6 ± 0.2	4.7 ± 0.1
	0.190	2.8 ± 0.05	3.1 ± 0.2	13.8 ± 0.3	6.6 ± 0.2	4.7 ± 0.1
	0.380	2.8 ± 0.05	3.1 ± 0.2	13.4 ± 0.3	6.6 ± 0.2	4.5 ± 0.1

[ab] Means within columns with different superscripts differ ($P < 0.05$).

Among the exceptions were the effects of the high dose of PME (0.40 U/g) on WHC and viscosity, and the effect of the two highest doses of PG (0.70 and 1.40 U/g) on pectin content.

However, the full dataset (Table 5.2) reveals evidence of synergistic interactions among the enzymes, with certain combinations of doses offering significant advantages over others. For example, the combination of higher dose of PG (0.70 or 1.40 U/g) with 0.40 U/g PMG and 0.190 or 0.380 U/g xylanase reduced both WHC and viscosity to the lowest levels observed.

Table 5.2. Effect of dose of enzyme combination on physico-chemical properties of lupin kernel *in vitro* (mean ± sem; n = 3). PG = polygalacturonase; PME = pectinesterase; Xyl = xylanase. Values in **bold text** are considered as informative with respect to detecting combinations of optimal doses.

PG (U/g)	PME (U/g)	Xyl (U/g)	WHC (g/g)	Viscosity (mPas/sec)	NDF (%)	ADF (%)	Pectin (%)
0.35	0.10	0.095	2.7 ± 0.04^{ef}	**2.5 ± 0.2^{lm}**	14.9 ± 0.5^{ab}	7.7 ± 0.4^{a}	5.1 ± 0.2^{abc}
		0.190	2.9 ± 0.04^{bcd}	3.8 ± 0.2^{cd}	14.6 ± 0.5^{abc}	**6.8 ± 0.4^{abcdef}**	5.0 ± 0.2^{ab}
		0.380	**2.6 ± 0.04^{fg}**	3.5 ± 0.2^{cdefg}	14.5 ± 0.5^{abcd}	7.4 ± 0. 4^{ab}	4.9 ± 0.2^{bcd}
	0.20	0.095	2.8 ± 0.04^{bcde}	3.4 ± 0.2^{defg}	**13.0 ± 0.5^{cde}**	**6.3 ± 0. 4^{bcdef}**	4.4 ± 0.2^{bcd}
		0.190	2.7 ± 0.04^{def}	2.9 ± 0.2^{hijkl}	**13.2 ± 0.5^{bcde}**	**6.6 ± 0.4^{abcdef}**	**4.5 ± 0.2^{cde}**
		0.380	**2.7 ± 0.04^{efg}**	3.6 ± 0.2^{cdef}	13.9 ± 0.5^{abcd}	**6.8 ± 0.4^{abcdef}**	4.7 ± 0.2^{abcd}
	0.40	0.095	**2.7 ± 0.04^{fg}**	2.9 ± 0.2^{hijkl}	**12.8 ± 0.5^{de}**	**5.9 ± 0.4^{ef}**	4.9 ± 0.2^{abcd}
		0.190	2.8 ± 0.04^{bcde}	3.0 ± 0.2^{hijk}	15.3 ± 0.5^{a}	7.2 ± 0.4^{abc}	5.2 ± 0.2^{abc}
		0.380	2.8 ± 0.04^{bcde}	**2.4 ± 0.2^{lm}**	14.6 ± 0.5^{abc}	**6.6 ± 0.4^{bcdef}**	5.0 ± 0.2^{bc}
0.70	0.10	0.095	2.9 ± 0.04^{ab}	2.7 ± 0.2^{jkl}	**13.1 ± 0.5^{cde}**	**6.1 ± 0.4^{cdef}**	4.4 ± 0.2^{bcd}
		0.190	3.0 ± 0.04^{a}	2.9 ± 0.2^{ijkl}	14.4 ± 0.5^{abcd}	**6.8 ± 0.4^{abcdef}**	4.9 ± 0.2^{abcd}
		0.380	3.0 ± 0.04^{a}	4.2 ± 0.2^{ab}	**13.2 ± 0.5^{bcde}**	**6.5 ± 0.4^{bcdef}**	**4.5 ± 0.2^{cde}**
	0.20	0.095	2.9 ± 0.04^{ab}	4.1 ± 0.2^{ab}	**13.5 ± 0.5^{bcde}**	**6.4 ± 0.4^{bcdef}**	4.6 ± 0.2^{abcd}
		0.190	2.8 ± 0.04^{def}	2.9 ± 0.2^{hijkl}	13.9 ± 0.5^{abcd}	7.2 ± 0.4^{abcd}	4.7 ± 0.2^{abcd}
		0.380	2.8 ± 0.04^{cde}	3.5 ± 0.2^{cdefg}	**13.0 ± 0.5^{cde}**	**6.8 ± 0.4^{abcdef}**	**4.4 ± 0.2^{bcde}**
	0.40	0.095	**2.6 ± 0.04^{fg}**	**2.6 ± 0.2^{klm}**	13.6 ± 0.5^{abcd}	**6.8 ± 0.4^{abcdef}**	4.6 ± 0.2^{abc}
		0.190	**2.7 ± 0.04^{efg}**	3.2 ± 0.2^{fghi}	**13.1 ± 0.5^{bcde}**	**6.0 ± 0.4^{cdef}**	**4.4 ± 0.2^{bcde}**
		0.380	**2.7 ± 0.04^{efg}**	**2.2 ± 0.2^{m}**	**12.8 ± 0.5^{cde}**	**6.1 ± 0.4^{def}**	**4.4 ± 0.2^{cde}**
1.40	0.10	0.095	2.9 ± 0.04^{ab}	3.1 ± 0.2^{ghij}	14.4 ± 0.5^{abcd}	7.0 ± 0.4^{abcde}	4.6 ± 0.2^{abcd}
		0.190	2.9 ± 0.04^{abc}	3.6 ± 0.2^{cde}	13.4 ± 0.5^{bcde}	**6.4 ± 0.4^{bcdef}**	4.6 ± 0.2^{abcd}
		0.380	2.9 ± 0.04^{bcde}	3.3 ± 0.2^{defgh}	**11.7 ± 0.5^{e}**	**5.7 ± 0.4^{f}**	**4.0 ± 0.2^{de}**
	0.20	0.095	3.0 ± 0.04^{a}	4.5 ± 0.2^{a}	**13.1 ± 0.5^{cde}**	**5.9 ± 0.4^{ef}**	**4.4 ± 0.2^{bcde}**
		0.190	3.0 ± 0.04^{a}	3.9 ± 0.2^{cdefg}	**13.0 ± 0.5^{cde}**	**6.5 ± 0.4^{bcdef}**	**4.4 ± 0.2^{bcde}**
		0.380	3.0 ± 0.04^{a}	3.5 ± 0.2^{cdefg}	**13.0 ± 0.5^{cde}**	**6.2 ± 0.4^{cdef}**	**4.4 ± 0.2^{bcde}**
	0.40	0.095	2.8 ± 0.04^{bcde}	3.8 ± 0.2^{bc}	14.5 ± 0.5^{abcd}	7.0 ± 0.4^{abcde}	4.9 ± 0.2^{abcd}
		0.190	**2.6 ± 0.04^{g}**	**2.4 ± 0.2^{m}**	13.6 ± 0.5^{bcd}	6.5 ± 0.4^{bcde}	**4.5 ± 0.2^{cde}**
		0.380	**2.7 ± 0.04^{efg}**	**2.7 ± 0.2^{jklm}**	13.6 ± 0.5^{abcd}	7.4 ± 0.4^{ab}	4.6 ± 0.2^{abcd}

^{abcdefghijklm} Means within columns with different superscripts differ ($P < 0.05$).

Neutral detergent fiber and ADF were also significantly affected by several of the enzyme-dose combinations: for example, the lowest dose of xylanase (0.095 U/g) in a combination with 0.35 or 0.70 U/g PG plus 0.40 U/g PME was a better combination for reducing NDF and ADF than combinations using the other xylanase doses (0.190 or 0.380 U/g).

5.4. Discussion

The results suggest that an optimal dose of pectinase and xylanase can be found that will break down the NSP in lupin kernel, thus improving the physical and chemical properties of lupin meal, and improving the nutritive value of lupin-based diets. However, in the present experiment, the optimal dose was not easy to identify because the differences between treatments were very small and, in many situations, increasing or decreasing the dose of one enzyme led to opposite outcomes for the physical and chemical properties. For example, 0.70 U/g PG and 0.40 U/g PME in combination with 0.38 U/g xylanase reduced both WHC and viscosity as effectively as the higher doses of PG (1.40 U/g) and the lower dose of xylanase (0.190 U/g).

To understand why there was no clear dose-response, we need to choose among two alternative scenarios: i) All the doses were too small to exert a significant effect; ii) All doses were sufficiently high to exert a maximal effect. A comparison of the data presented here with that presented in Chapter 3 supports the second scenario. For example, WHC was consistently reduced to a range of 2.6 – 3.0 g/g, below the values of 3.5 – 3.6 g/g observed in the no-enzyme control in Chapter 3. Similarly, for pectin content, for all the doses tested in this experiment, the values were 4.0 – 5.2%, well below the values achieved in Chapter 3 (9% for pectinase alone; 9.3% for xylanase alone; 10.8% for no-enzyme control). We therefore conclude that the range of doses

chosen was too high, with even the lowest dose inducing maximum responses, so a dose-response would be difficult to demonstrate.

The lack of major effects agrees with Malik & Bandla (2010) who also studied enzyme combinations *in vitro*, but they used xylanase and cellulase to treat wheat straw. However, a more useful comparison is with the study by Ali *et al,* (2009) who worked with lupin meal in our laboratory and found that the optimal combination for reducing WHC and viscosity was 1.4 U/g PG plus 0.20 U/g PME. This conflict can be explained by the inclusion of xylanase in the present study because this enzyme allows lower doses of PG and PME to have the same effect as the higher doses used by Ali *et al,* (2009). Moreover, in comparison with the study by Ali *et al,* (2009), we used different sources of enzyme, derived from different types of microorganism, and produced through different manufacturing conditions, raising the possibility of differences in specific activity (Wang & McAllister 2002). Indeed, using different enzyme sources, even from the same, single microbial species, can result in different enzymatic acidities (Gashe 1992). Thus, Beauchemin *et al,* (2003) found that the dose and type of enzyme are one of the factors affecting the response to enzyme supplementation for ruminant animal.

5.5. Conclusion

The protocol used in this study can lead to the definition of an optimal dose of pectinase and xylanase for breaking down the NSP in lupin kernel, thus improving the physical and chemical properties of lupin meal, outcomes that would most likely improve the nutritive value of lupin-based diets. However, it appears that the enzymes were all more effective than expected, particularly in combination, so we were not able to demonstrate clear dose-response relationships for any of the physical and chemical proper and thus

not able to define the optimal doses. This conclusion shows that defining an optimal dose is not easy.

On the other hand, it seems likely that all of the dose combinations tested were in the optimal range, because they all appear to have greatly improved all of the physico-chemical properties measured. Therefore, the lowest doses should be suitable as a starting point for treating lupin-based feed, particularly as the entire enzyme preparations are inexpensive and unlikely to be a major factor in feed costs compared to the savings made by using lupin meal. Future studies should test a wider range of doses, with at least one dose that is much lower than the lowest one used here.

Chapter 6

General Discussion

The general hypothesis of this thesis was that the synergistic effect between pectinase and xylanase will improve the physico-chemical properties of lupin meal more than pectinase or xylanase individually and hence lead to greater improvements in growth performance and FCR for Japanese quail fed lupin-based diets. The results clearly support this hypothesis with respect to improvements in water holding capacity, viscosity and pectin content *in vitro*, and these improvements were translated into better growth and FCR for Japanese quail fed lupin-based diets.

These studies add to the overwhelming evidence from studies with chickens showing that the poor performance of lupin-based diets without enzyme supplementation are caused by the high concentrations of soluble NSPs that have anti-nutritive effects on digestion and absorption in poultry because birds lack the enzymes required to break down NSPs (Choct & Annison, 1992). Also, we are now in a strong position to translate these concepts to the quail industry. However, the performance of quail was lower with a diet containing 20% lupin meal than with a diet containing 10% lupin meal, independently of the enzyme treatments tested in these studies. Clearly, before quail producers can adopt diets with more than 10% lupin meal, more research is needed on doses and combinations of enzymes.

In the second *in vitro* study in this thesis, we attempted to identify the optimal combined dose of pectinase and xylanase. All combinations improved the physico-chemical properties of lupin meal, but no dose-response for any of the enzymes tested was evident. Comparison with other *in vitro* studies, including that in Chapter 3, suggests

that that all the enzyme-dose combinations that we tested were exerting a maximal effect, so could be considered to be within the ‘optimal’ range. Therefore, further studies need to be conducted to determine a clear optimal dose, with a wider range of doses, with particular emphasis on searching for lower doses that are effective.

In conclusion, supplementing lupin with a combination of pectinase and xylanase improves the physical and chemical properties of lupin meal and thus leads to improvements in growth and FCR in Japanese quail fed a lupin-based diet. Producers can include 10% lupin meal in the diets of Japanese quail if they use a combined supplement of pectinase and xylanase, thus significantly reducing total production cost without compromising production performance or bird welfare.

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