MAXIMIZING THE NUTRITIONAL VALUE OF UNPROCESSED SOYBEAN MEAL THROUGH SUPPLEMENTATION WITH COMPLEX MICROBIAL ENZYME PRODUCTS

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SUMMARY

This thesis presents findings of a comprehensive research project on the potential of raw soybean meal (RSBM) as partial replacement of commercial soybean meal (SBM) in diets for broiler chickens. There was an extensive review of literature on the subject, followed by one *in vitro* experiment and five feeding trials.

The first experiment of this thesis (Chapter 3) investigated the physico-chemical properties of RSBM as a feed ingredient and the influence of the ingredient on the physical and chemical properties of broiler diets. The effects of heating (95^0C) the soybean samples over different durations on the nutrient composition and concentrations of anti-nutritive factors (ANF) were assessed. The enzymatic *in vitro* digestibility of nutrients in the samples was also evaluated. The results of the *in vitro* study showed that heating the soybean samples at 95^0C for up to 60 min was not sufficient to reduce the levels of ANF, particularly the trypsin inhibitors (TI). Replacing commercial SBM with RSBM (up 30%) in diets reduced the quality of the diets; for example, the urease activity (UA) and nitrogen solubility index (NSI) were increased. The *in vitro* digestibility of DM and CP as well as phytate were improved when the samples were incubated in a cocktail of protease and phytase compared to when these individual enzymes were used or not used.

In addition to the pellet durability index (PDI), the effects of two pelleting methods on diets containing graded levels of RSBM were assessed in another experiment (Chapter 4). The concentrations of ANF, particularly TI and performance of birds were evaluated. Feed particle distribution and dietary electrolyte balance (DEB) were also investigated in this experiment. Steam-pelleting the diets containing high levels of RSBM improved the PDI, compared to cold-pelleting but the amino acid profiles were better in cold-pelleted and mash samples than the steam-pelleted diets. Increasing the level of RSBM, particularly replacing 30% of commercial SBM (9% of diet) reduced the feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR) of broilers when fed from one to 14 days of age. Birds fed on the steam-pelleted diets had reduced BWG. Increasing RSBM in diets affected the development of internal organs, particularly the weight of the pancreas and duodenum, which was increased. The response of broilers fed on cold-pelleted diets containing graded levels of RSBM (replacing 0, 10 or 20% of SBM) and supplemented with increasing levels of microbial protease is reported in Chapter 5. The gross response of the birds, in terms of FI, BWG, and FCR; development of internal

organs, and meat yield were assessed. Furthermore, the activities of digestive enzymes, ileal nutrient digestibility, intestinal mucosal morphometry and concentration of DNA in the pancreas were also investigated in this experiment. Although the contents of ANF, especially TI exceeded the threshold level for poultry, the gross response (BWG, FI and FCR) of birds on the tested diets was statistically similar to that of bids fed on the RSBM-free diets. These results may be in response to microbial protease supplementation, ameliorating the adverse effects of ANF, particularly TI. The activities of some digestive enzymes at 24 d of age, for example trypsin and chymotrypsin improved in response to protease supplementation. The apparent ileal digestibility (AID) of CP and amino acids (AA) were reduced with increased levels of RSBM in diets.

These same parameters were further evaluated in another trial when diets contained a relatively higher level of RSBM (replacing commercial SBM at 25%) and extra-dosing with microbial protease and phytase (Chapter 6 of this thesis). Feeding the birds on diets containing this higher level of RSBM did not statistically reduce the gross response of broilers over 1-35 d, and these results further suggest the positive effects of extra-dosing of the diets with microbial protease and phytase. The villus height and crypt depth of broilers at 10 d of age were increased when the diets were extra-dosed with phytase. A preliminary cost-benefit analysis also showed that the use of the enzyme supplements even at extra-dose levels did not substantially increase the cost of the diets.

The effects of the test ingredient (RSBM) on dietary protein utilization were evaluated in Chapter 7. This involved measurements of AID of protein and AA. Endogenous nitrogen secretion at the ileum was also measured and used to calculate the standardized ileal digestibility's (SID) of protein and AA. The test ingredient reduced both the AID and SID of protein and AA but these were improved by supplemental protease.

The wellbeing of broilers, in terms of mortality, footpad dermatitis, intestinal lesions, tibia bone characteristics and litter quality were assessed and reported in Chapter 8. These traits were all not significantly different in chickens on the test diets compared to those on the RSBM-free diet. The concentrations of plasma myo-inositol, Na and Cl at 24 d of age were not affected by RSBM or protease supplementation. The results confirmed what was observed in previous Chapters in terms of productivity and health of the birds on the RSBM-containing diets.

A major finding of this project is that although the dietary concentration of TI was substantially increased with increase in level of RSBM, there was no drastic impact on productivity or health

of the birds. This may be due to the effect of the supplemental enzymes included in the diets. It can be concluded that RSBM could replace commercial SBM at levels beyond what was previously thought possible provided the diets are supplemented with appropriate microbial enzymes. The preliminary cost-benefit analysis of using RSBM indicate good returns but further studies are required into the behaviour of proteins in RSBM, especially in the presence of the test protease.

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PUBLICATIONS

Studies completed during candidature, some of which are reported in this thesis have been presented in the following scientific communications:

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CANDIDATE'S CERTIFICATION

I certify that the substance of thesis has not already been submitted for any degree and is not currently being submitted for any other degree or qualification.

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Signature

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AA	Amino acids
ANF	Antinutritional factors
AME	Apparent metabolizable energy
AP	Alkaline phosphatase
ASA	American Soybean Association
AVSA	Apparent villus surface area
BBI	Bowman-Birk inhibitors
BWG	Body weight gain
BSA	Bovine serum albumen
CP	Crude protein
CAES	College of Agriculture and Environmental Sciences
CRD	completely randomized design
d	days
DM	Dry matter
DEB	dietary electrolyte balance
DMD	Dry matter digestibility
DCP	Digestibility of crude protein
EE	Ether extract
FAO	Food and Agriculture Organization
FI	Feed intake
FF	Full-fat
FCR	Feed conversion ratio
g	Gram
GE	Gross energy
G+P	Gizzard + proventriculus
GP	General proteolysis
GLM	General linear model
h	Hours
J+I	Jejunum + ileum
KTI	Kunitz trypsin inhibitors
ME	Metabolizable energy
MJ	Mega joule
N	Nitrogen
NE	Net energy
NSI	Nitrogen solubility index
NSP	Non sugar polysaccharides
NRC	National Research Council
PDI	Pellet durability index
RSBM	Raw soybean meal
SBM	Soybean meal
SID	Standardized ileal digestibility
STD	Standards
TI	Trypsin inhibitors
Ti	Titanium
TIU	Trypsin inhibitor unit
UA	Urease activities

CHAPTER 1: GENERAL INTRODUCTION

Feed costs, including charges for processing and transportation constitutes a major expense in poultry production. After ingredients that provide energy, protein source are the next most important in poultry diet formulation. Due to its protein content and a well-balanced amino acid profile, soybeans meal (SBM) is a good source of protein for poultry. Soybean meal is available both as extracted (oil removed) product or a full-fat product (without extracting the oil). Full-fat soybean meal can also be prepared from the raw meal or after heat treatment. The raw soybean meal (RSBM), however, contains a variety of anti-nutritional factors (ANF) which limit the proper utilisation of nutrients in soybeans by non-ruminant animals. Protease inhibitors, lectins and phytate are the best characterised ANF in raw soybean seeds (Pettersson and Pontoppidan, 2013).

Protease (trypsin) inhibitors can interfere with the biological activity of endogenous protease and thereby reduce the digestion of proteins (Dourado *et al*., 2011; Nahashon and Kilonzo-Nthenge, 2013). Researchers, for example de Coca-Sinova *et al. (*2008) have tested different samples of SBM and found that the apparent digestibility of N, and AA in broilers varied widely with the highest values for samples with lower trypsin inhibitors (TI). The loss of endogenous protein has been found to be increased due to the activity of TI in the body consequently affecting the nitrogen balance (Barth *et al.,* 1993). Herkelman *et al.* (1992) noted that the presence of TI in diets can cause digestive diseases in non-ruminant animals. A high level of TI in diets, mostly of soya origin, has also been found to correlate with a rapid feed passage, which leads to greater nitrogen excretion, and hence poor litter quality (Ruiz and De Belalcázar, 2005; Ruiz, 2012).

Phytate (phytic acid) is another ANF that is present at high levels in soybeans. Phytate impedes digestion by forming protein-phytate or protein-phytate complexes and is resistant to digestion (Chen *et al*., 2013b). Phytic acids have a negative impact on amino acid digestibility and have recently been shown to increase the endogenous nutrient losses in pigs and poultry (Jongbloed *et al.,* 1996; Woyengo and Nyachoti, 2013). Supplementing the diets with microbial phytase however breaks the bonds of phytic acids to release the nutrients, such as minerals. Consequently the physical parameters and mineral composition of tibia bones of broilers were improved when microbial phytase is added to broilers' diets (Qian *et al*., 1996; Oluyinka *et al*., 2008; Slominski, 2011).

Apart from the effect of ANF, the nutritional values of SBM can also be affected by many other factors, such as the origin of the soybeans, the cultivars, and the way the meal is being processed (Nahashon and Kilonzo-Nthenge, 2013). Stakeholders therefore need to look at a range of options for improving both feed quality and animal performance. Doing this could enhance in broiler production as well as a reduction in pollution (Zohair *et al.,* 2012).

To improve the nutritional value of soybeans containing ANF for poultry, many strategies are currently being employed. These include development of new breeds of soybean with low anti-nutrient contents, treating the raw seeds with heat, or supplementation with microbial enzymes. Heat treatment is generally considered as the most effective approach to inactivate some of the TI, but not phytate, or Bowman-Birk inhibitors, oligosaccharides, and antigenic proteins. Furthermore, either under- or over-heating soybean seeds while processing has frequently been reported to reduce the nutritional values of SBM for poultry (Perilla *et al.,* 1997). The practice of using chemicals, such as solvents, to extract oil is questionable as the residual chemicals in the SBM may pose health risks.

The poultry industry therefore needs to utilise biotechnological techniques, such as genetic modification of feedstuffs, or feed additives (enzymes), to improve the nutrient balance of ingredients such as SBM. Before intervening with the application of biotechnological techniques (enzymes) to improve the nutritional value, information regarding the physical and chemical properties of feed ingredient are required (McDonald *et al*., 2002).

Evidence is steadily emerging that the use of new-generation enzymes is one of the most promising approaches to enhance the productivity of non-ruminant animals through improving the nutritional value of feeds (Adeola and Cowieson, 2011). Microbial proteases are protein-digesting enzymes that are used in pig and poultry diets to break down the stored proteins and proteinaceous anti-nutrients in various plant materials (Barletta, 2011; Ao, 2011). In addition to releasing phosphorus, supplementation of microbial phytase also improves the digestibility of CP and amino acids of plant-based proteins (Barletta, 2011; Guggenbuhl *et al.,* 2012).

There are few, if any, studies evaluating the response of raw soybean meal (RSBM) to microbial enzymes and subsequent performance and wellbeing of broiler chickens. Therefore the major objectives of this thesis were to:

- Assess the physico-chemical properties of RSBM as a feed ingredient as well as the diets containing RSBM.

- Test the enzymatic *in vitro* digestibility of RSBM, as an indication of *in vivo* nutritional values.
- Evaluate the potential of a new-generation microbial protease in reducing trypsin inhibitors, in diets containing RSBM for broilers.
- Examine the performance of broilers fed diets containing relatively higher level of RSBM and extra-dosed with microbial protease and phytase.
- Testing effects of graded levels of RSBM in diets and supplemented with protease on standardized ileal digestibility of CP and AA.
- Assess the wellbeing of broilers fed on diets containing RSBM with evidence of high levels of trypsin inhibitors.

The hypotheses tested were:

- Raw soybean meal in broilers diets could negatively affect the birds, including their productivity and health.
- The negative effect of RSBM in diets could be reduced through supplementation with phytase and highly potent protease, and
- The cost of production could be reduced or at least not altered due to inclusion of RSBM and high doses of microbial enzymes**.**

CHAPTER 2: LITERATURE REVIEW

ANSTRACT

The increasing worldwide population and improvements in living standards are driving forces for increased meat product demand. Chicken meat is more readily available than other meats due to several factors, including a short cycle of production, low costs of production, low impact of production on green-house gases and high efficiency of production. Soybean meal (SBM) remains the most important and preferred protein feed source for poultry. However, the supply of SBM fluctuates, and the meal is expensive due to processing and transportation costs. On the other hand, although there is a growing interest in the use of raw SBM for birds, its nutritive value is negatively affected by the presence of anti-nutritive factors (ANF). Heat treatment is applied to alleviate some of the ANF, such as trypsin inhibitors and lectins, but both under- and over-processing of soybeans can reduce the nutritive value of the meal. Feed supplementation with exogenous enzymes, such as phytase and protease enzymes, is becoming a common biotechnological option for improving the nutritional values of SBM and other ingredients. Proteases are protein-digesting enzymes that break down both stored proteins and proteinaceous anti-nutrients in feeds. Various studies have shown that the performance of birds can be improved through dietary supplementation with new-generation protease enzymes. Phytase is also effective in breaking down phytate (phytic acid), which chelates with mineral cations and other nutrients in soybeans. Recent *in vitro* and *in vivo* studies have shown that a cocktail (combined use of protease and phytase) is more effective in reducing ANF in soybean meal for birds than the use of single enzyme products. This review provides information on how microbial enzymes, particularly protease and phytase, contribute to the improvement of the nutritional values of different types of SBM for poultry.

2.1 INTRODUCTION

Poultry production is one of the most advanced industries in the livestock sector (Asyifah *et al.,* 2012) and relies on a complex feed production process. The ban on animal proteins in poultry diets, in some major production regions has led to an increase in demand for vegetable protein sources (Kocher *et al.,* 2002). Soybean meal (SBM) is the most commonly used vegetable protein source for poultry feed. Although soybean (*Glycine max*) is primarily grown for oil, the meal can be fed to poultry as full-fat or after oil extraction.

Soybeans, however, contain a number of anti-nutritive factors (ANF), such as trypsin (protease) inhibitors, lectins (Newkirk, 2010), phytate, non-starch polysaccharides (NSP) and oligosaccharides (Jezierny *et al.,* 2010), which contribute to a reduction in nutrient utilisation. Anti-nutritional factors elicit their negative effects via different mechanisms, including binding to digestive enzymes and nutrients or increasing gut viscosity (Ao, 2011). Due to carbohydrase inhibitors, such as pectins and NSP (Karr-Lilienthal *et al*., 2005; Choct *et al.,* 2010), energy from soybeans is poorly utilized by chickens (Ao, 2011). Pierson *et al*. (1980) noted that the low metabolizable (ME) of soybean meal in poultry is due to the poor digestibility of the carbohydrate fraction. Because of approximately 65-80% of the total phosphorus in the soybean seed is bound to phytic acid or phytate, most of the minerals, particularly phosphorus (P) is biologically unavailable because poultry do not possess enough endogenous phytase to digest the complex (Lall, 1991; NRC, 1994).

Heat treatment is generally considered an effective approach to inactivate ANF, but some of the ANF, e.g., phytate, oligosaccharides and antigenic proteins, cannot be reduced or decreased by heating (Ao, 2011). Although Campbell and Schöne (1998) suggested that trypsin inhibitors in general and lectins in soybeans could be inactivated by heat, Clemente *et al*. (2008) reported that Bowman-Birk Inhibitors (BBI), which are a group of trypsin inhibitors, exhibit considerable resistance to heat treatment. The nutritional value of commercial SBM is also occasionally affected by the use of excessive heat (Moure, *et al.,* 2006). A further problem is the health risks associated with the residual solvents that are used to extract the oil (O'Quinn *et al.,* 1997). The price of SBM could also be affected by the degree of processing and transportation costs (Newkirk, 2010; Shi *et al*., 2012). The solution to these problems may lie with the optional use of microbial feed enzymes, e.g., phytase and protease, which have been developed and used to improve the digestibility of nutrients through reducing the impact of ANF (Yadav and Sah, 2005; Ao, 2011). Microbial enzymes are also routinely used by the

poultry meat industry to improve the quality of diets and growth and feed efficiency (Amerah *et al.,* 2011). There has been a considerable amount of research regarding the use of microbial enzymes in poultry nutrition (Abudabos, 2010), but there is still limited information on their effects in diets containing raw full-fat soybeans (ASA, 2004). The objective of this paper is to review the findings on the efficacy of supplementing soybean-containing diets with feed enzymes, particularly protease and phytase, in an effort to reduce the impact of anti-nutrients and thereby improve the nutritional value of such diets.

2.2 Physiochemical properties of soybeans in relation to the nutritional values

Information regarding the physical and chemical properties of feed ingredients in general enables the development of interventions to improve the value of the ingredients (McDonald, et *al.,* 2002). Soybeans are leguminous plants with numerous uses. Although the crop is primarily grown for oil, soybean meal can also be fed to animals, especially non-ruminants, as full-fat or after extracting the oil. Of all the legume seeds, soybean meal has the highest level of crude protein and has the best amino acid profile, with only 6% crude fibre (Dei, 2011). The amino acids of SBM are highly digestible by all types and ages of poultry (Newkirk, 2010). Although SBM has highly digestible amino acids and a high lysine content (Pettigrew *et al*., 2008), there are still variations in the chemical and nutritional composition among the sources and the varieties of soybeans (Grieshop *et al*., 2003). The nutrient composition and quality assessment values for conventional, full-fat and raw whole SBM are shown in Table 2.1. The maximum (50.0%) and minimum (36.5%) CP values are usually associated with conventional and raw full-fat SBM, respectively. As expected, full-fat meal contains more (20.9%) fat (ether extracts) than other preparations, and crude fibre is the lowest (3.0%) in commercial SBM.

2.3 The importance of soybeans meal in poultry nutrition

Soybean cake or soybean chips are the direct product after most of the oil is extracted from whole soybeans by pressure or solvents, and soybean meal (SBM) is derived from grinding the soybean cake, soybean chips or soybean flakes. Soybean meal is usually classified for marketing by its crude protein content or by the sum of protein and oil (Heuzé *et al.,* 2015). Soybean meal may be prepared from dehulled material prior to oil extraction, and the hulls may be added back at the end of the process.

Table 2.1 The Nutritional composition and some quality parameters of full-fat, raw whole soybean and conventional soybean meals

Types of meals	CP	EE	Starch† (sucrose††)	CF	UA	KOH	ME	References
	%	%		%	ΔpH	%	kcal/kg	
Conventional SBM	44 -50	2-3	-	3	-	-	-	Heuzé *et al.(*2015)
	46.3-48.1	0.9 -2.9	-	3.9-5.2	0.0-0.01	68.1-84.0	3524	Serrano *et al*. (2012)
	46.4-48.2	1.09-2.05	††5.42-8.29%	3.63-6.08	0.007-0.081	69.7-74.3	2,000-2,375	Ravindran *et al*. (2014b)
Full-fat SBM	36-42	18-22	-	-	-	-	-	(Stein *et al*., 2008)
	38.0	20.9	-	5.1	0.28	-	-	Senkoylu *et al*. (2005)
	35.1- 39.9	17.7- 19.2			0.30-0.02	81.1-63.1	3016-3695	Ravindran et al. (2014a)
Raw full-fat SBM	37.08	18.38	-	-	-	-	-	Van Eys *et al*.(2004)
	36.5-43.2	20.7-22.2		2.5-8.3	-	-	-	Sharma *et al.* (2013)
	39.4-44.4	14.0-18.7	†4.3-6.7, g/kg	-	1.99	-	-	Sharma *et al.* (2014a)

KOH = protein solubility testing method; UA= urease activities; †= starch; ††= sucrose.

It is an established fact that soybean meal is the best and the most commonly used vegetable protein for poultry. However, due to events in the major production areas of the world, the supply of SBM fluctuates (Shi et al., 2012), and due to transportation and long processing procedures, the cost of SBM is also continuously rising (Newkirk, 2010; Shi et al., 2012). In addition, SBM is usually extracted with hexane, which is non-biodegradable, and this has associated with health risks for consumers (O'Quinn et al., 1997). Although typical SBM could have up to 85% ileal digestibility of CP, the average values from different batches and origins ranged between 75.2 and 82.3%. The reason for this variation might be due to differences in quality (Pettersson and Pontoppidan, 2013).

Due to differences in the origin of SBM, a wider variability in amino acid digestibility was also reported by Ravindran *et al*. (2014b). Other research scholars (Zanella *et al.,* 1999; Ao, 2011) have however suggested that by supplementing the selected exogenous enzymes the digestibility of SBM could be improved.

2.3.1 Socioeconomic and biophysical benefits of improving nutritional value of soybeans for poultry

The increasing worldwide population and an improvement in living standards are driving forces behind the quest to provide adequate animal protein (Boland *et al.,* 2013). To meet the double demand of mitigating climate change and improving feed efficiency, poultry production is the preferred system over other animal industries (Mengesha, 2011). Feed cost is considered the primary variable cost in poultry production (Ravindran, 2013). Due to the ban of animal proteins from inclusion in feed in some regions (Jan and Teum, 2011), vegetable protein, particularly soybean-based feeds are becoming more popular, as it is the best vegetable protein source in terms of quality (Banaszkiewicz, 2011). Thus, SBM is becoming an important source of feed for poultry and remains the most important and preferred source of high quality protein for animal feed manufacturing (FAO, 2014).

Conventional SBM is defined as the product that remains after the extraction of oil by either a solvent or expeller process and constitutes approximately 69% of the total world output of protein feedstuffs (Oil-World, 2010; Crowell, 2012). Approximately 85% of the world's soybeans are processed into SBM and oil (Soya-tech, 2003).

The inclusion level of SBM ranges from 25% in chick diets to 30–40% in diets for older broilers and laying hens (McDonald et al., 2002; Willis, 2003). According to Araba and Dale (1990), proper processing of vegetable proteins like SBM could result in improved growth when fed

to non-ruminant animals. Performance and cost-benefit results of broiler chicks fed processed soybean diets were better than those of birds fed unprocessed soybeans (Aletor and Olonimoyo, 1992). However, Mojtaba (2008) questioned whether or not the benefit always outweighs the processing cost.

Although soybean products are considered the best protein source based on the quality (Banaszkiewicz, 2011), the nutritive value is negatively affected by the presence of ANF (Liener, 1995; Liu *et al*., 1998), especially trypsin inhibitors and lectins (Newkirk, 2010). This problem is addressed mainly by heat treatment, as was previously highlighted. Researchers are also of the view that future improvements in the nutritional value of soybeans for birds will be achieved using alternative methods, such as genetic engineering of enzymes (Bharathidhasan *et al*., 2009; Ao, 2011).

2.4 Extruded full-fat soybean meal

Feed intake and weight gain of chicks fed the diet containing 15 % of extruded full-fat SBM were significantly reduced compared with those fed the control diet during 0–10 days of age, but were not influenced during grower and finisher stages and the whole period (Zhaleh *et al*., 2015). According to Mirghelenj et al. (2013), growth performance of broiler chickens was not influenced when birds were fed a diet containing up to 15 % extruded full-fat SBM in place of dehulled SBM. Inclusion of extruded full-fat SBMB at 120 g/kg in grower broiler diet had no adverse effect on performance (Foltyn *et al*., 2013).

By increasing temperature of soybean extrusion from 118 to 140°C the value of trypsin inhibitors, urease activities and protein solubility were decreased and thereby the performance of chicken was also improved (Perilla et al., 1997). Clarke and Wiseman (2007) added also that by increasing extrusion temperature of soybean from 90 to 160°C, the concentration of ileal apparent digestible lysine was increased from 10·53 to 17·63 g/kg. However, Leeson and Atteh (1996) reported that extrusion of soybeans at 140°C did not significantly improve the body weight gain and feed efficiency or mortality rate.

2.5 Full-fat soybean meal

Full-fat soybean meal typically contains hulls and is produced by different methods, including extrusion, cooking/autoclaving, roasting/toasting, micronizing and jet-sploding, to reduce/inactivate the ANF from the whole seed (Dei, 2011). Full-fat soybean is characterized by its low crude protein content (36-42%) and high level of fat (18-22%), which make it a good source of quality protein and energy for non-ruminant animals (Stein *et al*., 2008). Apart from being high in oil, full-fat soybean meal has almost the same excellent amino acid (AA) profile as conventional SBM, and sometimes the digestibility of AA in full-fat soybeans is greater than that of extracted SBM (Pahm and Stein, 2007).

Because full-fat soybeans contain more energy (15 to 27%) than extracted soybean meals (Newkirk, 2010); they could be an excellent source of energy for poultry in addition to being used as a protein source (ASA, 1997; Willis, 2003). Woodworth *et al.* (2001) also reported that the concentrations of DE and ME in full-fat soybeans are greater than in extracted SBM.
Due to the development of new varieties of soybeans with limited or lower levels of ANF, the use of full-fat soybean meal is increasing (Gu *et al.,* 2010). Partially replacing commercial SBM with full-fat meal obviates the cost of transporting the beans to the oil-extraction plant and shipping the meal back to distributors and to the end users (Newkirk, 2010). Full-fat SBM is becoming more popular in soybean-growing regions where there are no local oilseed processing plants.

The oil that is extracted from soybeans is widely used to supplement soybean-based diets for broiler chickens and growing turkeys as a feed-grade fat to increase energy density and improve the efficiency of feed utilisation (Sell *et al.,* 1979). This is costly for producers. Hence, it would be more cost-effective to use full-fat SBM, which can replace both conventional SBM and the soybean oil supplement. However, users are still wary of using full-fat meal for birds because chickens may deposit the fat that they consume with little or no modification (Newkirk, 2010). MacIsaac *et al*. (2005) suggested that properly heated full-fat soybeans can be used in all poultry diets, but excessive heating denatures important nutrients, especially amino acids (Lokuruka, 2011). Treating whole soybeans with heat is expensive and time consuming.

There is considerable information regarding the use of raw beans in feeds for poultry, and research indicates that tolerance to ANF depends partially on the age of the birds (Johri, 2005).

Barletta (2011) reported that exogenous feed enzymes could help to break down the ANF present in many feed ingredients for poultry and pigs and promote the use of raw full-fat SBM for birds.

2.6 Raw, full-fat soybeans meal

Although full-fat SBM has a good nutrient composition (Willis, 2003; Dei, 2011), the nutritive value of raw full-fat soybeans is negatively affected by the presence of ANF (Liu *et al*., 1998), especially by trypsin inhibitors and lectins (Newkirk, 2010). Various scholars (Liu *et al*., 1998; Newkirk, 2010; Erdaw *et al*., 2015a & b) have reported that feeding raw SBM with high levels of trypsin inhibitors and lectins negatively affects pancreatic function, the growth of birds and feed efficiency. Mogridge *et al.* (1996) reported that the consumption of raw beans increased the size of the pancreas (0.80 vs 0.37% of live weight) and the duodenum (1.35 vs 1.06% of live weight) and reduced feed consumption and growth of the chicks (66 vs 97 g/14 d). Similarly, ASA (2004) reported that diets based on raw beans reduced feed consumption and live weight and also decreased the feed conversion indices by 14, 35, and 53%, respectively.

Because some of the ANFs in soybeans are heat-labile, such as Kunitz trypsin inhibitors and lectins, heat treatment is always recommended before feeding to improve the nutritional value of raw full-fat soybeans (ASA, 1997). However, some of the other ANFs are heat-stable (BBI, oligosaccharides and phytate) factors (Clemente *et al.,* 2008; Ebrahimi-Mahmoudabad and Taghinejad-Roudbaneh, 2011). Although Rackis and Gumbmann (1981) reported that the activity of trypsin inhibitors in soybeans can be readily be eliminated by ordinary cooking and moist-heat treatment, Clemente *et al*. (2008) suggested that BBI, a group of trypsin inhibitors, are heat-stable. Sometimes, due to over- or under- heating of soybeans during roasting and oil removal, the nutritional quality is reduced (Perilla *et al*., 1997; Newkirk, 2010). Different types of commercial SBM from different sources, which might be processed at different heating intensities, were recently tested for their protein quality parameters, such as TI, UA and KOH, and they were found to be significantly different (Ravindran *et al*., 2014b).

Another method that has been suggested to reduce ANF in grains, including soybeans, is grinding. This type of process primarily disrupts the cell-wall structure of oilseeds and thereby increases the exposure of nutrients to digestive enzymes (Meng *et al.,* 2006). Huang *et al.* (2006) also found that altering the feed structure improved disease resistance in non-ruminant

animals. Moreover, Ravindran and Son (2011) reported that exogenous enzymes are now routinely used as feed additives in diet formulations for poultry and pigs to overcome the negative effects of several ANF.

2.7 Anti-nutrients and their importance

Soetan and Oyewole (2009) suggested that factors that determine the nutritive value of foods and feedstuffs are very complex. Anti-nutritional factors or anti-nutrients are compounds that interfere with the intake, availability, or metabolism of nutrients in the animal (Campbell and Schöne 1998; Soetan and Oyewole, 2009). Many protein-rich oilseeds/feedstuffs contain anti-nutrients that prevent their proper utilisation. Problems associated with soybeans are mainly due to the presence of ANF. Soybeans contain anti-nutrients, such as protease inhibitors and lectins, which have toxic effects when ingested by humans or non-ruminant animals (Nahashon and Kilonzo-Nthenge, 2013). Inhibitors that prevent protein digestion mainly target trypsin and chymotrypsin (Jiao *et al.*, 1992). In such situations, pigs and poultry cannot digest approximately 15–25 % of the feeds they eat.

In addition to developing crop varieties with low anti-nutritional factors and treating with heat (Gu *et al.*, 2010; Newkirk, 2010), dietary supplementation with enzymes can reduce anti-nutrients in SBM for poultry (Ao, 2011). The intensity of the negative impact on non-ruminant animals will expectedly vary, and the biotechnological intervention, for example, the supplemental level of microbial enzymes, must be considered. As indicated in Table 2.2, the maximum protein solubility (KOH) value of raw full-fat SBM is 98%, whereas the maximum value of KOH for commercial SBM is approximately 87%. The concentration of trypsin inhibitors (TI) in raw full-fat soybeans ranges from 23.9 to 85.0 mg/g, whereas the maximum TI value for commercial SBM is 3.45 mg/g. Such variations in TI content of raw full-fat SBM may arise either from differences in soybean crop varieties or origins.

2.7.1 Development, mode of action and use of exogenous enzymes

The first exogenous enzyme was synthesized by Schweiger and Gold (1969) but commercial utilization of enzymes in animal nutrition has been used less than 30 years. Their original purpose was to degrade so-called anti-nutritional molecules from grains used in feedstuffs, such as wheat, barley and wheatgrass. A unique feature of enzymes is their high substrate-specificity. Each enzyme breaks down highly specific substrates at specific reaction sites while different feed enzymes (Table 1) will have different modes of action, including degradation of

antinutritional factors that limit nutrient digestion and directly increase intestinal digesta viscosity indirectly, or both, augmentation of endogenous digestive enzymes, which are either insufficient or absent in the bird, resulting in improved digestion. This will be especially true for newly hatched chicks with immature digestive systems and reductions in endogenous secretions and protein losses from the gut resulting in reduced maintenance requirements (Bedford, 1998; Cowieson and Ravindran, 2007; Cowieson et al., 2009).

2.8 Effects of dietary anti-nutrients on poultry

Anti-nutritional factors cause depression in growth, performance and can negatively affect the health of animals. Although tolerance depends on the age of the birds (ASA, 2004), excessive levels of ANF reduce the nutritional quality of feeds (Stein *et al.*, 2008). However, some of the ANF can be denatured by heating (Newkirk, 2010); this process is discussed elsewhere in this review.

When animals lack specific enzymes to break down certain feed components, ANF interfere with the normal digestion process when they are present in the diet (Barletta, 2011). Birds fed on diets containing raw soybeans do not thrive. Their feed consumption rate is reduced by as much as 14%, their live weight gain drops by up to 15%, and the feed conversion rate decreases by approximately 53% (ASA, 2004). Palacios *et al.* (2004) added that chicks fed on diets containing any variety of raw soybeans gained less weight.

2.9 Dietary protease inhibitors in soybeans and their response to microbial proteases

Natural trypsin inhibitors, also known as serine protease inhibitors (serpins), are the largest and most diverse family of protease inhibitors. There are two main types of trypsin inhibitors: Kunitz trypsin inhibitors (KTI), which are the larger and stronger inhibitors of trypsin, and Bowman-Birk inhibitors (BBI), which are much smaller and inhibit both trypsin and chymotrypsin (DiPietro and Liener, 1989).

Table 2.2 Response of birds fed diets supplemented with different microbial enzymes.

Enzyme types	Supplementation (g/kg)	Base of diets	Type of chickens	Improved on	References

Roxazyme G®	2.0	Corn-ground nut	Broilers	Dressed percentage	Omojola and Adesehinwa (2007)
Multi-Carbohydrase	0.1	Corn/SBM	Broilers	No carcass part was improved	Vieira *et al*. (2006)
Multi-Enzymes		Corn	Broilers	Carcass yield was improved	Bamgbose *et al.(*2005)
Carbohydrolase	0.05	Corn/SBM	Broilers	Breast was highly improved	Ceccantini and Montanhini (2011)
Xylanase and β-glucanase	0.5	Corn/SBM	Broilers	Carcass, thigh & drumstick percentages were improved	Hajati (2010)
Protease and phytase	5,000 units/kg of diet	Corn/SBM	Broilers	Activity of ileal maltase, sucrase, and L-alanine aminopeptidase were increased	Murugesan *et al.* (2014)

Trypsin inhibitors and lectins are found in raw SBM and inhibit protease enzymes in the digestive tract, thereby reducing the activity of trypsin and chymotrypsin and reducing protein digestion in non-ruminant animals as well as in young ruminants (Liener, 1994). Protease inhibitors cause an increase in both pancreatic cell number (hyperplasia) and size (hypertrophy) (Liener, 1995). Protease inhibitors in raw soybeans negatively affect endogenous proteases (Mogridge et al., 1996). Birds fed diets containing raw SBM had low performance and hypertrophy of the pancreas (Mogridge *et al*., 1996; Perez-Maldonado *et al*., 2003; Newkirk, 2010). Trypsin inhibitors are rich in sulphur-containing amino acids and thus can create stress and cause a deficiency of methionine, which is the first limiting amino acid in soybeans. In a trial on different varieties of commercial soybeans and raw full-fat SBM, Clarke and Wiseman (2005) demonstrated that amino acid digestibility varied widely, but it did not correlate with TIA levels. Barth *et al.* (1993) also reported that a diet with raw soybean meal caused significant endogenous protein loss.

Proteases are protein-digesting microbial enzymes that break down both stored proteins and proteinaceous anti-nutrients in vegetable proteins (Barletta, 2011). Regardless of dietary protein or energy concentrations, protease supplementation has been shown to improve the feed efficiency and digestibility of CP and fat (Freitas *et al*., 2011) and thereby reduce the nitrogen excretion (Oxenboll *et al*., 2011). Protease supplementation in diets improves the digestibility of protein and reduces the impact of anti-nutritional proteins on non-ruminant animals (Pettersson and Pontoppidan, 2013). Yadav and Sah (2005) added that the inclusion of dietary protease improves the digestibility of crude proteins in the diet and improves body weight gain in broilers. Furthermore, Rada *et al*. (2014) reported that supplementation of protease enzymes improved the carcass yield of broilers. Protease also has the capacity to improve amino acid digestibility (Liu *et al*., 2013) and thereby improve the feed: body gain ratio of birds (Freitas *et al*., 2011).

The digestibility of CP and starch increases in broilers fed diets supplemented with protease at most concentrations (Angel *et al*., 2011; Selle *et al*., 2013). However, each protease type has its own specificity and mode of action, and hence the yield patterns of amino acids vary widely among the feedstuffs. Cowieson and Adeola (2005) found that a combination of phytase and protease improved nutrient retention and performance by 14%. Murugesan *et al*. (2014) also noted an increase in nutrient utilisation when broiler diets were supplemented with protease and phytase (cocktail). However, Cowieson and Ravindran (2008) reported that there was no interaction between diets and enzyme products containing xylanase, amylase and protease in terms of ileal digestibility of nitrogen and amino acids.

Jiang *et al*. (2008) reported that endogenous pancreatic proteases, e.g., trypsin and lipase, were not affected by supplementing exogenous amylase; rather, their intestinal activity was improved. As shown in Figure 2.1, the mechanism of substrate hydrolysis, especially protein by protease, is initiated by break-up of the large molecules into peptides and then into amino acids. Amino acids are metabolised into different end-products, e.g., ammonia, amines and organic acids. Although the majority of amino acids are synthesised into animal proteins, some of the end-products, including phenolic compounds, ammonia, amines, sulphides, organic acids and other gases, are excreted by the animal.

Phytate or phytic acid (Myo-inositol hexaphosphate) is a compound that contains bound phosphorus and other minerals, and it is found in most plants, including corn and soybeans (Raboy, 2009). Pallauf and Rimbach (1997) reported that most of the P in plants is contained

in phytic acid or its salts, which are known as phytates. Phytate is also associated with a number of anti-nutritional effects; for example, phytate chelates with minerals (cations) and proteinaceous anti-nutrients and thereby reduces protein availability (Liu et al., 1998). Phytate and non-starch polysaccharides (NSP) in plant-based diets are the two major anti-nutrients that are poorly digested by poultry and swine. They also interfere with the digestion of other nutrients (Ravindran et al., 1999).

Because phytic acid and NSP are not heat-labile, unlike some protease inhibitors and lectins, supplementing diets with exogenous phytase is very necessary (Dourado et al., 2011). However, the efficacy of supplementation in chicken feeds depends mainly on the rate of inclusion rate as well as the age and genotypes of the birds (Singh, 2008). Feed enzymes are used to assist the breaking down of ANF in many feed ingredients for poultry and pigs (Barletta, 2011), and generally, the pig and poultry industries are benefitting from the availability and use of phytase. Currently, around two-thirds of pig and poultry feeds contain supplemental phytase, and this is yielding economic benefits (Barletta, 2011). Selle et al. (2000) suggested, however, that vegetable proteins and cereal sources are different in their response to phytase supplementation in terms of amino acid digestibility.

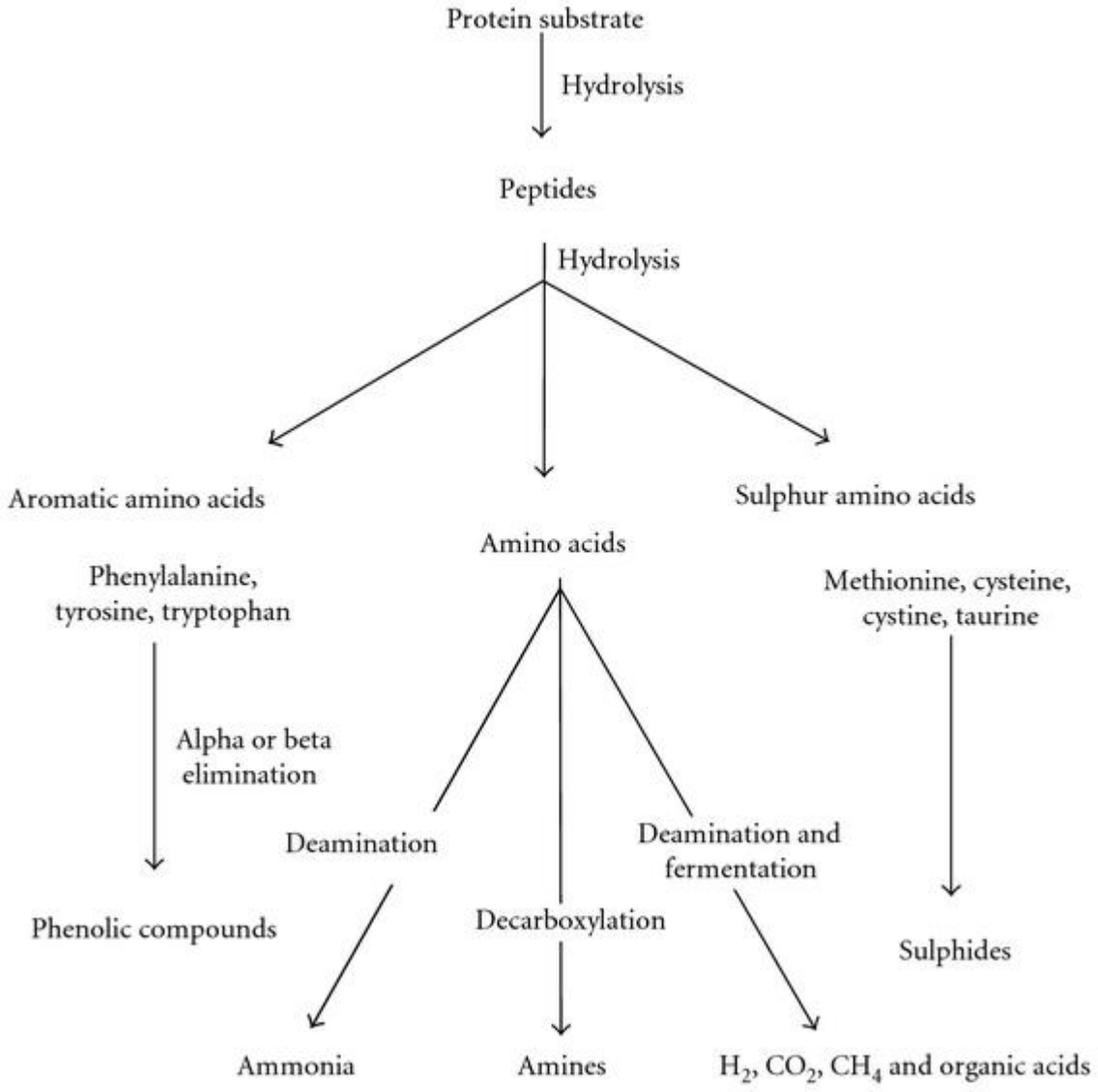


Figure 2. 1 Mechanism of protease hydrolysing its substrates. Adapted from: http://www.open-access-biology.com/probiotics/hughes/f1.gif

2.10 Dietary phytate (phytic acid) and the use of supplementary microbial phytase in soybean diets

Supplementing microbial phytase in corn–soybean based diets for poultry has proven effective in improving N, amino acid and energy utilisation (Rezaei *et al.,* 2007). Phytase supplementation in corn–soybean based broiler diets has been shown to improve the digestibility and intake of nutrients (Santos *et al*., 2008). Chen *et al*. (2013a) found that thermo-stable phytase supplementation can improve the growth, performance and nutrient utilisation of broilers.

Srinath *et al*. (2012) was able to show a higher release of inorganic phosphorus in an *in vitro* trial through the use of phytase. Good performance of broiler chickens fed phytase-supplemented diets might be due to an increased availability of phosphorus and other nutrients. This may help to reduce the excretion of proteins as waste and directly reduce the nitrate accumulation in the bedding materials, thereby minimizing ammonia production in poultry houses. Consequently, this contributes to the good health of birds and also reduces environmental pollution (Cabahug *et al*., 1999; Oxenboll *et al*., 2011).

However, Adeola and Sands (2003) reviewed the literature and reported that information regarding phytase-induced effects on protein and amino acid utilisation in pig and poultry feeds is conflicting. Selle and Ravindran (2007) added that there was no consensus among scholars as to whether phytase in poultry feed enhanced protein and energy utilisation or not, and Peter and Baker (2001) concluded that phytase supplementation, at any level, had no effect on any measure of chick performance, nor on any measure of protein utilisation. Kashani *et al.* (2014) added that phytase supplementation had no beneficial effect on the productive performances of laying hens and egg quality traits. These studies disagree regarding the effectiveness of microbial phytase on protein and amino acid utilisation and factors that regulate these effects, for example, types of feed ingredients, the dose of microbial phytase used in the diets or the type of microbial phytase used as a supplement.

The responses of birds fed diets supplemented with selected microbial enzymes are shown in Table 2.3. Birds fed diets supplemented with a cocktail (protease and phytase) show increased activities of ileal maltase, sucrase, and L-alanine aminopeptidase (Murugesan *et al*., 2014). Various researchers reported a positive response in meat part yield with the supplementation of different enzymes and enzyme combinations (Bamgbose *et al*., 2005; Omojola and Adesehinwa, 2007; Hajati, 2010).

2.11 Carbohydrase enzyme inhibitors in soybeans

Soybeans contain a high concentration of carbohydrates, including NSP and oligosaccharides (Zdunczyk *et al.*, 2011). Carbohydrates make up approximately 35% of soybean seeds and 40% of SBM. Half of percentage is non-structural, in the form of oligosaccharides, and a small amount is structural polysaccharides, known as pectic polysaccharides (Karr-Lilienthal *et al*., 2005). The fibre component of the seed also consists primarily of NSP, which have anti-nutritive effects (Choct *et al.*, 2010).

Raffinose and stachyose are two types of short-chain oligosaccharides that make up approximately 5-7% of the soybeans. They are not digested, and they are the cause of digestive disturbances and depressed growth (Choct *et al.*, 2010). The concentrations of galactosides vary from 0.5 to over 2.5% in SBM (Zdunczyk *et al*., 2011), and at high level, galactosides may produce anti-nutritional effects in poultry (Zdunczyk *et al.*, 2011).

Pectins are glycoproteins, which promote the binding of carbohydrate-containing molecules to the epithelial cells of the intestinal mucosa, which negatively affects the performance of birds. As shown in Table 2.4, supplementation of a wheat-based diet with a cocktail of enzymes (protease + carbohydrases) increased the AME value by 234.3 kcal/kg and N retention by 4.5%.

For corn/SBM based diets, supplementation with protease + phytase increased the AME value by 103.0 kcal/kg and N retention by 0.15%. Birds fed diets (corn/SBM based) supplemented with phytase + multi-carbohydrase improved their BWG by 45.1 g, AME value by 74.0 kcal/kg and N retention by 1.8%. From these research reports, one can conclude that microbial enzymes are most effective when they are combined; however, such results need to be supported by economic analyses.

Table 2.3 Some selected anti-nutrients in raw full-fat and commercial SBM.

Raw full-fat SBM					Commercial SBM				References
TI (mg/g)	UA (ΔpH)	KOH (%)	Lectins, (mg/gm)	Phytate (mg/gm)	TI (mg/g)	UA (Δ pH)	KOH (%)	Phytate (mg/gm)	
40.486	>0.20	90	3.7	-	-	0.05-0.20	85	-	(Gu et al. 2010)
30-102.5	-	-	-	5-25	-	-	-	14.5	(Sharma *et al*., 2013)
45-60	-	-	20-200 (ppm)	6	1.77	.02	-	-	(Van Eys *et al*.,2004)
26.48	-	-	-	-	1.8-2.9	-	-	-	(Serrano *et al*., 2012)
48.33 (TIU/mg)	-	-	-	11.2	-	0.2	-	-	(Crowell, 2012)
-	-	-	-	-	2.5-1.9(TIU/mg)	~0.01	69.7-74.3	-	(Ravindran *et al*., 2014b)
23.9	-	-	7.3	-	-		80-85	-	(Crowell, 2012)
50,800 (TIU/g)	1.99	98.0	-	-	3,000,TIU/g	0.08	82.0	-	(Ruiz *et al*., 2004)
41.5-85	-	-	-	2.3-5.6	1.50-3.45	-	77-87	-	(Sharma *et al*., 2014)

TI=trypsin inhibitors; UA=urease activity; KOH= protein solubility; TIU= trypsin inhibitor units.

Table 2.4 Response of birds fed diets supplemented with enzymes combinations (cocktail).

	Combined with	Base of diets	Type of chickens	BWG	AME (kcal/kg)	N (%)	References
Protease	Carbohydrases	Wheat	Broilers	-	234.2	4.8	Selle *et al.* (2010)
	Phytase	Corn/SBM	Broilers	14%	100		Cowieson and Adeola (2005)
	Phytase	Corn/SBM	Broilers	-	103.0	0.15	Murugesan *et al.* (2014)
	Xylanase + amylase	Corn/SBM	Broilers	-	64.53	0.6	Romero and Plumstead (2013)
Phytase	Multi-carbohydrase	Corn/SBM	Broilers	41.5 g	74	1.8	Woyengo *et al.* (2010)
	xylanase	Wheat	Broilers	15.4 %	0.67	2.1	Selle *et al.* (2003)

AME = Apparent Metabolizable energy; BWG= body weight gain; N= retained nitrogen.

2.12 Development, mode of action and use of exogenous feed enzymes

Enzymes are organic catalysts that can initiate or accelerate biochemical reactions (Santos and Ferket, 2006) and are categorized according to the substrates that they target, such as fibre, proteins, starch and phytate (Adams, 2004). Enzymes are proteins that have catalytic functions indispensable to the maintenance and activity of life. Enzymes become functional in a mild environment, where they are able to maximize the utilisation of nutrients (Cao et al., 2007).

Enzymes are naturally endolytic and are able to remove the diffusing constraints that can interfere with nutrient absorption (Campbell and Bedford, 1992). Enzymes can increase the partitioning of nutrients into edible substances by decreasing the size of the gastrointestinal

tract (McNab and Boorman, 2002). Bedford and Partridge (2010) also suggested that the development of more thermo-stable enzymes would allow their use in diets where they currently cannot be applied. Similarly, Campbell and Bedford (1992) predicted that enzymes with the desired activity and stability for feed applications will continue to be developed.
Enzymes that are naturally produced by non-ruminant farm animals are not adequate to break down fibre and other anti-nutrients, and that is why there is a need for exogenous enzymes in pig and poultry feed (Ani *et al.*, 2012). Supplementation of poultry feeds with enzymes has many advantages, including improvement of nutrient availability, reduction in feed costs and reduction in waste production (Santos and Ferket, 2006).

As cereal costs continue to rise, adoption of biotechnology to modify or design appropriate enzymes would be an option to improve the digestibility of nutrients in feed (Cao *et al*., 2007). Enzyme suppliers are also actively promoting the additive benefits of combining selected enzymes (Adams, 2004). Campbell and Bedford (1992) suggested that young chicks have a good response to an enzyme-induced reduction in viscosity. However, the efficacy of enzymes will vary, depending on the feedstuffs themselves (Adeola and Cowieson, 2011), and enzyme activity and stability can be affected by different parameters (Francesca and Perez-Vendrell, 1997). Scholars have argued that enzyme supplementation of poultry diets has nutritional, economic and environmental benefits (Dosković *et al.*, 2013). *In vitro* digestibility tests have shown that combining selected enzymes, particularly protease and phytase, is more effective than the use of single enzyme products (Malathi and Devegowda, 2001; Erdaw *et al.*, 2014 & 2016c). Future research and development will continue to be supported at an ever-increasing level by industry to improve the field (Brufau *et al*., 2006).

2.13 Options for improving the nutritional value of soybeans

The nutritional value of SBM can be affected by many factors, such as its origin, the cultivar, and the way the meal is processed (Nahashon *et al*., 2013). Therefore, stakeholders need to look at a range of options for improving both feed quality and animal performance, which could reduce the costs of broiler production and reduce pollution (Bharathidhasan *et al*., 2009).

In addition to developing new breeds of soybean crops with low anti-nutrient contents, treating the raw seeds with heat or supplementation with new-generation microbial enzymes are common options that are currently used to improve the nutritional value of SBM-containing feeds for poultry. The livestock industry needs to utilise biotechnological techniques, such as

genetic modification of feedstuffs or feed additives (enzymes), to improve nutrient balances for maximum feed utilisation. In addition to the processed (oil-extraction) meal, full-fat meal (without extracting the oil) can also be prepared. The full-fat SBM can be given as raw meal or a heat-treated preparation

2.14 CONCLUSION

Because the supply of processed SBM is fluctuating and the product is becoming increasingly expensive, there is a growing interest by farmers in minor production areas to use raw full-fat SBM for poultry. Supplementation of raw full-fat SBM with exogenous enzymes such as phytase and protease is an optional strategy to improve the nutritional value of such meals and to reduce production costs. Chickens fed diets containing raw SBM that are supplemented with microbial protease showed improved performance. The reason for this improvement might be due to the effects of proteases on stored proteins as well as proteinaceous anti-nutrients in raw SBM. The new-generation exogenous phytase products are also more effective in breaking down phytic acid. It is likely that there are differences in the quality of different sources of raw soybean seeds, as has been shown for commercial SBM. This is an area that needs to be further investigated.

CHAPTER 3: PHYSICOCHEMICAL PROPERTIES AND *IN VITRO* DIGESTIBILITY OF FULL FAT SOYBEAN MEAL

ABSTRACT

This study was conducted in order to investigate the enzymatic *in vitro* digestibility of nutrients and phytate in full-fat soybean. Raw full-fat (FF) soybean, heated at a range of temperatures, was compared to commercial SBM. The physiochemical properties, including nutrient composition were also assessed. Contents of phytate and its *in vitro* degradability in soybean samples were also measured in various enzyme combinations. Four seed samples of raw FF soybean were individually subjected to steam- or dry-heating for 15, 30, 45 or 60 minutes, all at 95 ^{0}C. After drying and grinding, subsamples of each of these meals alongside unheated full-fat and the commercial SBM were analysed for their nutrient composition and concentration of antinutritional factors (ANF). Other subsamples were also subjected to enzymatic *in vitro* nutrient digestibility testing. The results revealed that crude protein (CP) and dry matter (DM) contents ranged from 418.6 to 462.9 g/kg and 919.1 to 968.7 g/kg, respectively. On average the fat content was higher (157.4 g/kg) in FF samples than in the commercial SBM (19.2 g/kg), hence, on average; the calculated AME content of the FF samples was 13.1 MJ/kg compared to 9.0 MJ/kg for the commercial SBM. Starch (37.0 g/kg) and total sugars (107.6 g/kg) were highest in commercial SBM. Protein solubility (794.4 g/kg) and urease activity (0.09 ΔpH) were lower in commercial SBM than the average values of 893.4 g/kg and 2.1 ΔpH for FF samples, respectively. The concentration of trypsin inhibitors was highest in samples of FF heated for 15 minutes (13098.0 TIU/g) and lowest in commercial SBM (5743.0 TIU/g). On average, phosphorus (7.8 g/kg) and potassium (23.7 g/kg) were higher in the FF samples than in the SBM, but the reverse was the case for trace minerals. Except for arginine (33.5 mg/gm), the concentrations of other amino acid (AA) were higher in commercial SBM than average value in FF soybean. The steam-heated (15 min) FF soybean sample, incubated in a cocktail of enzymes (phytase + protease) had the highest (0.90) *in vitro* DM digestibility (DMD) compared to the lowest (0.57) DMD value when it was incubated only with microbial protease. The *in vitro* digestibility of crude protein (DCP) when samples were incubated for 30 min in the enzyme cocktail ranged from 0.60 for the unheated to 0.70 for the dry-heated FF soybean sample. The DCP of samples incubated in microbial protease was highest (0.67) in FF sample of steam-heated for 45 min, while the lowest value (0.56) was in unheated FF sample. Before incubating with enzymes, the original phytate contents of all samples were assessed with *in vitro* digestibility methods and the commercial SBM had the highest ($p<0.01$) value of all the samples. After incubating the remaining parts of these samples with different enzymes, the

residues were freeze-dried and were used to test *in vitro* digestibility of phytate. The degradability of phytate in the samples incubated in the enzyme cocktail (protease + phytase) was higher ($p<0.01$) than other media. Particle size in mash form of diets containing graded level of raw full-fat soybean meal (RSBM) was mostly between 1 and 2 mm. The pellet durability index (PDI) of diets containing graded levels of RSBM influenced ($p<0.01$) by pelleting conditions or inclusion rate of RSBM. Similar diets were used in feeding trials to assess their nutritive values and response to microbial enzymes when fed to broiler chickens. It can be concluded that except contents of ANF, the nutritional value of FF soybean is almost comparable with commercial SBM and enzymatic *in vitro* nutrient digestibility using a cocktail (protease + phytase) enzymes might be more efficient than the individual ones.

3.1 INTRODUCTION

Feed represents the primary variable cost of poultry production industry. Energy source ingredients constitute the largest component of poultry diets, followed by plant protein and animal protein sources, respectively. Due to scarcity, zoonotic diseases and the cost of animal protein sources, attention has been shifting to vegetable protein sources, particularly soybean meal (SBM) to formulate diets for poultry.

Processed SBM is the product that remains after extraction of the oil by either the solvent or expeller method. Soybean meal represents around 69% of the world's total output of protein feedstuffs (Oil-World, 2010; Crowell, 2012). And around 85% of the world's soybeans are processed into oil and soybean meal (Soya-tech, 2003). Soybean meal is the best vegetable protein source for poultry (Cromwell, 1999; Banaszkiewicz, 2011) due to its availability and quality. The inclusion rate of SBM in poultry diets is ranging from 25 % for chicks to 30-40% for older broilers and laying hens (McDonald et al., 2002; Willis, 2003). Based on its crude protein content, SBM can be classified as either high protein (49-50%) or low protein (44-46%).

However, either under- or over-heating during processing frequently reduces the nutritional value of SBM for birds (Perilla *et al.*, 1997). The consequence of the long process involved in extracting the oil from soybean and the costs of transporting the raw seed to the plant and then transporting the by-products, such as SBM, to the end-users is making SBM increasingly expensive (Newkirk, 2010; Shi *et al.*, 2012). Mojtaba (2008) questioned whether the benefit would outweigh the processing cost. Additionally, due to the events in the major producing regions of the world, in North and South America, conventional SBM supply is fluctuating (Shi

et al., 2012), and is also usually extracted using chemicals such as hexane, which are a non-biorenewable, and can pose health risks (O'Quinn et al., 1997).

On the other hand, various scholars (Willis, 2003; Meng *et al*., 2006; CAES, 2013) have indicated that there is an interest in using full-fat soybeans in poultry and livestock rations. Full-fat SBM can fully replace the commercial SBM as well as vegetable oil for birds (Popescu and Criste, 2003). Meals of full-fat soybean can be prepared either after heat treatment or as a raw full-fat meal. Raw full-fat SBM, however, contains many anti-nutritional factors (ANF) such as protease inhibitors, lectins, phytates and non-starch polysaccharides (Wiryawan and Dingle, 1999; Jezierny *et al.*, 2010; Newkirk, 2010; Mosenthin and Jezierny, 2010). Various scholars (Liu, 1997; ASA, 2004; Newkirk, 2010) have also suggested that feeding birds with raw SBM negatively affects their feed efficiency, body weight gain and their pancreatic function. This is mainly due to the presence of trypsin inhibitors and lectins in the raw SBM.

In addition to heating, the nutritive value of SBM can be improved by supplementing with microbial feed enzymes such as phytase and protease, which have been developed and are used primarily to improve digestibility of nutrients or to reduce the ANF (Farrell *et al.*, 1993; Yadav and Sah, 2005; Ao, 2011). It is important to fully identify the nutrient profiles and ANF in such ingredients prior to biotechnological intervention. The physical properties of ingredients have also a direct impact on the quality of diets, for example on both pellet durability (Thomas *et al.*, 1998; Briggs et al., 1999) and particle distribution in the diets. Moreover, Svihus and Zimonja (2011) reported that the conditioning heat used before the process of pelleting could improve the nutritional value of feed through inactivation of the proteinaceous anti-nutrients. These measures of physical quality and nutrient compositions can be combined with *in vitro* digestibility, as predictor of the nutritive values. The objectives of the current study were to assess the physical and chemical properties of raw full-fat (FF) soybean, heated at a range of temperatures alongside unheated once, in order to understand what interventions could be applied when it is fed to broiler chickens.

3.2 MATERIALS AND METHODS

3.2.1 Heat treatment

The test raw soybean seeds were harvested in July 2013 and purchased from a local supplier in northern New South Wales, Australia. The seeds were cleaned and subjected to either dry- or steam-heating.

3.2.1.1 Dry-heating

Four empty stainless-steel trays (38 x 43 cm) were pre-heated to 95°C in an oven. About 0.5 kg of raw whole soybean seed was poured into each tray, spread evenly and then placed in back into oven, set at 95°C. One tray was randomly selected and removed from the oven after 15, 30, 45 or 60 min. All samples were then allowed to cool and ground to pass through a 1.0 mm sieve.

3.2.1.2 Steam (moist)-heating

A sheet of wire mesh (32 x 26 cm) was suspended standing on its legs in a closed steam-heating machine and heated to a temperature of 95°C. About 0.5 kg of whole soybean seeds was evenly spread over the mesh, and allowed to heat using steam-heating at a temperature of 95°C for 15 min before removal. Three other samples were similarly treated for 30, 45 or 60 min. The samples were air-dried for 5 days and ground to pass through a 1.0 mm sieve.

3.2.2 Chemical analysis

3.2.2.1 Dry matter and ash determination

For dry matter (DM), sub-samples of ground seeds (induplicate) were weighed into crucibles of known weights and placed in a forced air convection oven (Qualtex Universal Series 2000, Watson Victor Ltd, Perth, Australia) at 105 °C for 24 h. The weight of the sample was then recorded after being cooled for 50 minutes in desiccators at room temperature.

Calculation: DM% = (weight of a sample after oven-drying/weight of sample before oven-drying) x 100.

After recording the DM weight of the samples (in duplicate), the crucible with the oven-dried samples were placed in a Carbolite CWF 1200 chamber furnace (Carbolite, Sheffield, UK) and ashed at an initial temperature of 350 °C for 1 h followed by ashing at higher temperature of 600 °C for 2 h to determine the ash content of the sample. The ash (residue) was weighed, recorded and the ash content was calculated as follows:

Ash% = (weight of ashed sample/weight of its oven-dried content (DM)) x 100.

3.2.2.2 Mineral analysis

Nitric acid, hydrochloric acid and perchloric acid were added to analyses for mineral contents in samples. Diluted samples and the standard solution were separately put into a set of fresh tubes for spectroscopy (ICP) (Vista MPX, Melbourne, Australia). Copper, Fe, Mn, Zn, Ca, P and Mg were measured at 327, 238, 257, 213, 616, 231, 279 nm wavelength, respectively. Two millilitres of a mixture of $HClO_4$ (70%) perchloric acid and H_2O_2 (30 %) were added to each

tube. Each tube was loosely covered with a lid and set overnight. Then one ml of H_2O_2 was added and tubes were tightly sealed and placed in an oven set at 80^oC for 30 minutes. The bottles were allowed to cool slightly and a further 1 mL H_2O_2 was added before they were capped tightly and digested for 1 hour at 80^oC. The solution was made to a volume of 25 mL by adding distilled water and filtered through Whatman No.1 filter paper for ICP analysis (Anderson and Henderson, 1986).

3.2.2.3 Bomb calorimeters

The gross energy (GE) content of the samples was determined using an IKA bomb calorimeter (IKA-WERKE, C7000, and Staufen, Germany).

Values of metabolizable energy (ME) in MJ/kg of the sample were predicted from the equation described by McDonald *et al.* (1926) for poultry.

Calculation: ME (MJ/kg) = (0.01551 CP) + (0.03431 AEE) + (0.01669 ST) + (0.01301 S), where: CP, AEE, ST, and S indicate the crude protein, acid ether extract, starch, and total sugar contents, respectively. These components were measured as described below.

3.2.2.4 Determining the values of crude protein (CP), available lysine, and complete amino acid profiles

The total nitrogen content of differentially heated full-fat soybean and unheated and commercial soybean meal samples were determined by the combustion analysis technique (LECO) Official Method 990.03 (Kjeldahl), Official Method 984.13 (A-D), (AOAC, 2006a). This analytical method quantitatively determines the total amount of nitrogen and carbon in plant materials using sample combustion coupled with thermal conductivity/IR detection (LECO FP-528 and TruSpec CN Analyzers). The method is based on the oxidation of the sample by "flash combustion", which converts all organic and inorganic substances into combustible gases (N_2, NO_x, CO_2, and H_2O). The method has a detection limit of approximately 0.02% for nitrogen. The CP content of the sample is therefore equal to the multiple of total N X 5.71.

The complete amino acid profile for the samples was also measured using the Official Method 982.30, whereas available lysine was measured using the Official Method 975.44, (AOAC, 2006b).

3.2.2.5 Evaluation of urease activity

The urease activity of the samples was determined following the method described by Official Methods Ba, 9-58 (AOCS, 2006a). About 0.2 g of the finely ground sample was weighed into a test tube containing 10.0 mL of urea buffer solution. The tubes were then stoppered, mixed and placed in a water bath at 30^0C. Each tube was then agitated at 5 min intervals and removed from the water bath after 30 min. The supernatant was transferred into 5.0 mL beakers, maintaining a 5-min interval between the test and blank tubes. The pH was then determined exactly 5 min after removing sample and the blank from the bath.

Calculations: The difference between the pH of a test sample and the pH of blank is an indication and an index of urease activity.

3.2.2.6 Determination of protein solubility (KOH)

The protein solubility of the aforementioned samples of soybean meals was determined following the method described by Araba and Dale (1990). After grinding, 1.5 g of each soybean meal sample was mixed with 75.0 mL solution of 0.2% KOH. The mixture was stirred for 20 minutes on a magnetic stir plate and then centrifuged at 2700 rpm for 15 min. The supernatant was decanted and filtered through glass wool, and 15.0 mL duplicates from a single filtrate were transferred to Kjeldahl tubes, and then 25/2 mL of concentrated H_2SO_4, Kjeltab, and 2.0 mL of 30% H_2O_2 were added to each tube. Total nitrogen was determined by the Kjeldahl method, and the protein content was calculated. Protein solubility was expressed as percentage of the total protein soluble in a 0.2% solution of potassium hydroxide.

3.2.2.7 Determining the trypsin inhibitors activities (TIA) of samples

The activity of trypsin inhibitors of the samples was measured using the procedure of Official Method 22-40 (AOCS, 2006c). Around 0.5 g of the sample was extracted with 25 mL of 0.01 M NaOH while stirring for 3 h at room temperature. Two mL of trypsin solution (4 mg trypsin in a 200 mL of 0.001 M HCl) was added to each tube and the mixture was placed in a water bath at 37°C. Five mL of the BAPNA substrate solution was pre-warmed to 37°C and added to the tube and then the reaction was stopped after 10 min with one mL of 30% acetic acid solution. The mixture was then filtered through Whatman No. 2 paper, and the absorbance was measured at 410 nm.

Trypsin inhibitors activity (TIA) was calculated as:

Trypsin inhibitor units (TIU) per mL diluted sample suspension as assayed = TIU/ml = 100 x (absorbance of B) - (absorbance of S)/Y
where - B = blank ("O mL assay").

S = sample solution.
Y = number of mL of diluted sample suspension used in assay.
2. Calculate TIU/mL for each of the assays. Use the simple mean of the T1U/ mL obtained.
3. TIU/g = TIU x dilution factor x 50

3.2.2.8 Crude fibre determination

The crude fibre content was analysed using the AOAC (1979) methods. After extracting 2.0 g (W1) of ground sample using ether, it was transferred into a 600 mL reflux beaker. Then, 0.5 g bumping granules and 200 mL of near-boiling 1.25% H_2SO_4 were added one after the other. The beaker was then placed on a digestion apparatus and was boiled for exactly 30 min while rotating it at 5 min intervals.

The filtration was adjusted with a vacuum to ca 25 mm Hg (735 mm pressure), and the boiled liquid was decanted through a funnel for filtration and then the residue was washed with near-boiling 1.25% NaOH. The crucible with the residue was then dried for 2 h at 130 ± 2°C, cooled and weighed (W2). The sample was then ashed for 2 h at 550°C, cooled and weighed (W3).

Crude fibre% = [(W2 – W3) – (B2 – B3)]/W1 x 100; where B2 and B3 are average weights of all blanks after oven drying and ashing, respectively.

3.2.2.9 Determination of ether extract

Ether extraction (EE) contents of the samples were measured according to the AOAC (2009) methods. A sample of about 2 g was dried and then Ca was extracted with hydrous ether. A thimble with high porosity was used to permit a rapid passage of the ether. The extraction period was around 4 h at a condensation rate of 5–6 drops. The extract was dried for 30 min at 100 °C, and then it was cooled, and weighed.

3.2.2.10 Total sugars

The total sugar content of the samples was determined using the procedures described by Munson and Walker (1929). After around 25 mL of $CuSO_4$ and alka tartrate solution were transferred into a 400 mL beaker of alkaline-resistant glass; a 50 mL of reducing sugar solution was added and the total volume was made up 100.0 mL with water. The solution was then heated on an asbestos pad on a gauze burner. After 4 min, boiling point was reached and the solution was boiled for a further 2 min. The hot solution was filtered using suction. The precipitate of Cu_2O was thoroughly washed with H_20 at ca 60° and the Cu_2O was directly weighed. The weight of the content of reduced sugar was calculated (considered) as equivalent to the weight of Cu_2O.

3.2.2.11 Starch content

The starch content was determined following the procedures described by AOAC (1984). Around 2.5 g of finely ground sample was added to 50 mL lipped centrifuge tubes, washed with ether to remove the fats and then with 10.0 mL of 65% by weight of alcohol. After centrifuging, the residue was stirred with 10 mL H_2O and this was all poured into a 250-mL Erlenmeyer flask. The content was completely transferred by thoroughly washing with 60 mL of $CalCl_2$ solution containing 2 mL 0.8% HOAC, while stirring with a glass rod to remove the fats. After transferring the rod to the flask, the content was heated quickly while still stirring and then briskly boiled for 17 min. The residue was then quickly cooled with running water and poured into a 100-mL volumetric flask that had also been rinsed thoroughly with $CaCl_2$ solution. One drop of alcohol was added and mixed with the samples, and 10 mL solution was poured onto fluted filter paper (Whatman No. 42). The filtrate was run and 45 mL was collected. The liquid was polarised in a 10-cm tube, taking 2 sets of 10 readings each.

Calculations: Percentage of starch = 100 x R x 100/1 x 203 x W= 49 x R/W; where R is observed angular rotation and W is weight of a sample; and 203 is the specific rotation for all starches.

3.2.2.12 Evaluation of phytate concentration in samples

The concentration of phytate in the samples was determined following the procedure described by Haug and Lantzsch (1983). After extracting 60 mg sample with 10.0 mL of 0.2 M HCl solution, around 0.5 mL was pipetted into a new test tube (fitted with a ground glass stopper) containing 1.0 mL of a solution (0.2 g ammonium iron sulphate.$12H_20$ in 100.0 mL 0.2 M HCl topped to 1.0 L with Milli-Q water). The mixture was heated for 30 min, cooled and centrifuged for 30 min at 3000 x *g*. One mL of supernatant was transferred into a fresh test tube, into which 1.5 mL of another solution (10.0 g of 2, 2'-bipyridine (BP) dissolved in 10.0 mL thioglycolic acid and topped to 1000 mL with ROH) was pipetted.

The standard curve of phytate was constructed with 10, 20, 40, 80, and 160 µL of sodium phytate solution. The standard solution contained 0.15 g Na phytate in 100.0 mL Milli-Q water. The blank had about 100 µL Milli-Q water.

The concentration of phytate in the sample was then calculated using steps "a" and "b" below:

a) Sample phytate (µg) = (STD absorbance/Sample Absorbance) x [STD] x (10.0/0.5)
b) Sample phytate (g/kg) = a (µg)/sample weight (mg).

3.2.3 *In vitro* nutrient digestibility

The laboratory procedures used in the enzymatic *in vitro* digestibility testing were as described by Babinszky *et al*. (1990). Chemicals and reagents: Pepsin from hog porcine (PL082- Chem-Supply, Australia), pancreatic lipase (1494079-Sigma Aldrich-USA), bile salt (B8756-Sigma Aldrich, USA), alpha-amylase from porcine pancreas (Aldrich, USA), and HCL, 37% concentration (from Chem-Supply, Australia). Enzymatic *in vitro* nutrient digestibility was tested by incubating the samples with different microbial enzymes as described follows.

3.2.3.1 *In vitro* digestibility in response to microbial phytase

Five hundred milligrams of ground sample (triplicate) were digested in 50-mL centrifuge tubes with 12.5 mL of 0.1 M HCl solution containing 4 g pepsin/L and incubated at 40 ºC for 1.5 h. The sample was then dissolved into 2.0 mL of 0.65M $NaHCO_3$ (110 mg/L) and thoroughly mixed, and 12.5 mL of potassium phosphate buffer (pH 6.8) containing 40 mg pancreatic lipase, 80 mg of bile salt, 4 mg phytase (DSM Nutritional Products, Australia Pty. Ltd), and 4 mL of amylase/L were added. The mixture was then incubated at 40 ºC for 3 h. After this incubation period, 2.5 mL of Na_2CO_3 (100 g/L) was added to each tube and the mixture was centrifuged at 3500 x g for 15 min. The residue was repeatedly rinsed with Milli-Q water and was then freeze-dried. The concentrations of DM and CP were determined and *in vitro* digestibility was calculated using the equation:

$$\text{Digestibility \%} = \frac{\text{Weight of sample or nutrient-Weight of dried sample or nutrient (mg)}}{\text{Weight of DM or nutrients in a sample (mg)}} \times 100$$

To correct for enzyme and buffer residues, blank samples were incubated without the feed sample. A control group without microbial enzyme inclusion was also incubated.

3.2.3.2 *In vitro* digestibility of CP and DM in response to microbial protease

All steps and reagents used were the same as mentioned above except that 8 mg of microbial protease enzyme (Ronozyme® ProAct) (DSM Nutritional Products, Australia Pty. Ltd) was added in place of microbial phytase.

3.2.3.3 In vitro digestibility of CP and DM in response to cocktail (protease and phytase) of microbial enzymes

All steps and reagents used were the same as mentioned above except that a cocktail (8 mg protease + 4 mg phytase) of microbial enzyme was used in place of individual enzymes.

3.2.4 Enzymatic *in vitro* degradability of phytate

Following the *in vitro* digestion of samples, and as described in Sections 3.2.3.1, 3.2.3.2, 3.2.3.3, the residues were pooled and frozen. Each sample was then freeze-dried. The concentration of phytate in the residue was determined as described in Section 3.2.2.12 above. The *in vitro* phytate degradability was then calculated as:

= Phytate content of sample-phytate content of residue/Phytate content of sample.

3.2.5 Evaluation of particle distribution of diets

The particle distribution of the diets was evaluated using a Bygholm Feed Sieve Particle Size Tester (Vaucluse Livestock Equipment and Animal Production Services, Inglewood, South Australia). Each sample of the diets, in mash form was assessed. The compartment with the largest pore size was filled and then shaken for 5 min. Particles were then distributed to their appropriate compartment, after which the height of the sample in each compartment was recorded (see Plate 3.1). The proportion of material in each compartment was calculated as:

Amount of particles per compartment = [Height of the sample in compartment/sum of height in all compartments] x 100 .

Plate 3.2: Typical distribution of feed particles in the test equipment

3.2.6 Evaluation of pellet durability index of steam- or cold-pelleted diets

Four diets were formulated to contain 0, 10, 20 or 30% (eqivalenet to 0, 30, 60 and 90 g/kg of diet) of raw soybean meal (RSBM) replacing commercial SBM. One half of each diet was steam-pelleted (94-96 ^{o}C) and the other half was cold-pelleted to yield 8 sample groups. The pellet durability was determined using Seedburo's pellet tester (Seedburo Equipment Company West Jackson Blvd, Chicago, USA). A representative sample was taken from each pelleted dietary treatment and the broken pellets were screened (removed) by a hand held sieve, American Society for Testing and Materials (ASTM E 11-61 Specification for Sieves for Testing Purpose). Five replicates of 500 g of screened (intact) pellet samples were weighed and

were tumbled by machine for 10 min. The tumbled sample was re-screened using the same sieve and once again the intact pellets, which did not pass through the sieve, were weighed. Pellet durability index was calculated as:

= (weight (g) of the intact pellets/ 500 g sample) x 100.

3.2.7 Statistical analysis

Descriptive statistics and general linear model (GLM) were used to analyse the data with Minitab software version 17 (Minitab, 2013). The differences were considered to be significant at $p < 0.05$, and the significant differences between mean values were separated using the Duncan's test.

3.3 RESULTS

3.3.1 Proximate nutrient composition of samples

The nutrient composition of SBM samples is shown in Table 3.1.The CP and dry matter (DM) contents ranged from 382.4 to 422.9, and 919.1 to 968.7 g/kg, respectively. The highest DM value (968.7 g/kg) was found in the sample that was dry-heated for 6o min, while the lowest DM value (919.1 g/kg) was from commercial SBM. The highest CP content (422.9 g/kg) was recorded for the commercial SBM, while the lowest value (382.4 g/kg) was for the unheated SBM. Fibre content was lowest in the commercial SBM while the highest value (72.3 g/kg) was found in the full-fat sample that had been dry-heated for 60 min. The lowest ether extract (EE) was found in the commercial SBM.

3.3.2 Nutritional quality of samples

Starch and total sugar contents generally were higher in commercial SBM than values for differently heated samples of full fat SBM (Table 3.2). The average protein solubility (KOH) recorded in the full-fat soybean samples (heated and unheated) was 893.4 g/kg; whereas, the commercial SBM had a value of 794.4 g/kg.

Table 3.1 Effect of steam- or dry-heating soybean seeds at 95^{0}C for different durations (min), and unheated and commercial SBM) on nutrient composition (g/kg) of the samples.

	RSBM[1]	Duration of dry heating (min)				Duration of steam heating (min)				Commercial SBM
		15	30	45	60	15	30	45	60	
Composition:										
Dry matter	923.6	919.1	951.3	962.9	968.7	963.2	958.7	943.9	952.2	914.8
Crude protein	382.4	392.0	392.1	395.4	396.9	393.9	391.8	399.2	394.2	422.9
Crude fibre	62.0	61.5	71.7	71.1	72.3	71.4	62.4	63.2	61.5	37.9
Ether extract	147.3	152.2	154.7	147.6	146.5	154.7	157.4	155.8	156.7	19.2
Total starch	26.1	26.1	28.3	32.6	28.3	34.8	31.5	34.8	31.5	37.0
Total sugars	95.0	78.9	100.5	97.6	94.6	98.2	98.7	98.8	98.9	107.6

[1]RSBM= raw soybean meal. Number observations = 3.

Table 3.2 Effect of steam- or dry-heating soybean seeds at 95^{0}C for different durations (min), and unheated and commercial SBM on nutritional qualities of the samples.

	RSBM[1]	Duration of dry heating (min)				Duration of steam heating (min)				Commercial SBM
		15	30	45	60	15	30	45	60	
Protein solubility (KOH)	898.6	945.5	859.7	871.0	828.0	939.0	915.2	908.8	875.2	794.4
Available lysine	26.4	26.9	26.4	26.9	26.3	26.8	26.2	26.7	26.1	28.4
Trypsin inhibitor (TIU/g)	13498	12833	12655	12578	12516	13098	12105	11162	10326	5743
Urease activity (ΔpH)	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	0.09
Gross energy (MJ/kg)	21.4	21.5	21.3	21.5	21.0	21.5	21.5	21.3	21.4	17.5
Calculated AME (MJ/kg)	12.6	12.7	13.2	13.0	12.9	13.2	13.6	13.5	13.3	9.0

[1]RSBM= raw soybean meal. Number of observations = 3. .

The available lysine of the commercial SBM was 28.4 g/kg for commercial SBM compared to 26.5 g/kg for the full-fat samples. The highest concentration of trypsin inhibitors was 13098.0 TIU/g in the steam-heated (for 15 min) full-fat soybean sample, while the lowest value (5743.0 TIU/g) was in the commercial SBM. Similarly, urease activity (UA) was lower in the commercial SBM sample than in the full-fat samples, regardless of heat treatments. The calculated AME content of the test sample ranged from 13.6 in the full-fat that heated for 30 min to 9.0 MJ/kg in the commercial SBM sample.

3.3.3 Mineral contents

As indicated in Table 3.3**,** the highest ash and calcium contents were recorded for commercial SBM, while the average lowest value were for samples of differentially heated full-fat SBM. On the average, the highest phosphorus (7.5 g/kg) and potassium (23.7 g/kg) contents were recorded in the heated full-fat SBM while the lowest values (5.7 and 21.1 g/kg, respectively) were in the commercial SBM. Magnesium content was lower in the full-fat SBM than in the commercial SBM.

Table 3.3 Effect of steam- or dry-heating at 95^{0}C, over different durations on mineral composition of samples.

	RSBM[1]	Dry-heating (min)				Steam-heating (min)				Commercial SBM
		15	30	45	60	15	30	45	60	
Ash (g/kg)	76.9	48.5	75.5	78.5	55.9	55.9	49.7	75.5	75.5	85.1
Macro minerals (g/kg)										
Calcium	1.5	1.2	1.3	1.7	1.5	1.3	1.5	1.4	1.0	2.9
Phosphorus	7.9	6.5	7.4	7.5	7.8	7.5	7.7	7.8	7.7	5.7
Potassium	23.8	20.1	22.6	26.7	23.5	26.2	23.2	23.7	23.6	21.1
Magnesium	2.4	2.1	2.3	2.7	2.4	2.7	2.4	2.4	2.4	2.8
Trace minerals (mg/kg)										
Manganese	28.3	24.6	27.4	33.1	29.2	31.9	27.4	29.0	28.5	39.5
Copper	6.9	5.6	6.4	7.9	7.0	7.3	6.4	6.4	7.7	10.7
Iron	84.7	69.1	74.9	87.8	84.8	95.0	70.9	78.7	78.3	89.3
Zinc	49.0	41.2	46.3	55.0	47.1	53.3	46.4	49.3	48.8	36.7

[1]RSBM= raw soybean meal. Number observations = 3.

The commercial SBM had the higher manganese, copper and iron contents than the heated full-fat SBM samples. In contrast, zinc content was higher in the SBM than in the commercial SBM.

In general, the commercial SBM had higher amino acid contents than the full-fat SBM samples (Table 3.4). The commercial SBM had methionine and lysine contents of 6.2 mg/g and 29.0 mg/g, while the heated full-fat samples averaged 5.7 mg/g and 26.9 mg/g, respectively. The

average values of threonine, valine, leucine, isoleucine and Phenylalanine were 16.2, 18.9, 31.5, 18.0 and 21.1 mg/g, respectively. These were lower than the value obtained in commercial SBM samples. However, average arginine value was higher (33.5 mg/g) in the full-fat samples than in the commercial SBM.

[1]RSBM= raw soybean meal. Number of observation was 3.

Table 3.4 Effect of steam- or dry-heating at 95^{0}C, over different durations on amino acid profiles (mg/g) of full-fat soybean meals, and commercial SBM.

	RSBM[1]	Dry-heating (min)				Steam-heating (min)				commercial
		15	30	45	60	15	30	45	60	SBM
Aspartic Acid	45.8	46.7	45.9	46.5	45.3	46.0	46.0	47.1	46.0	51.0
Threonine	16.1	16.4	16.2	16.3	15.9	16.2	16.1	16.5	16.2	18.2
Serine	19.4	19.4	19.0	19.3	18.6	18.8	19.1	19.6	19.0	20.2
Glutamic Acid	73.3	74.2	72.7	73.8	71.6	73.0	73.0	74.7	73.0	82.5
Proline	20.2	20.8	20.5	20.8	20.0	20.4	20.4	20.9	20.5	22.7
Glycine	17.3	17.6	17.3	17.6	17.2	17.1	17.5	17.7	17.3	19.5
Alanine	17.3	17.6	17.2	17.6	17.0	17.2	17.4	17.6	17.3	20.3
Cysteine	6.0	6.2	6.0	6.1	6.0	6.0	6.0	6.1	5.8	6.0
Valine	18.7	19.0	18.6	18.9	18.8	19.1	18.8	19.0	18.9	21.9
Methionine	5.6	5.7	5.6	5.7	5.6	5.6	5.6	5.8	5.7	6.2
Isoleucine	17.9	18.1	17.7	18.1	17.9	18.3	18.0	18.2	18.1	20.5
Leucine	31.0	31.9	31.3	31.8	31.0	31.8	31.3	32.0	31.6	35.6
Tyrosine	13.5	14.9	14.7	15.0	14.6	14.9	14.8	14.9	14.9	16.1
Phenylalanine	20.6	21.1	21.0	21.4	20.6	21.4	21.0	21.2	21.3	23.1
Lysine	26.6	27.2	26.6	27.1	26.5	27.1	26.7	27.2	26.7	29.0
Histidine	10.9	11.3	11.0	11.2	11.0	11.2	11.0	11.3	11.1	12.1
Arginine	32.9	34.3	33.3	33.8	32.7	33.6	33.3	34.3	33.4	32.9
Tryptophan	4.7	5.6	4.5	5.6	3.7	5.3	6.3	6.6	5.8	6.5

3.3.4 *In vitro* CP and DM digestibility of DM and CP in samples

Results of *in vitro* DM digestibility (DMD) and CP (DCP) of steam- or dry-heated full-fat soybean, and unheated and commercial SBM samples incubated in different enzymes are shown in Table 3.5. The DMD values of samples incubated in the cocktail of microbial enzymes (phytase + protease) ranged from 0.71 to 0.90, being for full-fat SBM sample that was dry-heated for 15 min and the lowest was for the steam-heated samples (45 min).

The DMD value of those samples that were incubated with microbial phytase alone ranged from 0.66 to 0.82 with the highest values recorded for both the unheated and commercial SBM samples. The lowest digestibility of DM was in the sample that was dry-heated for 30 min. After incubating samples with microbial protease alone, the highest DMD (0.73) was recorded for unheated full-fat SBM, while the lowest value (0.60) was in one of the steam-heated (15 min) full-fat soybean sample.

Table 3.5 *In vitro* digestibility of DM and CP in samples incubated in different microbial enzymes.

	RSBM	Dry heating (min)				Steam heating (min)				SBM
		15	30	45	60	15	30	45	60	
Dry Matter:										
Control[1]	0.72	0.68	0.67	0.67	0.67	0.59	0.71	0.64	0.67	0.70
Phytase	0.82	0.80	0.66	0.77	0.78	0.78	0.77	0.78	0.76	0.82
Protease	0.73	0.70	0.69	0.69	0.66	0.60	0.72	0.64	0.72	0.71
Phytase + protease	0.84	0.90	0.80	0.80	0.82	0.79	0.74	0.71	0.76	0.78
Pooled SEM	0.024	0.042	0.034	0.023	0.038	0.068	0.018	0.030	0.013	0.068
Crude Protein:										
Control[1]	0.51	0.53	0.53	0.53	0.54	0.53	0.57	0.55	0.54	0.55
Phytase	0.66	0.69	0.59	0.64	0.61	0.64	0.66	0.64	0.65	0.54
Protease	0.56	0.66	0.61	0.62	0.61	0.62	0.65	0.67	0.57	0.63
Phytase + protease	0.66	0.68	0.70	0.66	0.69	0.66	0.65	0.66	0.69	0.59
Pooled SEM	0.022	0.007	0.024	0.013	0.022	0.043	0.035	0.038	0.052	0.043

[1]Control samples were incubated in enzyme-free buffers. Number observations = 3.

Without any enzymes in the media, DMD ranged from 0.59 in the sample that was steam-heated for 15 min to 0.72 for the unheated full-fat SBM. The *in vitro* DCP of samples incubated in the cocktail of microbial enzymes (phytase + protease) ranged from 0.59 to 0.69, the highest value being in the full-fat sample that was dry-heated for 60 min and the lowest value was in the commercial SBM.

The DCP of samples incubated with phytase was between 0.54 and 0.69 with the highest value in the full-fat sample that was dry-heated for 15 min and the lowest value was for commercial SBM. For samples incubated with protease alone, the DCP value was highest (0.67) in the full-fat sample that was steam-heated for 45 min while the lowest value (0.56) was in the unheated samples. Samples that were incubated in enzyme-free buffers had DCP values between 0.51 and 0.57 with the highest value recorded for the sample that was steam-heated for 30 min and the lowest value in the unheated sample.

3.3.5 Particle distribution in diets

The particle size distribution of mash-form diets containing different levels of RSBM is shown in Figure 3.1a.

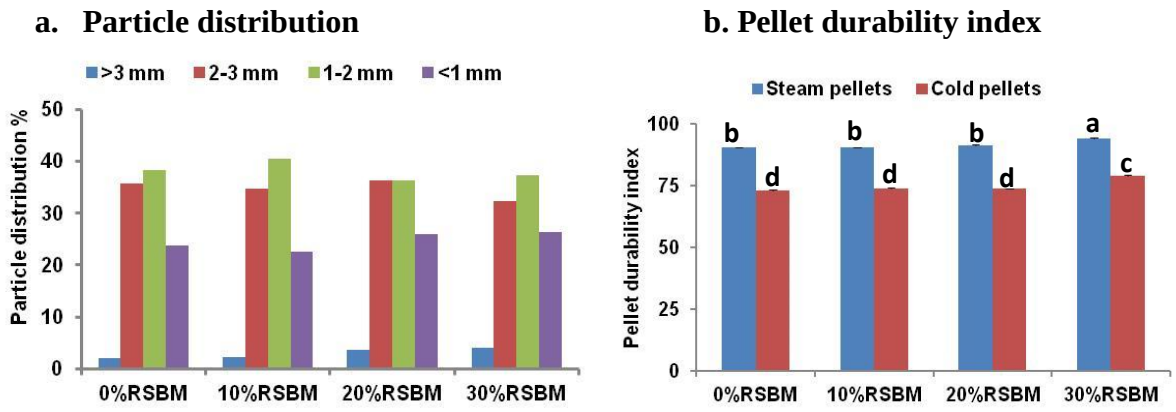


Figure 3.1 (a) the particle distribution in a mash form diets (b) the PDI value of steam- or cold-pelleted of diets containing graded levels of raw full-fat soybean meal (RSBM)

Bars bearing different letters, in the figure are significantly different (*p< 0.05;**p< 0.01;***p<0 .001).

Most of the particles fell within <1-3 mm regardless of RSML levels. Particle > 3 mm were minimal (around 3%) although slightly more in diets containing increasing RSBM supplementation.

3.3.6 Pellet durability index of steam- or cold-pelleted diets

The highest pellet durability index (PDI) value was recorded for diets supplemented with 30% RSBM; whereas, the lowest value was found for diets supplemented with RSBM-free diets. Regardless of changing the pelleting methods, increasing supplementation of RSBM in diets, particularly at 30%, increased ($p<0.001$) the PDI values as shown in Figure 3.1b. Changing the pelleting methods of diets containing graded levels of raw full-fat SBM (RSBM) had significant ($p<0.05$) interaction effects on PDI values. Regardless of the RSBM supplementation rate, changing the pelleting method alone affected ($p<0.05$) the PDI value, with steam-pelleted diets gaining the highest score.

3.3.7 Concentration of phytate in samples and in vitro digestibility of phytate

As shown in Table 3.6, the concentration of phytate in original samples was significantly ($p<0.01$) different with commercial SBM having the highest (4.1 mg/g) value. The enzymatic *in vitro* digestibility of phytate (EDP) of samples incubated in the cocktail of microbial enzymes was higher ($p<0.01$) in the full-fat SBM that was steam-heated for 30 min than in the other samples. The EDP value of samples incubated in phytase alone was also highest ($p<0.001$) in the full-fat SBM that was steam-heated for either 30 or 60 min.

Table 3.6 Phytate concentrations in original samples and *in vitro* digestibility of phytate after incubation with samples in different microbial enzymes.

	RSBM[1]	Duration of dry heating (min)				Duration of steam heating (min)				Commercial SBM	SEM[1]
		15	30	45	60	15	30	45	60		
Concentration of phytate (mg/g)	4.3^{a}	3.6^{b}	3.9^{b}	3.5^{bc}	3.9^{b}	3.3^{c}	3.5^{bc}	3.9^{b}	4.1^{b}	4.4^{a}	0.08
In vitro degradation coefficient of phytate											
Phytase	0.34^{d}	0.49^{c}	0.54^{b}	0.56^{ab}	0.54^{b}	0.53^{b}	0.57^{a}	0.52^{b}	0.57^{a}	0.38^{d}	0.07
Protease	0.31^{c}	0.41^{bc}	0.41^{bc}	0.43^{b}	0.41^{bc}	0.47^{a}	0.48^{a}	0.43^{b}	0.45^{ab}	0.36^{c}	0.03
Phytase + protease	0.59^{c}	0.61^{bc}	0.60^{c}	0.66^{bc}	0.61^{c}	0.72^{ab}	0.75^{a}	0.73^{a}	0.68^{b}	0.38^{d}	0.01
Negative control	0.31^{c}	0.32^{c}	0.40^{b}	0.40^{b}	0.41^{b}	0.45^{a}	0.40^{b}	0.41^{b}	0.40^{b}	0.28^{c}	0.07

[a, b, c] Means bearing uncommon superscripts within a row are significantly different at the levels shown ;***P < .001; [1]SEM= pooled standard error of means; negative control= samples were incubated in enzyme-free buffers; RSBM= raw soybean meal.

3.4 DISCUSSION

3.4.1 Nutrient composition and nutritional quality of samples

The nutrient and anti-nutrient contents of soybean were measured primarily to provide data for formulation of the diets that were required for the subsequent feeding trials. These data were then compared to those of heat-treated and commercial SBM samples. Although there were slight variations between samples of FF, the materials which were heat-treated, their CP values were generally lower than that of commercial SBM. The lower values of CP and amino acid contents of full-fat meals are largely due to the fat (oil) content of full-fat seeds.

However, the crude protein content for FF soybean found in this study is comparable to that reported by Nahashon and Kilonzo-Nthenge (2013). The variations in nutrient contents between this and other studies may be due to various reasons such as processing, variety of soybean crops or the geographical origins (Swick, 2007, de Coca-Sinova, 2010; Baker *et al.*, 2011).

On average, the gross as well as the calculated metabolizable energy and the crude fat contents of FF soybean was greater than that of the value for commercial SBM. However, values of metabolizable energy and the levels of crude fat content that were found in this study were generally lower than those found by previous researchers (Nahashon and Kilonzo-Nthenge, 2013). The total sugars and starch contents of commercial SBM were found to be higher than the average values of the FF SBM samples, heated or raw, although the value was lower than that reported by Hou *et al*. (2008).

In this study, the average concentration of trypsin inhibitors (TI) for raw and differentially heated full-fat soybean was higher than that of the commercial SBM sample. The TI values however are much lower than those researchers (Ruiz *et al*., 2004 and Sharma *et al*., 2013) who reported values that were as high as 50 000 TIU/g. The lower values of the TI in the current study may be due to differences in varieties or sources. The major factors that could determine TI value are soil properties and agronomic practices.

The urease activity (UA) and protein solubility (KOH) of the test samples (differently heated) were higher than from among other researchers (Căpriţă *et al*., 2010). Although UA is widely used to measure the soybean quality (CAES, 2013), Palić *et al*. (2008) has questioned about its reliability.

Although the KOH values were decreasing as the duration of heating time increases, particularly at steam-heating, generally the values in this study are not in agreement with those of CAES (2013) who reported the values between 78 and 84%. The higher values of UA and KOH in the current study may be due to either under-heating or a shorter heating duration than previously applied by other researchers (Herkelman *et al.*, 1991; Whittle and Araba, 1992). The present data are however close to those of Carvalho *et al.* (2013).

The phytate contents of differentially heated samples was on average lower than that of the commercial SBM, but the total phosphorus (P) content was averagely higher for samples of FF SBM. Tahir *et al*. (2012) reported that the phytate concentration and the total P of feed ingredients are mostly proportional. This was not the case in this research although phytate values recorded are similar to the values of other researchers (Anderson and Wolf, 1995). The total P value of commercial SBM was lower than the value reported by Batal *et al.* (2010). The reason for these variations might be due to the origin or from crop variety.

The profiles of amino acids in general are better in the commercial SBM than in the full-fat SBM as has been previously observed (Swick, 2007; de Coca-Sinova, 2010; Baker *et al.*, 2011). Available lysine was also higher in commercial SBM than in the full-fat SBM. This may suggest the need for higher levels of supplementation with lysine when such SBM are included in diets.

3.4.2 Physical quality of diets

Although most of the particles of the mash form of the diets fell within <1-3 mm sieve sizes of a tester, amounts of particles falling in larger sieve size (>3 mm) were increased as supplementation of RSBM increased. Aderibigbe *et al*. (2013) also suggested that particle size has also an effect on the feed quality for poultry. The reason for the current value of feed particle distribution may be due to the coarse grinding of supplemental RSBM.

In this study, changing the pelleting methods for diets containing graded levels of RSBM had an interactive effect on PDI values. Moreover, it was found that steam-pelleting produced stronger and more durable pellets than cold-pelleting. This result agrees with Skoch *et al. (*1981) who reported that steam-pelleting improves the PDI of poultry diets.

The result of this study revealed that regardless of the pelleting methods, the PDI value improved as RSBM supplementation increased. The reason for these are unclear although PDI could be affected by grain hardness and degree of grinding (Briggs *et al*., 1999; Amerah *et al*.,

2007) both of which are likely favoured by lack of previous processing for oil, in case of full-fat SBM.

3.4.3 Enzymatic *in vitro* digestibility of nutrients and phytate in soybean samples

On average, *in vitro* digestibility of DM and CP was higher for samples incubated in a cocktail (phytase + protease) of microbial enzymes. This result partially agrees with those of previous researchers who reported that composite enzymes more effectively hydrolyse nutrients in the feed (Malathi and Devegowda, 2001). Such response may be due to synergistic effects of enzyme activities, as they work on different components of the diet or ingredients. Phytase would enable the release of nutrients that are bound by phytic acids (Mittal *et al.,* 2013). The response to protease alone is in contrast to the findings of Yu *et al.* (2007) who reported that protease supplementation did not improve *in vitro* digestibility of DM and protein. The response observed in this study may be due to the nature of protease being tested, but this will require further confirmation.

There was some response to heat treatment, especially illustrated by higher *in vitro* digestibility of CP in the FF soybean steam-heated for 45 min and incubated in microbial protease. This improvement may be due to break down of both stored proteins and proteinaceous antinutrients by the microbial protease, a process that may be aided by prior application of heat to the nutrients structure. The results obtained in this study showed a reduction in residual phytate contents due to digestibility of the phytate molecule. Although the phytase enzyme alone reduced the much phytate contents, phytate digestibility was higher when protease was also included in the mixture. It is probably the first time that the direct effects of these enzymes on phytase are being tested. The results suggest that the enzyme cocktail could be useful in diets containing RSBM. This will be tested in feeding trials.

3.5 CONCLUSION

Most of the nutrients profiles of full-fat SBM when heat-treated are comparable to those of commercial SBM. However, the concentration of trypsin inhibitors, KOH, and urease activity were averagely higher in the full-fat samples. These higher values might be due to under-heating of the whole seeds by heat. Moreover, the response to *in vitro* tests on nutrients and phytate were slightly different. The combination phytase and protease appears to be effective on nutrients and phytate digestibility, but it is not known if this response will be reflected in *in vivo* systems.

CHAPTER 4: RESPONSE OF BROILER CHICKS TO COLD- OR STEAM-PELLETED DIETS CONTAINING RAW, FULL-FAT SOYBEAN MEAL

ABSTRACT

This study was conducted to assess the effects of pelleting and the pelleting method on broiler chick diets containing raw soybean meal (RSBM). The chemical attributes and feeding value of the diets were assessed. Four diets containing RSBM, which replaced commercial SBM at 0, 10, 20 or 30% were prepared. One-half of each diet was steam-pelleted, and the other half was cold-pelleted. The composition of the subsamples was then analysed. A feeding trial was designed with a 2 x 4 factorial arrangement (RSBM 0, 10, 20 or 30%, and steam- or cold-pelleted), each with six replicates and eight birds per replicate. The chicks were housed in environmentally controlled rooms and were fed up to 14 days of age. The concentration of trypsin inhibitors (TI) in the diets ranged between 4,153.2 and 10,484.4 TIU/g, with the maximum TI value (10,484.4 TIU/g) recorded in the mash diets containing 30% RSBM. The maximum urease activity (UA) (1.83 ΔpH) was also recorded in the same diet. The available lysine values were nearly the same across all diets (15.4 g/kg). In general, the amino acid composition was better for both the cold-pelleted and mash diets than the steam-pelleted diets. There was no difference ($p>0.05$) in the mortality of birds between treatments. Both feed intake (FI) ($p<0.05$) and body weight gain (BWG) ($p<0.001$) decreased with increasing RSBM content. The BWG of the birds fed the steam-pelleted diets was less ($p<0.001$) than that of the birds fed the cold-pelleted diets, but the FI was not significantly ($p>0.05$) affected. The FCR was negatively affected ($p<0.05$) by increasing levels of RSBM. Changing the pelleting method of diets containing levelled RSBM had no ($p>0.05$) interactive effects on the FI, BWG, or FCR of birds. Increasing the inclusion rate of RSBM increased the weight of the gizzard + proventriculus (G+P) ($p<0.05$), pancreas ($p<0.001$), and duodenum ($p<0.01$). The steam-pelleted diets increased the weight of the G+P ($p<0.01$), jejunum + ileum (J+I) ($p<0.001$) of the birds. Neither the pelleting method nor the increased level of RSBM had any effects ($p>0.05$) on the weight of the other visceral organs. Cold-pelleting increased ($p<0.05$) the pancreatic protein content, whereas the activity of chymotrypsin amidase was reduced ($p<0.01$) when the RSBM level was increased. Although the differences were not significant ($p>0.05$), the mucosal morphometry of the jejunum of birds fed the RSBM-free diets had thicker muscle, longer villi, wider villus surface areas and higher villus to crypt depth ratios compared to those fed the other diets. Overall, this study showed that steam-pelleting is not sufficient to inactivate of ANF in diets containing RSBM.

4.1 INTRODUCTION

Soybean meal is extensively used to provide vegetable proteins to poultry worldwide. Although its supply fluctuates (Shi *et al*., 2012) due to the events in the major soybean-producing regions of the world, North and South America, it is generally expensive because it is the premier vegetable protein for poultry.

Soybean meal can be produced as a full-fat meal or an oil-extracted meal. Full-fat soybean meal is characterised by relatively low crude protein (CP) content (36-42%) and a high level of fat (18-22%) (Stein *et al*., 2008). In addition to its high oil content, full-fat soybean meal has the same excellent amino acid profile as conventional SBM, and sometimes, the digestibility of the AA in full-fat soybean meal is higher (Pahm and Stein, 2007). Consequently, Popescu and Criste (2003) suggested that full-fat SBM can successfully be used to replace the oil and commercial SBM in the diets of broilers.

The inclusion of full-fat SBM in broiler diets has been found to support high performance of the birds at lower costs (Subuh *et al.*, 2002; Newkirk, 2010). However, users are still wary of using full-fat meal and are concerned that chickens may deposit the fat they consume (Newkirk, 2010). However, MacIsaac *et al.* (2005) reported that lean birds could be produced when they were fed a roasted full-fat SBM.

Although full-fat SBM has a very good composition (Willis, 2003; Stein *et al*., 2008), the nutritive value of raw, full-fat soybean meal is negatively affected by the presence of anti-nutritional factors (Liener, 1994; Liu, 1997), particularly trypsin inhibitors and lectins (Newkirk, 2010). Various scholars (ASA. 2004; Newkirk, 2010) have reported that birds fed the raw, full-fat SBM exhibited poor performance and pancreatic hypertrophy due to the presence of trypsin inhibitors and lectins.

Grinding is the first method used to disrupt the cell wall structures of the ingredients, which increases the exposure of the nutrients to the animal's digestive enzymes (Meng *et al.,* 2006). The trypsin and lectins in raw soybean meal are heat-labile factors (Soetan and Oyewole, 2009; Ebrahimi-Mahmoudabad and Taghinejad-Roudbaneh, 2011); hence, the activity of trypsin inhibitors can readily be reduced by ordinary cooking and moist-heat treatments (Rackis and Gumbmann, 1981). Svihus and Zimonja (2011) also indicated that the conditioning temperature used while pelleting the diet can improve the nutritional value of the feed by inactivating the proteinaceous anti-nutrients. However, Perez-Maldonado *et al.* (2003) reported that birds fed steam-pelleted diets containing raw soybean meal (RSBM) did not show any

change in performance. However, limited information is available with regards to the advantages of steam-pelleting in reducing the impact of anti-nutrients in diets containing RSBM. The objectives of the current study were to assess the effects of RSBM on some nutrient and the anti-nutrient profiles of the diets. The relative benefits of cold- or steam-pelleting were also evaluated through a feeding trial.

4.2 MATERIALS AND METHODS

4.2.1 Soybean sample and diets

The tested raw soybean seed was harvested in July 2013 and purchased from a local farmer in northern New South Wales, Australia. The product was cleaned and the seeds were ground to pass through a 0.2-mm sieve. Four diets were then formulated containing RSBM, which replaced the commercial SBM at 0, 10, 20 or 30% (equivalent to 0, 30, 60 and 90 g/kg of diet). One-half of each diet was steam-pelleted, and the other half was cold-pelleted. In addition to evaluating the pellet durability index (PDI) of the steam- or cold-pelleted diets, subsamples were collected from all steam- or cold-pelleted diets, as well as from the mash diets (from the same diets, but before pelleting), and subjected to chemical laboratory analysis.

4.2.2 Nutrient composition and anti-nutritional factors in the diets

The samples were ground to pass through a 1-mm screen and analysed for the CP and complete amino acid profiles using methods, Kjeldahl 990.03 and 984.13, respectively (AOAC, 2006a), available lysine contents (methods 982.30 and 975.44, AOAC, 2006b), urease activity (method Ba, 9-58, AOCS, 2006a), trypsin inhibitor concentration (method 22-40, AOAC, 2006c), protein solubility (KOH) (Araba and Dale, 1990), and nitrogen solubility index (method Ba 11-65, AOCS, 2006b).

Dietary electrolyte balance of the diets (DEB): To determine the DEB, sub-samples were taken from each diet and ground with a coffee grinder. The samples were analysed to determine the Na, K, and Cl contents, which are the most important regulators of the acid-base balance in animals. The DEB for the dietary treatments was then calculated using the following formula.

DEB (mEq/kg) = % Na x 434.98 + % K x 255.74 - % Cl x 282.06.

4.2.3 Feeding trial

A completely randomized design (CRD) with a 2 x 4 factorial arrangement was employed in the feeding trial to evaluate the response of broiler chicks fed steam- or cold-pelleted diets (corn-soybean-based) containing a gradient of RSBM concentrations.

Table 4.1 Composition of the starter diets fed during the study (0-14 d) used.

	Raw soybean meal (RSBM) (%)			
	0	**10**	**20**	**30**
Ingredient composition (g/kg)				
Corn (Rolled)	564.0	563.8	563.4	562.8
Soybean meal	300.0	270.0	240.0	210.0
Meat meal	97.2	101.2	105.2	109.2
Raw soybean meal	0	30.0	60.0	90.0
Canola oil	16.3	13.3	10.4	7.5
TiO_2	5.0	5.0	5.0	5.0
DL-Methionine	3.9	3.9	4.1	4.1
L-Lysine	3.9	3.5	3.0	2.9
Salt	3.0	3.0	2.9	2.5
Limestone	2.5	2.5	2.5	2.4
L-Threonine	1.5	1.5	1.5	1.6
UNE TM conc 0.75 kg/mt	0.8	0.8	0.8	0.8
UNE Vit conc 0.5 kg/mt	0.5	0.5	0.5	0.5
Choline Cl 70%	0.5	0.5	0.5	0.5
Sodium bicarbonate	0.5	0.4	0.3	0.3
Dical Phos 18P/21Ca	0.4	0.2	0.2	0.1
Phytase	0.1	0.1	0.1	0.1
Nutrient composition (g/kg)				
ME Poultry (MJ/kg)	12.66	12.66	12.66	12.66
Crude protein	249.0	248.6	248.3	247.9
Crude fat	43.8	45.3	46.8	48.4
Arginine	15.8	15.8	15.8	15.8
Lysine	15.1	15.0	15.0	15.0
Methionine	7.4	7.4	7.4	7.4
Methionine + Cysteine (M+C)	10.7	10.7	10.7	10.7
Threonine	9.9	9.9	10.0	10.0
Calcium	10.5	10.5	10.5	10.5
Phosphorus (avail)	5.0	5.0	5.0	5.1
Sodium	2.0	2.0	2.0	2.0
Chloride	3.1	3.1	3.1	3.1
Choline	1.6	1.5	1.4	1.3

Trace minerals supplied per kilogram of diet: Cu (sulphate), 16 mg; Fe (sulphate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulphate and oxide), 120 mg; Zn (sulphate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg. Vitamins supplied per kilogram of diet: Vitamin A (retinol), 12,000 IU; Vitamin D3 (cholecalciferol), 5,000 IU; Vitamin E (tocopheryl acetate), 75 mg, Vitamin K (menadione), 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; Vitamin B12 (cyanocobalamin), 16 µg; biotin, 200 µg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg. The commercial SBM was replaced by RSBM at 0, 10, 20 and 30%, equivalent to 0, 30, 60 and 90 g/kg of diet.

A total of eight starter diets were prepared as iso-caloric and iso-nitrogenous dietary treatments (Table 4.1), and each was replicated six times with eight birds per replicate.

4.2.3.1 Animal husbandry and sampling

One-day-old male broiler chicks (Ross 308) with an average body weight of 44.98±0.14 g were obtained from a local commercial hatchery (Baiada Poultry Pty. Ltd., Tamworth, Australia). On arrival, the chicks were weighed and distributed to the eight dietary treatment groups in a completely randomized design. The birds were housed from 1 to 14 days of age in a cage-

rearing system in climate-controlled rooms. Each pen was furnished with a feeding trough and drinker points. The feeding troughs were cleaned before supplying the diets and whenever fresh feed was supplied. The drinkers were also washed to maintain hygienic conditions. The excreta on the trays were cleaned whenever necessary.

For the first 2 days, the birds were housed at a temperature of 33 °C. The temperature was then gradually reduced by 1 or 2 °C every 1 or 2 days. Twenty-four hours of light were provided on the first day, which was reduced to 23 h for the next 6 consecutive days, followed by 20 h for the remaining days. Feed was provided *ad libitum* in a crumbled form and birds had free access to water. Mortality was recorded when it occurred.

At day 14, one bird from each pen was randomly selected, weighed and killed by cervical dislocation to assess the visceral organ development. Tissue samples from the proximal part of the jejunum and the entire pancreas were collected and snap-frozen in liquid nitrogen before transferring to a freezer (-20^{0}C) to assay the mucosal protein concentrations and digestive enzyme activities. The remaining birds and the leftover feed were weighed to calculate the average body weight gain (BWG) and feed intake (FI), from which the feed conversion ratio (FCR) was calculated.

4.2.4 Pancreas tissue processing

The entire pancreas was placed on ice and cut into small pieces and then transferred into a centrifuge tube containing cold Ringer's solution, pH 7.4, at a ratio of 15.0 ml Ringer's solution to 1.0 g of tissue (Susbilla *et al*, 2003). The sample was homogenized at a speed of 4 for about 60 sec using a homogenizer (Ika Ultra Turrax ®T25, Germany). The homogenate was then centrifuged at a speed of 30,000 × g for 10 min and then duplicates of the supernatant in Eppendorf tubes were taken and stored in a freezer (-20^{0}C) for subsequent analysis. Another sample was collected from proximal part of jejunum, and fixed with formalin solution for next histological analysis.

4.2.4.1Tissue protein contents in pancreas

Tissue protein concentration of the pancreatic tissue was determined using the method of Bradford (1976). The tissue homogenate was diluted 10 times with ROH, then 40 μL of this dilute was pipetted into culture tubes and 2.0 mL of Bradford reagent (BR) was added. The reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue in 50 mL of 95% EtOH + 100 mL of 85% phosphoric acid, and topped to 1.0 L with Milli-Q water. The solution was filtered through Whatman paper, No 24. The mixture (dilute homogenate and BR) was vortexed

and the absorbance was read at 595 nm. A standard curve with concentrations of 6.25, 12.5, 25, 50, and 100 µg protein /mL was prepared using bovine serum albumen (BSA).

4.2.4.2 Pancreatic enzyme activities

The method of Tarvid (1992) and Susbilla *et al.* (2003), with slight modification by Delia, (2008) was used to determine the pancreatic enzyme activity. After diluting 5 times (only for trypsin or chymotrypsin) the pancreatic homogenate was mixed with enterokinase solution (1.0:0.1 v/v) (enterokinase solution contained 5 mg enterokinase from porcine intestine [Sigma E-0632]/10 mL of Tris-HCl buffer (3.025 g Trizma base Tris [hydroxymethyl] amino-methane [Sigma T3253] + 2.95 g Tris-HCl/500 mL of deionized water, pH 7.4). The procedures used to determine individual enzyme activities are described below.

***General proteolytic (GP) enzyme activities*:** The homogenate plus enterokinase solution (1.0:0.1) was incubated on ice for 2 h, to activate the zymogen granules (Susbilla *et al.,* 2003)**.** Around 25 µL of this activated sample were then transferred into a fresh test tube and 2 mL of 1% casein solution (1.0 g casein [Sigma C-8654) /100 mL of 0.1 M sodium phosphate buffer, pH 8.0) were added (León *et al*., 2014). This mixture (solution) was incubated in a shaking water bath at 41^0C for 20 min.

After filtering the incubated mixture through Whatman filter paper No. 24, a 0.4 mL aliquot was transferred to a fresh test tube containing 2.0 mL of 0.2 M Na_2CO_3 and allowed to stand for 5 min at room temperature. The reaction was terminated with 1.0 mL of 5% trichloroacetic acid (TCA) (w/v) and 0.4 mL of Folin-Ciocalteu's solution was then added and allowed to stand for 90 min at room temperature after which the absorbance was read at 625 nm. Folin-Ciocalteu's solution was prepared by mixing Folin-Ciocalteu's reagent (Sigma F-9252) with deionised water in a 1:5 (v/v) ratio.

The standard curve for GP was prepared with varying concentrations of tyrosine (0, 0.05625, 0.1125, 0.225, 0.45, 0.9, and 1.8 mg/mL). Tyrosine was dissolved in 0.1 M sodium phosphate buffer, pH 8.0. About 0.4 mL of each concentration of tyrosine standard solution was pipetted into individual fresh test tubes and 2.0 mL of 0.2 M Na_2CO_3 solution were added to each tube and the mixture was allowed to stand for 5 min at room temperature. Then one mL of 5% TCA and 0.4 mL of Folin-Ciocalteu's solution were added and the mixture was allowed to stand for 90 min at room temperature, after which the absorbance was read at 625 nm.

***Activities of trypsin and chymotrypsin amidase*:** The activities of pancreatic trypsin and chymotrypsin amidase in the homogenate were determined following the procedures described by Erlanger *et al. (*1961) and modified by Caviedes-Vidal and Karasov (2001). The homogenate was diluted five times with Milli-Q water. The dilute homogenate plus enterokinase solution (1:0.1 v/v ratio) was incubated for one h in a shaking water bath (JULABO GmbH**,** Germany) at 25^0C (Erlanger *et al.,* 1961; Caviedes-Vidal and Karasov, 2001). The detailed methods for determining the activities of trypsin amidase or chymotrypsin amidase are described below.

***Trypsin amidase activity*:** Approximately 0.8 mL of one mM Nα-Benzoyl-DL-arginine-p-nitroanilide (DL-BAPNA, Sigma B4875) solution was transferred to a test tube containing 25 µL of pre-incubated sample (dilute homogenate + enterokinase solution) and the mixture was further incubated in a shaking water bath (40 °C) for 20 min. The reaction was terminated by pipetting in a 0.16 mL of 30% acetic acid, and the absorbance was read at 410 nm. The DL-BAPNA solution contained 43.49 mg DL-BAPNA dissolved in a dimethylsulfoxide solution (one mL dimethylsulfoxide [Sigma D2650] + 100 mL 0.05 M Tris-HCl buffer, pH 8.2. Tris-HCl buffer was prepared with 3.54 g Trizma HCl (Sigma T3253) + 3.34 g Tris (hydroxymethyl) aminomethane (Boehringer Mannheim Cat. No. 604205) + 2.94 g calcium chloride/1000 mL of deionised water.

***Chymotrypsin amidase activity*:** Approximately 0.8 mL of one mM N–glutaryl- L - phenylalanine-p-nitroanilide (GPNA, Sigma G2505) solution was transferred to a test tube containing 25 µL of pre-incubated sample. The mixture was further incubated in a shaking water bath (40 °C) for 20 min, and the reaction was terminated by adding 0.16 mL of 30% acetic acid, and the absorbance was read at 410 nm. The GPNA substrate solution contained 39.90 mg GPNA dissolved in one mL dimethylsulfoxide (Sigma D2650) +100 mL of 0.05 M Tris-HCl buffer, pH 7.6. The Tris-HCl buffer, pH 7.6, contained 6.06 g Trizma HCl +1.39 g Tris (hydroxymethyl) aminomethane (Cat. No. 604205) + 2.94 g calcium chloride/1000 mL of deionised water.

Using 0.3 mM p-nitroaniline, a standard curve was produced for either trypsin or chymotrypsin amidase activity with concentrations of 0, 0.0175 0.035, 0.07, and 0.14 mg/mL. The p-nitroaniline was dissolved in Tris-HCl buffer, pH 8.2 for trypsin, and pH 7.6 for chymotrypsin activities. About 0.8 mL was transferred from each of these solutions into fresh test tubes containing 16 µL of Tris-HCl buffers with pH 8.2 for trypsin and pH 7.6 for chymotrypsin

activity. After adding around 0.16 mL of 30% acetic acid to each of these test tubes, the absorbance of each standard was read at 410 nm.

***Lipase activity*:** The lipase activity in the pancreas was determined according to the procedure described by Winkler and Stuckmann (1979). Approximately 200 µL of lipase substrate (P-nitrophenylpalmitate) and blank were transferred into test tubes and then pre-warmed, at 39 C. The lipase substrate was prepared by dissolving 30 mg of p-nitrophenylpalmitate in 10 mL of isopropanol and then mixed with 100 mL of 50 mM Sorensen phosphate buffer (containing Na deoxycholate). Around 50 µL of homogenate samples were pipetted into the pre-warmed lipase substrate and further incubated at 39 °C for 15 min. The reaction was terminated with 100 µL of 40% TCA. After cooling, 2 mL of 0.4 M NaOH solution were added, then mixed and the absorbance was read at 410 nm.

The standard solution, consisting of different concentrations of 2 mM P-Nitrophenol (0.174, 0.348, 0.696, 1.391, and 2.782 mg/mL) was prepared. Around 350 µL of each of these concentrations were transferred into fresh test tubes and 2 mL of 0.4 M NaOH were added. The absorbance was read at 410 nm to produce a standard curve.

4.2.5 Mucosal protein contents and enzyme activities in the jejunum

Mucosal protein concentration in the jejunum tissue was determined using the same method as described in Section 4.2.4.1 above.

Alkaline phosphatase activity: The activity of alkaline phosphatase was determined using the procedure described by Forstner *et al.* (1968) and Holdsworth (1970) and standardised for poultry by Iji *et al*. (2001a). The reaction mixture consisted of 20 µL of dilute sample + 800 µL of 50 mM Tris buffer (7.88 g/L, pH 10.1) +100 µL of 50 mM $MgCl_2$ (2.54 g/250 mL) + 100 µL of 10 mM phosphatase substrate (Sigma 104; MW 263.1). The blank contained all buffers and substrates, but no samples. After standing for 20 min, the reaction was terminated by rapidly pipetting in 100 µL of 40% TCA. Then, 100 µL of the mixture was transferred into new tubes and 2 mL of 0.4 M NaOH (16 g/L) solution were added.

The standard solution, containing different concentrations of 2 mM p-nitrophenol (0.174, 0.348, 0.696, 1.391, and 2.782 mg/mL) plus all other incubation solutions, except the sample and substrates, were vibromixed and read at 410 nm for absorbance along with the samples.

***Sucrase activity*:** To determine the activity of sucrase in the jejunum, 25 µL of homogenate was incubated in a solution (containing 100 mg sucrose [MW 342.2] + 4 mM sodium succinate

[1.08 g] + 90 mM NaCl [5.26 g]/L of milli-Q water, pH 6.0). The reaction was then terminated with 2.5 mL Triton X-100 solution (a solution contained 0.2% Triton X-100 w/v [about 0.2 g] + 0.5 M Tris-HCl buffer [78.8 g Tris/L], pH 7.02 at 37 °C). Approximately 0.4 mL of this incubated sample was mixed with 2.5 mL of GoPoD solution and then further incubated at room temperature before reading the absorbance at 610 nm.

***Maltase activity*:** The activity of maltase, in the jejunum was determined using the same procedure described above for sucrase activity, except that maltose was used as substrate.

***Mucosal morphometry of the jejunum*:** The mucosal morphometry was measured using the method described by Iji *et al*. (2001b). The samples were excised from the proximal part of the jejunum. After flushing with saline water, the samples were immediately fixed in formalin solutions and then in 70% ethanol alcohol for storage. Two pieces of tissue were excised from each fixed sample and enclosed in tissue cassettes (Bayer Diagnostics, Australia Pty Ltd) containing ethanol solution. The tissue samples were then processed in an automatic tissue processor (Shandon, Pittsburgh, USA) over 17 h during which the tissue samples were passed through a series of baths of increasing concentrations of ethanol, followed by xylene solutions. The cassettes containing the samples were then immersed in 2 separate baths of a 100% solution of paraplast. The samples were then embedded in a single mould containing paraffin wax. Sections were cut from the tissue using a microtome equipped with a feather blade. After the sections were floated on warm water, they were mounted on microscopic slides, checked for quality, and then allowed to dry on a hotplate.

Before staining, the sections were deparaffinised by immersing the slides in three consecutive solutions of SOLV21C (United Biosciences Pty Ltd, Australia) and then dehydrated by passing them through a series of alcohol concentrations. The sections were stained with Harris' haematoxylin and eosin solutions. These stained sections were immersed in a series of solutions containing increasing concentrations of alcohol, followed by three separate baths of SOLV21C, and then mounted in LEICACV (Leica Biosystems, Nussloch GmbH, Germany).

The slides with stained tissue were viewed on an Olympus BH-2 microscope and digitized using video image software (Video Pro, Leading Edge, Bedford Park, South Australia). The images were viewed to measure the muscle depth, crypt depth, basal width, villus height, and villus apical width. The apparent villus surface area was calculated as: ([apical width + basal width] /2) x villus height. The mucosal depth was derived by adding the villus height and crypt depth.

4.2.6 Statistical analysis

Descriptive statistics and general linear model (GLM) were used to analyse the data with Minitab software version 17 (Minitab, 2013). The data from the feeding trial were analysed using general linear model (GLM) to evaluate the RSBM and enzyme supplementations as main factors and their interactions. The differences were considered to be significant at $p < 0.05$, and the significant differences between mean values were separated using the Duncan's test.

4.3 RESULTS

4.3.1 Composition and quality of diets containing raw, full-fat soybean meal

As indicated in Table 4.2, the highest level of potassium (11.09 g/kg) was recorded in the diet containing 10% RSBM, while the lowest value (10.44 g/kg) was found in the diet containing 30% RSBM.

Table 4.2 Dietary electrolyte balance of diets containing different levels of RSBM.

	Diets			
Levels of RSBM in diets (%)	0.0	10.0	20.0	30.0
Potassium (g/kg)	10.88	11.09	10.57	10.44
Sodium (g/kg)	1.88	1.84	1.84	1.76
Total chloride (g/kg)	2.92	2.99	3.39	2.93
Calculated values of DEB (mgEq/kg)	280.0	282.4	254.8	260.0

RSBM = raw soybean meal (SBM was replaced by RSBM at 0, 10, 20 and 30%, equivalent to 0, 30, 60 and 90 g/kg of diet, respectively); DEB= dietary electrolyte balance

Sodium content was highest (1.88 g/kg) in the non-RSBM supplemented diet, while the lowest value (1.76 g/kg) was found in the diet containing 30% RSBM. The concentration of Cl ranged between 2.92 and 3.39 g/kg, with the lowest value (2.922 g/kg) in the control diet. The highest calculated DEB (282.4 mEq/kg) was recorded in the diet containing 10% RSBM, while the lowest value (254.8 mEq /kg) was recorded in the 20% RSBM diet.

The values associated with protein quality and how the protein is used are shown in Table 4.3. On average, the available lysine content was higher in the mash and the cold-pelleted diets than in the same steam-pelleted diets. Regardless of the processing methods applied to the diets, increasing the RSBM in the diets increased the nitrogen solubility index (NSI). For example, the results of the analysed samples taken from the mash diets showed that when the RSBM

increased from 0 to 30%, the value of NSI increased by 33.7%. There was no consistent trend in the effects of increasing levels of RSBM on the protein solubility (KOH) values.

The concentration of trypsin inhibitors (TI) ranged from 1,742 to 10,484.4 TIU/g and was lowest in samples from the steam-pelleted control diet and highest in 30% RSBM of mash diet. Regardless of the pelleting method used for the diets, increasing the RSBM supplementation, particularly at 30%, promoted a several fold increase in the TI concentration in the diets compared to the TI values in the diets with no RSBM supplementation.

Regardless of the RSBM level in the diets, changing the pelleting method alone altered the concentration of TI. When the diets containing RSBM were steam-pelleted, the average TI concentrations were reduced by 7.2% compared to the TI values of the same diets that were cold–pelleted. The urease activity (UA) in the diets containing RSBM ranged from 0.13 (steam-pelleted) to 1.83 ΔpH (30% RSBM mash).

The complete amino acid (AA) profiles of the diets containing increasing levels of RSBM are presented in Table 4.4. In the steam-pelleted diets, particularly those that included 30 % RSBM, the analysed AA content was approximately 3.6% less than the AA composition in the diets with 10% RSBM. The opposite result was observed for the cold-pelleted and mash diets, in which the AA content in the diets containing 30 % RSBM increased by 0.64 and 0.94 %, respectively, compared to the cold-pelleted and mash diets containing 10% RSBM.

Cold-pelleting increased the AA contents by 2.6% compared to the values for the steam-pelleted diets, regardless of the RSBM content. On average, the AA contents of the mash diets were 3% higher than the steam-pelleted diets.

Table 4.3 Effects of the cold-pelleted, steam-pelleted or mash-form diets on the values of the quality parameters (g/kg) of diets containing increasing amounts of raw, full-fat soybean meal.

	Steam-pelleted				Cold-pelleted				Mash			
Diets with RSBM[1] %	0	10	20	30	0	10	20	30	0	10	20	30
Available lysine	14.8	15.5	14.7	14.9	16.2	14.8	15.6	15.3	15.4	17.6	15.0	15.5
NSI	138.6	132.9	156.5	178.5	155.3	169.0	206.1	239.6	129.1	158.2	170.9	204.2
KOH	540.9	587.6	548.9	538.4	670.3	502.5	603.4	667.8	584.4	538.1	535.6	554.3
Trypsin inhibitor (TIU/g)	1,747	7,136.4	7,509.6	9,913.2	1,940	5,881.2	9,913.2	10,473.6	1,961	6,908.4	8,910	10,484.4
Urease activity (ΔpH)	0.13	0.55	0.77	1.70	0.16	0.60	1.40	1.65	0.28	0.74	1.26	1.83

[1]RSBM= raw soybean meal (SBM was replaced by RSBM at 0, 10, 20 and 30 %, equivalent to 0, 30, 60 and 90 g/kg of diet, respectively); NSI= nitrogen solubility index; KOH= protein solubility in 0.2% KOH

More methionine (3.1 and 2.4%), lysine (5.6 and 3.8%) and threonine (1.4 and 3.1%) were observed in the mash and cold-pelleted diets, respectively than in the same diets that were steam-pelleted.

Table 4.4 Effects of the steam-pelleted, cold-pelleted or mash-form diets on the amino acid profiles (mg/g) of starter diets containing increasing levels of RSBM.

	Steam-pelleted				Cold-pelleted				Mash			
RSBM[1] %	**0**	**10**	**20**	**30**	**0**	**10**	**20**	**30**	**0**	**10**	**20**	**30**
***Amino acids*:**												
Taurine	1.2	1.2	1.3	1.2	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.4
Hydroxyproline	2.0	2.6	1.9	2.4	1.7	1.9	2.3	2.5	1.9	2.6	1.9	2.8
Aspartic Acid	22.2	23.6	22.2	22.4	23.3	22.3	23.5	23.8	23.2	24.8	22.0	22.6
Threonine	10.5	10.6	10.5	10.6	11.2	10.4	11.0	11.0	10.7	11.7	10.4	10.0
Serine	10.1	10.4	10.2	10.2	10.5	10.2	10.6	10.7	10.6	11.1	10.1	10.2
Glutamic Acid	40.4	42.2	40.1	40.7	41.9	41.0	42.1	43.0	42.3	43.7	40.1	41.7
Proline	14.8	15.7	14.7	15.2	15.0	14.9	15.4	15.8	15.3	16.2	14.7	15.9
Lanthionine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Glycine	13.4	15.1	13.3	14.5	13.7	13.5	14.6	15.2	13.9	16.0	13.5	15.3
Alanine	13.6	14.2	13.6	13.8	14.1	13.8	14.2	14.5	14.2	15.0	13.7	14.2
Cysteine	3.2	3.3	3.2	3.5	3.3	3.3	3.5	3.5	3.4	3.6	3.3	3.5
Valine	11.3	11.7	11.2	11.2	11.9	11.4	11.7	11.8	11.6	12.6	11.2	11.2
Methionine	6.7	7.4	7.1	7.2	7.5	6.6	7.6	7.4	7.1	8.7	7.0	6.5
Isoleucine	9.5	9.9	9.4	9.5	9.9	9.5	9.8	10.0	9.7	10.5	9.3	9.5
Leucine	21.1	21.4	20.8	20.8	21.8	21.4	21.6	21.8	22.0	22.6	21.0	21.0
Tyrosine	7.2	7.4	7.2	6.8	7.6	7.1	7.4	7.5	7.5	8.3	7.2	7.1
Phenylalanine	11.3	11.8	11.2	11.4	12.0	11.4	11.8	11.9	11.9	12.8	11.3	11.4
Hydroxylysine	0.5	0.5	0.4	0.6	0.5	0.4	0.4	0.5	0.4	0.6	0.4	0.5
Ornithine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Lysine	15.4	16.3	15.4	15.5	16.8	15.4	16.4	16.5	16.0	18.5	15.7	16.1
Histidine	6.2	6.3	6.1	6.0	6.5	6.2	6.4	6.4	6.4	6.8	6.1	6.1
Arginine	15.0	16.6	15.1	15.6	15.6	15.2	16.1	16.9	15.7	17.2	15.1	16.1
Tryptophan	2.6	2.3	2.5	2.3	3.0	2.5	2.2	2.3	2.7	2.3	2.1	2.1

[1]RSBM = raw soybean meal (SBM was replaced by RSBM at 0, 10, 20 and 30%, equivalent to 0, 30, 60 and 90 g/kg of diet, respectively).

4.3.2 The gross response of chicks to the diets

The results of the gross responses of the broiler chicks fed the test diets between hatch and 14 days of age in terms of feed intake (FI), body weight gain (BWG), and feed conversion ratio (FCR) are shown in Table 4.5. Increasing the RSBM supplementation, particularly at 30%, significantly ($p<0.05$) reduced the birds' feed consumption. The birds' BWG decreased ($p<0.001$) with increasing levels of RSBM in the diets. Regardless of the increasing RSBM supplementation, cold-pelleting had beneficial effects ($p<0.001$) on BWG. The birds' feed efficiency decreased ($p<0.05$) with increasing levels of RSBM in the diets.

Table 4.5 Feed intakes (g/b), live weight gain (g/b), and the feed conversion ratio (FCR) of broiler chicks on different diets between hatch and 14 days of age.

Pelleting method	RSBM[1] (%)	Feed intake	Body weight gain	FCR
Steam	**0**	582.4	514.9	1.13
	10	526.0	487.6	1.15
	20	554.7	471.6	1.17
	30	496.9	426.1	1.21
Cold	**0**	556.2	517.1	1.11
	10	562.2	516.1	1.14
	20	554.3	503.6	1.13
	30	544.0	479.8	1.17
Pooled SEM		6.05	4.9	0.01
Main effects:				
RSBM[1] level (%)				
	0	569.3[a]	516.0[a]	1.12[b]
	10	544.1[ab]	501.9[ab]	1.14[ab]
	20	554.5[a]	487.6[b]	1.15[ab]
	30	520.5[b]	459.7[c]	1.19[a]
Pelleting method				
Steam		540.0	482.1[b]	1.17
Cold		554.2	505.4[a]	1.14
Sources of variation				
RSBM level		*	***	*
Pelleting method		NS	***	NS
RSBM x pelleting		0.07	NS	NS

[a,b,c]Means bearing uncommon superscripts within a column are significantly different at *p<0.05; ***p<0.001; [1]RSBM = raw soybean meal (SBM was replaced by RSBM at 0, 10, 20 and 30%, equivalent to 0, 30, 60 and 90 g/kg of diet, respectively); NS= not significant; SEM= pooled standard error of means.

However, changing the pelleting method had no (p>0.05) effect on the feed efficiency. With the exception of FI, which tended to be significant (p=0.07), pelleting method had no (p>0.05) interaction effect on the birds' BWG and FCR. Mortality was negligible; only two of the initial 384 birds died, and this was not treatment-specific

4.3 3 Influence of diets on the development of internal organs

The relative weights of the visceral organs (g/100 g of body weight) of the 14-day-old broiler chicks fed on different diets are shown in Table 4.6. Increasing the RSBM in the diets, particularly in the birds fed diets containing 30% RSBM, increased (p<0.05) the G+P weight. The relative G+P weights of the birds fed the steam-pelleted diets were also increased (p<0.01) compared to the birds fed the cold-pelleted diets.

Table 4.6 Effects of changing the pelleting methods for the chicks' diets containing RSBM on the weight gain of the visceral organs (g/100 g of body weight) at 14 days of age.

Pelleting method	RSBM[1] (%)	G+P	Pancreas	Duodenum	J+I	Heart	Liver	Bursa	Spleen
Steam	**0**	3.3	0.46	1.41	6.1	0.82	3.4	0.23	0.08
	10	3.4	0.48	1.48	6.1	0.88	3.4	0.22	0.09
	20	3.5	0.53	1.52	6.4	0.87	3.4	0.23	0.09
	30	3.8	0.66	1.67	6.7	0.85	3.4	0.27	0.08
Cold	**0**	2.9	0.32	1.29	5.1	0.80	3.2	0.25	0.09
	10	3.1	0.42	1.37	5.7	0.84	3.5	0.21	0.08
	20	3.1	0.56	1.56	5.7	0.85	3.3	0.18	0.08
	30	3.4	0.64	1.60	5.8	0.93	3.4	0.24	0.10
Pooled SEM		0.05	0.02	0.03	0.09	0.02	0.04	0.01	0.00
Main effects:									
RSBM[1] level (%)									
	0	3.1^{b}	0.39^{c}	1.35^{b}	5.6	0.81	3.3	0.24	0.08
	10	3.2^{b}	0.45^{c}	1.43^{b}	5.9	0.86	3.4	0.21	0.08
	20	3.3^{ab}	0.54^{b}	1.54^{b}	6.1	0.86	3.4	0.20	0.08
	30	3.6^{a}	0.65^{a}	1.63^{a}	6.2	0.89	3.4	0.25	0.09
Pelleting method									
Steam		3.5^{a}	0.53	1.52	6.3^{a}	0.85	3.4	0.24	0.08
Cold		3.1^{b}	0.49	1.45	5.6^{b}	0.85	3.4	0.22	0.09
Sources of variation									
RSBM level		*	***	**	NS	NS	NS	NS	NS
Pelleting method		**	NS	NS	***	NS	NS	NS	NS
RSBM x pelleting		NS	NS	NS	NS	NS	NS	p=0.05	NS

[a, b, c] Means bearing uncommon superscripts within a row are significantly different at the levels shown; *p≤0.05; **p<0.1; ***p<0.001; SEM= pooled standard error of the means; [1]RSBM=raw soybean meal (SBM was replaced by RSBM at 0, 10, 20 and 30%, equivalent to 0, 30, 60 and 90 g/kg of diet, respectively); G+P= gizzard + proventriculus (empty); J+I = jejunum + ileum (weighed with the internal contents).

Increasing the RSBM level in the diets resulted in a significant ($p<0.001$) increase in the weight of pancreas; whereas, changing the pelleting methods had no significant effect ($p>0.05$). Increasing the level of RSBM in the diets significantly ($p<0.01$) increased the weight of the duodenum, but the pelleting method had no effect ($p>0.05$). Birds fed the steam-pelleted diets exhibited an increase ($p<0.001$) in the weight of the jejunum + ileum (J+I), although this did not occur with the birds that were fed diets containing 10% RSBM. Increasing the RSBM supplementation had no influence ($p>0.05$) on the J+I weight.

Neither pelleting method nor increasing levels of RSBM had significant ($p>0.05$) effects on the weights of the heart, liver and spleen of the experimental birds. With the exception of the increased weights of the bursa ($p<0.05$), there were no interactions ($p>0.05$) between pelleting

method and rising RSBM levels with the weights of the other internal organs that were assessed.

4.3.4 Mucosal morphometry and protein concentration and pancreatic and jejunal enzyme activities

The results of the mucosal protein concentration and pancreatic and jejunal enzyme activities are shown in Table 4.7. Increasing the amount of RSBM included in the diets had no significant ($p<0.05$) effects on either the mucosal protein concentrations in the pancreas or jejunum. The protein content of the pancreatic tissue of the chicks fed the cold-pelleted diets was higher ($p<0.05$) than that of the chicks fed the steam-pelleted diets, but the jejunal protein content was unaffected.

Chymotrypsin activity in the pancreas was negatively affected ($p<0.01$) by the increasing RSBM level in the diets. However, neither the increasing RSBM level nor pelleting method had any effects ($p>0.05$) on the activities of other enzymes, such as general proteolytic activity, lipase, trypsin, maltase, sucrase, and alkaline phosphatase, or on the mucosal protein concentration of the jejunal mucosa.

The mucosal morphometries at the jejunum are shown in Table 4.8. Although there were no significant differences ($p>0.05$), the birds fed diets containing 0% RSBM had a relatively thicker muscle, longer villi, a larger apparent villus surface area and a higher villus: crypt ratio.

Table 4.7 Effects of the pelleting method on diets containing RSBM on mucosal protein concentrations and pancreatic and jejunal enzyme activities of chicks at 14 days of age.

Pelleting method	RSBM[1] (%)	Protein conc. (mg/g)		Enzyme activities in selected tissues (nmol/mg protein/min)						
				Pancreas				Jejunum		
		Pancreas	Jejunum	Trypsin	Chymotrypsin	GP	Lipase	Maltase	Sucrase	AP
Steam	**0**	32.97	22.3	4.4	4.2	5.0	4.1	1.2	0.23	0.21
	10	31.63	27.2	4.1	4.3	5.0	3.3	1.2	0.25	0.24
	20	37.23	25.0	4.5	4.0	5.1	3.5	1.3	0.23	0.32
	30	34.54	27.1	4.4	3.9	4.9	3.6	1.4	0.22	0.17
Cold	**0**	37.29	25.9	4.3	4.1	5.0	3.1	1.3	0.28	0.18
	10	36.18	27.6	4.5	4.2	4.8	3.4	1.3	0.23	0.27
	20	31.01	20.2	4.4	4.0	5.3	3.1	1.5	0.26	0.36
	30	39.95	23.6	4.5	3.9	4.8	3.6	1.4	0.28	0.33
Pooled SEM		1.7	0.95	0.06	0.05	0.06	0.3	0.04	0.01	0.02
Main effects:										
RSBM[1] level, (%)										
	0	36.9	24.1	4.3	4.2[a]	5.0	3.6	1.29	0.25	0.20
	10	34.2	27.4	4.3	4.3[a]	4.9	3.3	1.26	0.24	0.25
	20	41.4	22.6	4.5	4.0[ab]	5.2	3.3	1.36	0.24	0.34
	30	36.8	25.2	4.4	3.9[b]	4.9	3.6	1.39	0.25	0.25
Pelleting										
Steam		34.2[b]	25.3	4.3	4.1	5.0	3.6	1.28	0.23	0.23
Cold		40.8[a]	24.5	4.4	4.1	5.0	3.3	1.38	0.26	0.28
Sources of variation										
RSBM		NS	NS	NS	*	NS	NS	NS	NS	NS
Pelleting		*	NS	NS	NS	NS	NS	NS	NS	NS
RSBM x pelleting		NS	NS	NS	NS	NS	NS	NS	NS	NS

[a, b, c] Means bearing uncommon superscripts within a row are significantly different at the levels shown; *p≤0.05; **p<0.1; SEM= pooled standard error of the means. GP= general proteolytic; AP= alkaline phosphatase; NS= non-significant; * P<0.05; RSBM= raw soybean meal (SBM was replaced by RSBM at 0, 10, 20 and 30 %, equivalent to 0, 30, 60 and 90 g/kg of diet, respectively).

Table 4.8 Mucosal morphometry (µm) of the jejunum tissue of broiler chicks fed different diets between hatch and 14 days of age.

Pelleting method	RSBM[1] (%)	Muscle Depth	Crypt depth	Villous length	Mucosal depth	AVSA (mm^2)	Villus: Cry
Steam	**0**	163.2	136.2	1,439.2	1575.4	0.06	10.6
	10	115.9	138.7	1,376.5	1,515.4	0.06	9.9
	20	167.6	134.3	1,169.4	1,305.8	0.05	8.6
	30	168.3	133.1	1,342.7	1,475.7	0.05	10.3
Cold	**0**	162.0	135.5	1,349.4	1,484.8	0.06	10.1
	10	144.0	135.0	1,390.7	1,525.7	0.05	10.3
	20	215.7	129.0	1,225.0	1,356.5	0.05	9.3
	30	138.0	116.1	1,257.9	1,390.3	0.05	9.5
Pooled SEM		14.2	2.9	32.7	29.2	0.03	0.3
Main effects:							
RSBM[1] level (%)							
	0	162.6	135.8	1,400.8	1,478.0	0.06	10.4
	10	129.9	136.8	1,385.4	1,521.8	0.06	10.2
	20	191.6	126.5	1,207.6	1,331.1	0.05	9.8
	30	148.1	121.8	1,308.8	1,441.6	0.05	10.0
Steam		152.4	132.3	1,345.0	1,442.0	0.05	10.3
Cold		166.2	128.0	1,322.5	1,456.4	0.05	9.9
Sources of variation							
RSBM		NS	NS	NS	NS	NS	NS
Pelleting		NS	NS	NS	NS	NS	NS
RSBM x pelleting		NS	NS	NS	NS	NS	NS

[a, b, c] Means bearing uncommon superscripts within a row are significantly different at the levels shown; NS= non-significant; SEM= pooled standard error of the means VSA= apparent villous surface area; Villus Cry = villous length to crypt depth ratio; RSBM= raw soybean meal.

4.4 DISCUSSION

4.4.1 Composition and quality of the diets

The results showed that the protein, amino acid and available lysine contents were higher in the cold-pelleted and mash-form diets than the steam-pelleted diets. These results are partially in accord with the findings of various scholars (Swick, 2003, de Coca-Sinova, 2010; Baker *et al.*, 2011) who reported that the protein and amino acid contents of soybean meal could vary as a result of the processing method. The reduction in CP and the AA content in the steam-pelleted diets might be a reflection of their reduced digestibility, due to the impact of the conditioning temperature used before steam-pelleting.

Regardless of the nature of the diets (steam- or cold-pelleted or mash), increasing the RSBM content in the diet increased the TI concentrations in the diets. The TI concentration exceeded

the threshold level for birds stipulated by Hong *et al*. (2004) who reported that diets containing TI concentration exceeding 0.77 mg/g (equivalent to 1,463.0 TIU/g) can affect the performance of non-ruminant animals. The urease activity (UA) and nitrogen solubility index (NSI) increased nearly proportionally to the RSBM level. This result agrees with the findings of Ruiz *et al*. (2004) who reported NSI and UA values for the raw soybean meal to be 98% and 1.99 ΔpH, respectively. The urease activity test measures the increase in pH when SBM is placed in a solution containing urea and is the most widely used method to determine whether SBM has been properly treated by heat and is suitable as poultry feed (CAES, 2013).

The reduction in DEB with the increase in dietary RSBM may have some implications on the birds' performance. There are conflicting reports on the effects of DEB on poultry growth. Borges *et al.* (2003) reported that birds fed diets with 240 mEq/kg DEB exhibited the best BWG and FCR. However, Arantes *et al.* (2013) evaluated the effects of different levels of DEB (ranging between 200 and 320 mEq/kg) in broilers diets and found that the DEB variations had no influence on the birds' performance. It is likely that the rearing environment may have some effect on the birds' response to DEB. In the present study, the birds were housed in climate-controlled rooms.

4.4.2 Gross response to RSBM and the pelleting method

Feed intake and BWG were negatively affected by the increasing RSBM level in the diet. This result agrees with ASA (2004) who reported that birds fed diets containing RSBM consumed less feed, gained less weight and had poorer feed conversion indices. The reduction in BWG is likely the result of the poor feed consumption, but could also be due to interferences of the ANF with nutrient metabolism in the animals. Mirghelenj *et al.* (2013) have shown that the growth performance of broiler chickens was not influenced by diets containing up to 15% extruded full fat soybean.

The effect of the pelleting method was obvious in the present study. For example, birds consumed more of the cold-pelleted feed and gained more weight than birds fed on the same formulation of steam-pelleted feed. These results agree with those of Cutlip *et al*. (2008) who reported that a high steam pressure combined with high conditioning temperature improved the pellet quality, but broilers fed these pelleted diets exhibited decreased feed intake and feed efficiency. Although it is generally believed that a high pellet durability index (PDI) is associated with better bird performance, researchers (Greenwood *et al.,* 2004; Cutlip *et al.* (2008) reported that pellet durability has no bearing on the negative effects of the anti-nutrients.

In contrast to the current results, various scholars (Salmon *et al.*, 1988; Mbugua *et al*., 2002) have reported that steam-pelleted diets were efficiently used by birds. In the current study, the nutrients in the diets might have been denatured by the conditioning temperature used in steam-pelleting. The PDI was improved when the diets were steam-pelleted, particularly in diets containing higher RSBM levels. However, in the current study, the gross responses in terms of the FI, BWG, and FCR of the birds fed the steam-pelleted diets with better PDI values did not improve as expected. These results disagree with those of previous researchers (Choi *et al*., 1986; Serrano *et al*., 2012) who reported that broilers fed quality pellets had improved performance, as well as carcass quality. The unexpected results of the current study might be due to the conditioning temperature used on the diets before steam-pelleting or the nature of the feed ingredients. In a previous study, Erdaw *et al*. (2015a) observed that the pellets from the steam-pelleting process were firmer and harder, particularly those with high RSBM levels.

4.4.3 Visceral organ development

Birds fed diets containing increasing levels of RSBM, particularly at 30%, had heavier gizzard + proventriculus (G+P) than the birds fed the other diets. This result agrees with that of Barletta (2011) who reported that ANF interferes with the birds' digestive system. This might be due to the negative impact of the antinutrients in unprocessed RSBM.

Regardless of the pelleting method, birds fed diets with higher levels of RSBM had a heavier pancreas than those fed the RSBM-free diets. The current result agrees with those of other scholars (Mogridge *et al*., 1996; Beukovic *et al.*, 2012; Mirghelenj *et al.*, 2013) who have reported that the protease inhibitors in RSBM negatively affect the physical development of the pancreas in birds. Mayorga *et al.* (2011) associated this observation with the increasing urease activity in the birds' diets and with some effects on the duodenum. The enlargement in the pancreas may be because the organ is striving to produce adequate enzymes to digest the nutrients and overcome the inhibition of these enzymes by the dietary ANF.

The current study shows that regardless of the increase in RSBM levels, birds fed steam-pelleted diets exhibited increased G+P weights than the birds fed the same formulation of cold-pelleted diet. This is similar to the reports by Rezaeipour and Gazani (2014) who found that the gizzard weight was influenced by the form of the feed. This is most likely a result of the textural changes in the diets.

However, changing the pelleting temperature had no effect on the weight of pancreas. This result disagrees with that of Svihus and Zimonja (2011) who reported that the conditioning

temperature used prior to pelleting the diets could inactivate the proteinaceous anti-nutrients. The reason why the pelleting method had no effect on the pancreas weight gain in this study may be that the conditioning temperature used during steam-pelleting was too low to inactivate the protease inhibitors and other antinutrients in the diet.

Regardless of the pelleting methods, birds fed diets containing high levels of RSBM, particularly the 30%, had a heavier duodenum than the birds fed the other diets. This finding supports that of Mogridge *et al.* (1996) who reported that the consumption of raw soybeans increased the sizes of the pancreas and duodenum in birds. The duodenum is anatomically located near the pancreas; therefore it may also be indirectly affected by the negative impact of the protease inhibitors.

4.4.4 Tissue protein content, digestive enzyme activities, and mucosal morphometry

Although the pancreatic mucosal protein concentration of the chicks responded to the pelleting method, the increasing levels of RSBM in diets had no significant effect on either the pancreatic or jejunal protein concentrations. The reason for the lack of differences might be because all of the diets were formulated to be iso-nitrogenous and iso-caloric.

With the exception of chymotrypsin activity, which was affected by the increasing RSBM levels, the activities of the other pancreatic enzymes were not affected by the amount of RSBM included in the diet or by changing the pelleting method. This result indicates that although the weight of the pancreas was affected by the RSBM, this diet had no impact on the secretion of digestive enzymes. Similar results were observed in the jejunum, which did not show changes in enzyme activity in response to the RSBM or pelleting method.

Although there was no significant difference, birds fed diets containing no RSBM had a thicker intestinal muscle, longer villi, a larger apparent villus surface area, and an increased villus to crypt ratio than those fed the other diets. To the best of our knowledge, there have been no previous studies on the effects of RSBM on the intestinal mucosa.

4.5 CONCLUSION

Although the test diets contained RSBM, the chemical composition of both the cold-pelleted and the mash diets appear to be better than those of the steam-pelleted diets. Regardless of the processing method (steam- or cold-pelleting or mash) applied to the diets, increasing the RSBM level alone greatly enhanced the concentrations of trypsin inhibitors and other ANF above the threshold levels, and the subsequent impact of feeding diets was reflected in the reduced

performance of the birds. Steam-pelleting alone might not be sufficient to reduce the negative impact of ANF on birds.

Increasing the dietary contents of RSBM reduced the feed intake, body weight gain, and feed efficiency. However, cold-pelleting tended to improve the BWG of the birds. On the other hand, the high levels of RSBM in the diets led to an increase in the weights of the gizzard+provetriculus, duodenum and pancreas. However, most of these changes did not affect digestive function or morphometry. Subsequent studies are planned to investigate the benefits of supplementing similar diets with high potency microbial enzymes.

CHAPTER 5: REPLACEMENT VALUE OF RAW FULL-FAT SOYBEAN FOR COMMERCIAL SOYBEAN MEAL WITH SUPPLEMENTATION OF HIGH-IMPACT PROTEASE FOR BROILER CHICKENS

ABSTRACT

This study was conducted to evaluate the effects on the performance of broiler chickens of diets containing graded levels of raw full-fat soybean meal (RSBM) and supplemented with increasing levels of microbial protease. A 3 x 3 factorial study, involving three levels of RSBM (0, 10 or 20%) and three levels of protease (0.1, 0.2 or 0.3 mg/kg), with six replication per treatment and nine birds per replicate was employed. The birds were housed in cages in a climate-controlled room and fed on starter (1-10 d), grower (11-24 d) and finisher (25-35 d), corn-soybean-based diets formulated to Aviagen standard for Ross 308 broiler. The analysed value of trypsin inhibitors (TI) in the diets ranged between 1730.5 and 9913.2 TIU/g. Increasing the levels of RSBM in the diets reduced ($p<0.01$) the feed intake (FI) during the period from 1 to 35 d. Except in the early periods, increasing the levels of RSBM had no significant ($p>0.05$) effects on body weight gain (BWG) of the birds. The BWG was improved during the periods of 1-10 d ($p<0.01$), 11-24 d ($p<0.05$) and 1-35 d ($p=0.06$) due to increasing levels of protease were added to the diets. Because of the increasing levels of RSBM, the feed efficiency was decreased ($p<0.001$) during the period from 1-10 d, but the inverse ($p<0.01$) was true while increasing the protease levels in the diets during the period from 1 to 24 d. There was no difference ($p>0.05$) in the mortality of the birds. The weight of the small intestine increased ($p<0.05$) due to increasing the level of RSBM, but was reduced ($p<0.05$) when protease was added to the diets during the period from 1 to 10 d. Increasing the level of RSBM increased the weight of the pancreases 10 d ($p<0.000$), 24 d ($p<0.001$) and 35 d ($p<0.05$). There were no significant ($p>0.05$) interaction effects, however, on the weight of the pancreas in any of the assessed periods. The weights of the drumstick and wing of the bird were similarly increased ($p<0.05$) by increasing levels of protease in the diets. Increasing the level of RSBM in the diets reduced the apparent ileal digestibility (AID) of CP and most of the amino acids (AA) at 24 day of age, but these were not statistically influenced ($p>0.05$) when the diets were supplemented with protease. Neither increasing the supplementation of RSBM nor protease had an influence ($p>0.05$) on the DNA concentration of the pancreas. Increasing the level of RSBM in the diets decreased ($p<0.01$) the pancreatic protein content, but this was increased ($p<0.05$) when protease was added to the diets during the early period (1-10 d). Neither increasing RSBM nor protease had an influence ($p>0.05$) on the contents of the mucosal

proteins and the activities of any endogenous digestive enzymes during the period from 1 to 10 d. Increasing the level of protease improved the activities of the pancreatic digestive enzymes, including trypsin ($p<0.05$), chymotrypsin ($p<0.01$) and general proteolysis ($p<0.05$), and also tended to improve ($p=0.06$) the lipase. The pancreatic protein content increased at low level of protease supplementation (0.1 g/kg), but the activity of alkaline phosphatase in the jejunum decreased with level of RSBM. It can be concluded that commercial SBM might be replaced by up to 20% by RSBM, provided that the right microbial protease is supplemented for broilers.

5.1 INTRODUCTION

It is a well-established fact that soybean meal (SBM) is an excellent feed ingredient used to formulate diets (Banaszkiewicz, 2011; Pettersson and Pontoppidan, 2013) and that it is the most important source of nutrients for poultry (Newkirk, 2010; Dei, 2011). However, in addition to fluctuation in supply and seasonal inaccessibility in some parts of the world, the price of SBM has been increasing over the years (Farrell, 2005; Shi *et al.,* 2012; Pettersson and Pontoppidan, 2013). The nutrients in SBM are also sometimes denatured when excessive heat is used during oil extraction or further processing. It is therefore more effective to use full-fat SBM to replace both commercial SBM and the oil in the diets for broilers (Popescu and Criste, 2003). Full-fat soybean meal can be prepared either after treating by heat or from the raw soybean seed. The problem with raw full-fat soybean meal, however, is the contents of anti-nutritional factors (ANF) (Chen *et al.,* 2013b). Friedman and Brandon (2001) reported that protease inhibitors constitute approximately 6% of soya protein while Pettersson and Pontoppidan (2013) stated that the amount of ANF altogether constituted approximately 5% of the CP fraction of SBM, and the large amounts of ANF are concentrated in the hull fractions of the seeds.

There are 3 well-known ANFs in soybeans-protease inhibitors (TI), lectins and phytate (Chen *et al*., 2013b; Pettersson and Pontoppidan, 2013), among which the most important in raw soybean meals are the TI (Newkirk, 2010; Dourado *et al*., 2011). Trypsin inhibitors in soybeans are a type of protein that can significantly reduce the digestibility and utilisation of proteins and amino acids (Rawlings *et al.,* 2004). Protein digestibility could be reduced by a multifaceted action of ANF, such as by binding various nutrients and increasing the gut viscosity (Ao, 2011). Due to the activity of TI, nitrogen retention can be negatively affected, and the metabolic nitrogen excretion is also increased (Banaszkiewicz, 2011; Dourado *et al.,*

2011). Hong *et al*. (2004) reported that diets containing TI concentration of 0.77 mg/g or more could reduce the performance of non-ruminant animals.

Some of these ANF can be reduced or eliminated by application of various methods. Pettersson and Pontoppidan (2013) suggested that de-hulling the soybean seed could reduce the levels of ANF substances in the meals. Another method that has been suggested to reduce ANF in grains, including soybeans, is grinding. Treating the soybean seed by heat prior to feeding is one of the most common approaches to reducing the risks of ANF (Cromwell, 1999; Akande and Fabiyi, 2010; Mayorga *et al*., 2011). However, heat treatment depending on the temperature, pressure and the duration may negatively influence the quality of the product (Căpriţă *et al.*, 2010; Newkirk, 2010).

Therefore, investigations into other techniques that can improve the nutritional value of raw soybeans for non-ruminant animals continue to attract the attention of researchers (Oluwafemi, 2009). Research scholars (Costa *et al*., 2008; Dosković *et al*., 2013) have suggested that supplementation of poultry diets with exogenous enzymes may be effective.

The concept of using exogenous microbial enzymes in the animal feed industry has already been established, and this method is used on a routine basis to improve the nutritive value of feed ingredients (Pettersson and Pontoppidan, 2013). However, adding selected feed enzymes into diets to reduce the negative impact of protease inhibitors and lectin on non-ruminant animals is a recent phenomenon (Enneking and Wink, 2000; Ao, 2011). Microbial proteases are protein-digesting enzymes that are used in pig and poultry nutrition to break down the stored proteins and proteinaceous anti-nutrients in various plant materials (Barletta, 2011; Ao, 2011). The use of new-generation enzymes is becoming one promising approach to enhance the productivity of non-ruminant animals through improvement in feed quality (Adeola and Cowieson, 2011).

Phytate is another ANF in soybeans that has the capacity to form complexes with minerals, proteins and starch components and thereby reduces their availability to the animal (Singh, 2008; Nahashon and Kilonzo-Nthenge, 2013). Supplementation with microbial phytase, however, enables an increase in the digestibility of CP and amino acids in plant-based proteins (Barletta, 2011; Guggenbuhl *et al*., 2012). The objectives of this study were to determine the composition of ANF in the diets and to evaluate the effects of microbial protease

supplementation on diets containing high levels of TI, with the possibility of improving the performance of broiler chickens.

5.2 MATERIALS AND METHODS

5.2.1 Experiment and diets

The experiment was conducted at the Animal House of the University of New England (UNE). It was approved by the Animal Ethics Committee (Authority No: AEC14-005) prior to commencement.

This experiment was designed with a 3 x 3 factorial arrangement, involving 3 levels of RSBM (0, 10 or 20%, equivalent to 0, 30 and 60 g/kg of diet, respectively), replacing the commercial SBM and 3 levels of mono-component protease (Ronozyme® ProAct) (DSM Nutritional Products, Australia Pty. Ltd) at 0.1, 0.2 or 0.3 g/kg. Phytase (1000 FYT/kg) (DSM Nutritional Products, Australia Pty. Ltd) was uniformly supplemented across all the diets (Tables 5.1, 5.2 and 5.3), and each was replicated six times with nine birds per replicate.

The potential efficacy of Ronozyme® ProAct has been demonstrated at a dose level of 15000 PROT kg^{-1} feed in chickens for fattening (EFSA, 2009). The activity of 6-phytase is expressed in phytase units (FYT). According to the producer, one FYT unit is defined as the amount of enzyme that releases one μmol of inorganic phosphate from phytate per minute under reaction conditions with a phytate concentration of 5.0 mM at pH 5.5 and 37°C (EFSA, 2012).

5.2.2 Animal husbandry and sampling

A total of 486 day-old Ross 308 male broiler chicks, with an average body weight of 40.24±0.097 g, were obtained from a local commercial hatchery (Baiada Poultry Pty. Ltd., Tamworth, Australia). To maintain uniformity, the chicks were weighed and randomly allocated into 54 multi-tiered brooder cages (600 x 420 x 23 cm), with nine chicks per cage. The birds were housed from 1 to 35 days of age in cages of climate-controlled rooms.

Every pen was equipped with a feeder and two nipple drinkers. The feeders were scrubbed and cleaned before providing the diets. The drinkers were also checked every time to determine whether they were working properly and they were also cleaned whenever they were dirty. The excreta trays were scrubbed and cleaned whenever they were filled with excreta materials.

Table 5.1 Ingredients and composition and basal starter (0-10 d) diets (as-fed basis).

	RSBM[1] (%)		
	0	10	20
Ingredients (g/kg)			
Corn (rolled)	598.1	595.8	614.9
Soybean meal	300.0	270.0	240.0
Raw soybean meal	0	30.0	60.0
Meat meal	53.0	62.8	43.7
Limestone	10.4	10.0	10.0
Dical Phos	6.5	4.0	7.1
Canola Oil	14.0	10.5	6.4
TiO_2	5.0	5.0	5.0
L-Lysine	3.0	3.0	3.5
DL-Methionine	3.0	2.2	2.8
Salt	2.1	2.0	2.0
L-Threonine	1.0	1.9	1.7
Sodium bicarbonate	2.0	1.0	1.1
Trace minerals, 0.75kg/mt[1]	0.75	0.75	0.75
Choline Cl	0.50	0.50	0.50
Vitamins, 0.5 kg/mt[2]	0.50	0.50	0.50
Phytase	0.10	0.10	0.10
Nutrients (g/kg)			
ME poultry (MJ/kg)	12.59	12.59	12.59
Crude protein	228.6	231.0	220.0
Crude fat	37.4	38.9	37.2
Arginine	14.4	14.6	13.8
Lysine	14.0	14.2	13.9
Methionine	6.2	5.4	5.8
Methionine + cystine	9.4	8.6	8.9
Threonine	8.9	9.0	9.4
Calcium	11.7	11.7	10.7
Phosphorus (avail)	5.4	5.2	5.0
Sodium	1.9	1.9	1.6
Chloride	2.6	2.6	2.6
Choline	1.5	1.4	1.3

[1]Trace mineral supplied per kilogram of diet: Cu (sulphate), 16 mg; Fe (sulphate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulphate and oxide), 120 mg; Zn (sulphate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg. [2]Vitamin supplied per kilogram of diet: Vitamin A (retinol), 12000 IU; Vitamin D_3 (cholecalciferol), 5000 IU; Vitamin E (tocopheryl acetate), 75 mg, Vitamin K (menadione), 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; Vitamin B_{12} (cyanocobalamin), 16 µg; biotin, 200 µg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg. [1]RSBM=raw soybean meal (SBM was replaced by RSBM at 0, 10 and 20%, equivalent to 0, 30 and 60 g/kg of diet, respectively) supplemented with 0.1, 0.2 of 0.3 g/kg protease..

Table 5.2 Ingredients and composition and basal grower (10-24 d) diets (as-fed basis).

	RSBM (%)		
	0	**10**	**20**
Ingredient s (g/kg)			
Corn (rolled)	607.4	606.7	605.9
Soybean meal	300.0	270.0	240.0
Raw soybean meal	0	30.0	60.0
Meat meal	25.0	30.0	35.0
Canola Oil	34.7	31.7	28.6
Limestone	8.9	8.3	7.9
Dical Phos	7.5	6.9	6.3
TiO2	5.0	5.0	5.0
DL-Methionine	3.3	3.3	3.4
Salt	2.5	2.5	2.5
L-Lysine HCl	2.4	2.3	2.2
L-Threonine	0.8	0.8	0.8
Trace minerals, 0.75 kg/mt[1]	0.75	0.75	0.75
Sodium bicarbonate	0.76	0.70	0.60
Vitamins, 0.5 kg/mt[2]	0.50	0.50	0.50
Choline Cl	0.50	0.50	0.50
Phytase	0.10	0.10	0.10
Nutrients (g/kg)			
ME poultry (MJ/kg)	13.18	13.18	13.18
Crude protein	212.4	212.5	212.7
Crude fat	55.1	56.6	58.1
Arginine	13.4	13.4	13.5
Lysine	12.7	12.7	12.7
Methionine	6.3	6.3	6.3
Methionine + cystine	9.4	9.4	9.4
Threonine	8.3	8.3	8.3
Calcium	9.0	9.0	9.0
Phosphorus (avail)	4.5	4.5	4.5
sodium	1.6	1.6	1.6
Chloride	2.6	2.6	2.6
Choline	1.3	1.3	1.3

[1,2]Composition as in Table 5.1**;** the dietary treatments were basal diets (SBM was replaced by RSBM at 0, 10 and 20%, equivalent to 0, 30 and 60 g/kg of diet, respectively) supplemented with 0.1, 0.2 of 0.3 g/kg protease. 1RSBM=raw soybean meal.

Table 5.3 Iingredients and composition and basal finisher (24-35 d) diets (as-fed basis).

	RSBM (%)		
	0	**10**	**20**
Ingredients (g/kg)			
Corn (rolled)	612.4	611.8	617
Soybean meal	300.0	270.0	240.0
Raw soybean meal	0.0	30.0	60.0
Canola oil	42.6	39.8	37.1
Meat meal	15.0	15.0	15.0
Limestone	9.3	10.0	9.4
Dical Phos	7.9	9.4	8.7
TiO_2	5.0	5.0	5.0
DL-Methionine	3.0	3.5	3.0
Salt	2.0	2.0	2.0
Trace minerals, 0.75 kg/mt[1]	0.75	0.75	0.75
Choline Cl 70	0.5	0.5	0.5
Vitamins, 0.5 kg/mt[2]	0.5	0.5	0.5
L-Lysine HCl	0.5	0.5	0.5
L-Threonine	0.2	0.5	0.2
Sodium bicarbonate	0.2	0.2	0.2
Phytase	0.1	0.1	0.1
Nutrients (g/kg)			
ME poultry(MJ/kg)	13.39	13.39	13.39
Crude protein	204.6	202.1	199.6
Crude fat	61.9	63.3	64.6
Arginine	13.0	12.9	12.8
Lysine	10.9	10.8	10.7
Methionine	5.9	5.8	5.8
Methionine + cysteine	8.9	8.8	8.7
Threonine	7.5	7.5	7.4
Calcium	8.5	8.5	8.5
Phosphorus (avail)	4.2	4.2	4.2
Sodium	1.2	1.2	1.2
Chloride	1.9	1.9	1.9
Choline	1.4	1.3	1.3

[1,2]Composition as in Table 5.1; the dietary treatments were basal diets (SBM was replaced by RSBM at 0, 10 and 20%, equivalent to 0, 30 and 60 g/kg of diet, respectively) supplemented with 0.1, 0.2 of 0.3 g/kg protease.
[1]RSBM=raw soybean meal.

The temperature of the rooms was set at 33^{o}C for the first two days with relative humidity ranged between 49 and 60%. This temperature was then gradually reduced to 24^{o}C at 19 days age and this was maintained for the remaining study periods. For the first 2 days, 24-h light, with intensity of 20 lux was provided. This was then reduced to 23 hr for the next 6 consecutive days, followed by 20-h light, with intensity of 10 lux for the remaining days. Feed was provided *ad libitum* (crumbled for starters), and the birds had free access to water. The birds were

provided with starter (0-10 d), grower (10-24 d) and finisher (24-35 d) diets (corn-soybean based) formulated to Aviagen standards for Ross 308 broiler. The leftover feed and live body weight of the birds were recorded at 10, 24 and 35 days of age (at the times of sampling). Mortality was recorded whenever it occurred.

5.2.3 Internal organs and tissue sampling

On the 10^{th}, 24^{th} and 35^{th} days of age, one bird per replicate was randomly selected and killed using cervical dislocation. The weights of the internal organs, such as the gizzard, proventriculus, small intestine, pancreas, liver, spleen and bursa, were recorded. Additionally, at the age of 35 d, the weight of the pancreas was also recorded from a randomly selected bird per cage, and these data were used to compare the magnitude of change in the organs over the study periods under the effect of dietary TI.

Pancreatic tissue sampling: The entire pancreas was collected from the sampled birds from each cage on the 10^{th} and 24^{th} d, then snap-frozen in liquid nitrogen and stored in a freezer (-20°C) until the next analysis of the tissue protein contents and the activities of the digestive enzymes. Pancreas was also collected from another sampled bird per cage on the 24^{th} and 35^{th} d (sampling days), snap-frozen in liquid nitrogen and stored in the freezer for the next evaluation of DNA concentration.

Jejunal tissue sampling: Samples from the proximal part of the jejunum were collected, snap-frozen in liquid nitrogen and then transferred into a freezer (-20°C) and used for analysis of the tissue protein content and the activities of the digestive enzymes. Another tissue sample was also collected from the same position (proximal) of the jejunum, just next to the previous samples (at 10 and 24 d) and fixed in formalin solution for the analysis of histological parameters.

Ileal digesta sampling: On day 24, samples of the ileal digesta were collected from at least 2 birds (depending on the amounts of digesta collected) per cage. The digesta were pooled and placed in ice and then stored in a freezer (-20°C) until analysis for nutrient contents, which were then used to calculate digestibility.

5.2.4 Gross response of the birds

On days 10, 24 and 35, the live weights of both the sampled and remaining birds (on each sampling day) were recorded per cage. The leftover feed was also weighed to calculate the feed intake (FI) of the birds for each phase, and the feed conversion ratio (FCR) was computed for each cage using the body weight gain (BWG) and the FI. The meat yield was measured at 35 day of age. After randomly selecting 2 birds from each cage, the birds were humanely killed,

scalded and plucked. The head, legs and all visceral organs of the birds were then removed, and the dressed carcass was weighed. The main carcass parts, such as the breast, drumsticks, thighs, wings and neck, were then cut out and weighed to evaluate the average meat yield.

5.2.5 Chemical analysis

Values for the GE, DM and ash contents of the diets and the ileal digesta were determined following the same procedures described in Section 3.2.2. The concentration of CP was determined following the same procedures described in Section 3.2.2.1.

The amino acid analysis was carried out by the Australian Proteome Analysis Facility (APAF), Macquarie University, NSW, Australia. Amino acid concentrations in the diet and ileal digesta samples were determined using pre-column derivatization amino acid analysis with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) followed by separation of the derivatives and quantification by reversed phase high performance liquid chromatography (HPLC), according to Cohen and Michaud (1993), and Cohen (2000).

***Concentration of anti-nutritional factors in the diets*:** The feed quality parameters, such as the available lysine, AA, KOH, TI, UA and NSI (in the diets containing RSBM), were evaluated using the same methods described in Section 4.2.2.

5.2.5.1 Analysis of the titanium contents in the samples and the ileal crude proteins and amino acid digestibility

The concentration of titanium (Ti) in the ileal digesta and diets was determined using the method described by Short *et al*. (1996). Two hundred and 100 mg samples of the freeze-dried ileal digesta and diets, respectively, were weighed in duplicates. These samples were ashed in porcelain crucibles for 13 hours at 580 °C, and then, after cooling, 5 mL of 7.4 M sulphuric acid were added. The mixture (contents) was then boiled on a heating plate for 30 minutes at 200 ^{0}C, and then the temperature was raised to 250 ^{0}C for another 30 minutes until the sample was completely dissolved. After cooling, the mixture was quantitatively transferred into a 50 mL volumetric flask through a filter paper (Whatman 541, hardened ashless, 90 nm ø Cat No. 1541 090, Whatman International Ltd., Maidstone, England). Then, 10 mL of hydrogen peroxide (30%, v/v) were added to each flask, and the content was further diluted up to 50 mL with Milli-Q water and thoroughly mixed. The colour of the solution in the flasks turned to orange, the intensity of which was proportional to the concentration of Ti. An aliquot of the solution was obtained alongside the standard solutions, and the absorbance was read using a Hitachi 150-20 UV spectrophotometer (Hitachi Science System Ltd., Ibaraki, Japan) at 410

nm. The Ti content was determined from the standard curve and converted to mg/g of the sample. The values of the nutrient and the Ti marker were used to calculate the ileal digestibility as follows:

$$\text{Digestibility coefficient} = 1 - \frac{\text{Digesta nutrient (g/kg)/ digesta Ti (g/kg)}}{\text{Diet nutrient (g/kg)/ diet Ti (g/kg)}}$$

5.2.6 Tissue processing and analysis

The pancreas and jejunum were processed following the same procedure described in Section 4.2.4. The contents of the pancreatic and jejunal tissue proteins were also analysed using the same method provided in Section 4.2.5.

***Pancreatic enzyme activities*:** Pancreatic enzyme activities, such as trypsin amidase, chymotrypsin amidase, general proteolysis and lipase, were determined following the same procedures described in Section 4.2.4.

***Activities of jejunal enzymes**:* The activities of the jejunal enzymes, such as alkaline phosphatase, sucrase and maltase, were determined using the methods described in Section 4.2.5.

5.2.7 Mucosal morphometry of the jejunum

The parameters of the mucosal morphometry were measured following the method described in the previous chapter (Section 4.2.5).

5.2.8 Determination of DNA contents of the pancreas

The concentration of DNA in the pancreas was determined following the same procedure described in the Kits (Isolate II Genomic DNA kit, Bioline, Pty Ltd, Alexandria, NSW 1435, Australia). Approximately 180 µL of buffer GL and 25 µL of proteinase K solution were pipetted into Eppendorf tubes containing 25 mg of the samples, and the mixture was then vortexed and incubated for 2 hrs at 56 °C for pre-lysis. Two hundred µL of lysis buffer G3 were added to the lysed mixture and then vigorously vortexed. The mixture was further incubated for 10 minutes at 70 ° C, followed by a brief and vigorous vortexing of the samples before and after adding ethanol (210 µL). Subsequently, the content was quantitatively transferred into a DNA spine-column tube (a tube placed into a 2 mL collection tube) and centrifuged at 11000 xg for 1 min. After including GW1 (500 µL) and GW2 (600 µL), one next to the other, the DNA in the tissue was washed by centrifuging at 11000 xg for 1 min each. The residual ethanol was removed from the tissues by centrifuging at 11000 xg for 1 min, leaving a dry silica membrane. The collection tubes were replaced by Eppendorf tubes (1.5

mL) and approximately 100 µL of preheated (70°C) Elusion Buffer G were added onto the silica membrane and centrifuged at 11000 xg for 1 min. The eluted DNA was then determined by the ratio of spectrophotometric absorbance of the sample at 260 nm to that of 280 nm (NanoDrop® ND-1000). The DNA content was calculated as follows:

DNA mg/g= (DNA concentration (ng)/ µL /wt of sample (g))* Elusion (µL).

5.2.9 Statistical analysis

Descriptive statistics and general linear model (GLM) were used to analyse the data using Minitab software version 17 (Minitab, 2013). The data were analysed using general linear model (GLM) to evaluate the RSBM and enzyme supplementations as main factors and their interactions. The differences were considered to be significant at $p < 0.05$, and the significant differences between mean values were also separated using the Duncan's test.

5.3 RESULTS

5.3.1 Composition and quality of diets

As shown in Table 5.4, the concentration of TI in the diets containing graded levels of RSBM ranged between 1747 and 9913.2 TIU/g. The highest TI value was found in the diets containing 20% RSBM. Urease activity in the diets ranged between 0.16 and 1.4 ΔpH. The value of NSI proportionally increased in the diets with increasing level of RSBM, but there was no consistency in KOH across the dietary treatment groups.

Table 5.4 Effect of supplementation of raw soybean on the diets and their qualities (g/kg).

	Diets		
RSBM[1] %	0	10	20
Available lysine	16.2	14.8	15.6
NSI	155.3	169.0	206.1
KOH	670.3	502.5	603.4
Trypsin inhibitor (TIU/g)	1747	5881.2	9913.2
Urease activity (ΔpH)	0.16	0.60	1.40

NSI= nitrogen solubility index; KOH= protein solubility; TIU= trypsin inhibitors units; [1]RSBM= raw soybean meal (SBM was replaced by RSBM at 0, 10 and 20%, equivalent to 0, 30 and 60 g/kg of diet, respectively).

5.3.2 The gross response to the diets

Results for the gross response of the birds, in terms of FI, BWG and FCR, are shown in Table 5.5. During the period from 1 to 35 d, feed consumption significantly ($p<0.01$) decreased with increasing level of RSBM in the diets, but this had no influence ($p>0.05$) during the early periods (1-10 d or 1-24 d). Increasing the level of protease positively influenced ($p<0.05$) the FI over the entire periods.

Table 5.5 Feed intake (g/b), body weight gain (g/b) and feed conversion ratio of broiler chickens between hatch and 10, 24 or 35 days of age.

RSBM[1] (%)	Protease (g/kg)	Feed intake (d)			Body weight gain (d)			FCR (d)		
		1-10	1-24	1-35	1-10	1-24	1-35	1-10	1-24	1-35
0	**0.1**	253.6	1895	3898	238.1	1411	2453	1.08	1.36	1.60
	0.2	261.6	1754	3670	242.8	1414	2475	1.07	1.29	1.48
	0.3	267.0	1818	3776	245.1	1442	2562	1.07	1.25	1.47
10	**0.1**	264.4	1839	3581	231.7	1383	2349	1.09	1.33	1.53
	0.2	267.6	1747	3419	241.8	1349	2351	1.10	1.30	1.46
	0.3	263.3	1734	3502	242.8	1408	2475	1.07	1.24	1.42
20	**0.1**	259.9	1736	3458	219.7	1356	2348	1.16	1.32	1.48
	0.2	245.7	1821	3586	220.1	1361	2437	1.14	1.31	1.47
	0.3	271.1	1786	3529	235.9	1419	2485	1.14	1.26	1.45
Pooled SEM		2.0	15.4	34.7	1.6	8.6	21.4	0.01	0.01	0.02
Main effects										
0		260.7	1822	3781^{a}	242.0^{a}	1423	2499	1.07^{b}	1.30	1.52
10		265.1	1774	3501^{b}	238.8^{a}	1380	2392	1.08^{b}	1.29	1.47
20		258.9	1781	3525^{b}	225.2^{b}	1379	2424	1.15a	1.29	1.47
	0.1	259.3	1823	3631	229.8^{b}	1384^{ab}	2381^{b}	1.11	1.34^{a}	1.54
	0.2	258.3	1774	3552	234.9^{ab}	1375^{b}	2421^{b}	1.11	1.30^{ab}	1.47
	0.3	267.1	1779	3603	241.3^{a}	14223^{a}	2509^{a}	1.09	1.25^{b}	1.45
Sources of variation										
RSBM		NS	NS	**	***	NS	NS	***	NS	NS
Protease		NS	NS	NS	**	*	*	NS	**	NS
RSBM x protease		NS	NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscript within a column are significantly different at * <0.05; **p<0.01; ***p<0.001; NS= nonsignificant;1RSBM = raw soybean meal; SBM was replaced by RSBM at 0, 10 and 20%, equivalent to 0, 30 and 60 g/kg of diet, respectively; SEM= pooled standards error of means

Table 5.6 Meat yield (g) of broiler chickens fed on different diets at 35 d of age.

RSBM[1] %	Protease g/kg	Dressed %	Breast	Thigh	Drumstick	Wing	Neck
0	**0.1**	75.8	676	266	245	184	110[a]
	0.2	77.6	712	284	255	181	109[ab]
	0.3	75.5	723	284	263	201	108[ab]
10	**0.1**	75.6	656	271	247	188	105[ab]
	0.2	75.0	683	273	242	180	116[a]
	0.3	75.8	673	266	253	188	97[bc]
20	**0.1**	76.4	654	262	233	189	90[c]
	0.2	75.1	655	260	250	186	104[ab]
	0.3	75.1	698	276	253	192	107[ab]
Pooled SEM		0.31	8.22	2.83	1.92	1.33	1.72
Main effects							
0		76.3	704	278	254	189	109
10		75.5	671	270	248	185	106
20		75.6	669	266	245	188	101
	0.1	75.9	662	266	242[b]	186[b]	101
	0.2	75.9	683	272	253[a]	183[b]	110
	0.3	75.5	699	276	253[a]	194[a]	104
Sources of variation							
RSBM		NS	NS	NS	NS	NS	NS
Protease		NS	NS	NS	*	*	*
RSBM x protease		NS	NS	NS	NS	NS	*

[a,b,c] Means bearing uncommon superscript within a column are significantly different at NS= non-significant;*p<0.05; SEM= pooled standards error of means; [1]RSBM = raw soybean meal (SBM was replaced by RSBM at 0, 10 and 20%, equivalent to 0, 30 and 60 g/kg of diet, respectively).

The BWG of the birds was negatively affected (p<0.001) with increase in the inclusion rate of RSBM in the early assessed period (1-10 d). Due to increasing the level of RSBM, the BWG decreased by up to 3.1% during the period from 1 to 24 days of age, but this was not significant (p>0.05). Supplementation with increasing levels of protease in the diets, particularly at 0.3 g/kg, improved the BWG during 1 -10 d (p<0.01 and 1- 24 d p<0.05).

The BWG also tended (p=0.06) to improve over the period from 1 to 35 d. The birds fed on diets containing 20% RSBM poorly utilised (p<0.01) the feeds during the early assessed period

(1-10 d).When the commercial SBM was replaced by increasing levels of RSBM, particularly at 20%, the feed efficiency was decreased by between 0.8 and 3.3%, but was not significantly different ($p>0.05$) during the periods from 1 to 24 d or 1 to 35 d.

Supplementation with increasing rate of protease in the diets improved ($p<0.01$) the feed efficiency during the 1-24 d period. The interaction between the main factors (protease x RSBM) had no significant influence ($p>0.05$) on the FI, BWG or FCR during any of the assessed periods (1-10 d, 1-24 d or 1-35 d). There was no significant ($p>0.05$) difference in the mortality of the birds between treatment groups during the entire study period.

The values for the meat yields are shown in Table 5.6. Replacing commercial SBM with increasing levels of RSBM in the diets had no significant effects ($p>0.05$) on any meat part yield at 35 d. Increasing the level of protease in the diets, particularly at 0.3 g/kg, significantly improved ($p<0.05$) the weights of the drumsticks, wings and neck of the birds. However, other measures, such as the dressed percentage, breast and thighs were not significantly ($p>0.05$) affected by increasing the inclusion rate of protease in the diets. The neck weight was significantly ($p<0.05$) affected by an interaction between the main factors (protease x RSBM), but this interaction had no ($p>0.05$) influence on any other meat parts at 35 d.

5.3.3 Development of internal organs

As the results are shown in Table 5.7, except for the small intestine ($p<0.05$), increasing the levels of RSBM in the diets had no influence ($p>0.05$) on the weight of any internal organs, such as the gizzard + proventriculus (G+P), liver, heart, bursa and spleen, during the starter phase (1-10 d). Supplementation with microbial protease, particularly at 0.3 g/kg, decreased ($p<0.01$) the weight of the small intestine. Increasing the level of protease in the diets had no influence ($P>0.05$) on the weight of the other internal organs. The weight of the bursa was significantly influenced ($p=0.05$) by the interaction between RSBM x protease, but this interaction had no significant influence ($p>0.05$) on any other weights of the internal organs at 10 d. Neither inclusion of RSBM nor protease supplementation had any effect ($p>0.05$) on visceral organ weights at 24 d.

Table 5.7 Weight of visceral organs (g/100 g of body weight) of broiler chickens on different diets at 10 days of age.

RSBM[1] (%)	Protease (g/kg)	S. intestine	Liver	G+P	Heart	Bursa	Spleen
0	**0.1**	8.5	4.3	4.0	0.98	0.15	0.09
	0.2	8.0	4.0	3.7	0.89	0.15	0.08
	0.3	7.0	4.3	3.7	0.90	0.16	0.08
10	**0.1**	8.7	4.3	3.8	0.10	0.20	0.09
	0.2	7.9	4.0	3.7	0.94	0.17	0.06
	0.3	8.1	3.9	3.9	0.99	0.17	0.08
20	**0.1**	8.7	4.3	4.0	0.82	0.17	0.09
	0.2	8.7	3.8	3.7	0.89	0.17	0.08
	0.3	8.3	4.0	3.7	0.90	0.19	0.08
Pooled SEM		0.02	0.26	0.28	0.02	0.01	0.00
Main effects							
0		7.8[b]	4.2	3.8	0.92	0.15	0.08
10		8.2[ab]	4.1	3.8	0.96	0.18	0.08
20		8.6[a]	4.0	3.8	0.87	0.18	0.08
	0.1	8.6[a]	4.3	3.9	0.92	0.17	0.09
	0.2	8.2[ab]	3.9	3.7	0.91	0.16	0.07
	0.3	7.9[b]	4.0	3.8	0.93	0.17	0.08
Sources of variation							
RSBM		*	NS	NS	NS	NS	NS
Protease		**	NS	NS	NS	NS	NS
RSBM x protease		NS	NS	NS	NS	P=0.05	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different at *p<0.05; **p<0.01; NS= nonsignificant; [1]RSBM = raw soybean meal (SBM was replaced by RSBM at 0, 10 and 20 %, equivalent to 0, 30 and 60 g/kg of diet, respectively); SEM= pooled standard error of means; S. intestine= small intestine (duodenum + jejunum and ileum were weighed with their contents); gizzard and proventriculus were emptied (no content inside); G+P = gizzard + proventriculus.

Although the data for visceral organ development in the period of 1- 24 d are not shown, increasing level of protease in the diets had no significant influence (p>0.05) on the weight of any internal organs, such as the small intestine, G+P, liver, heart, bursa and spleen. Interaction between RSBM and protease had no significant difference (p>0.05) in the weight of internal organs.

5.3.4 Changes in weights of pancreas

As presented in Table 5.8, increasing the level of RSBM in the diets, particularly at 20%, increased the weight of the pancreas by approximately 24% (p<0.001), 32% (p<0.001) and 26% (p<0.01) at 10, 24 and 35 days of age, respectively. Although not statistically different (p>0.05), increasing the level of microbial protease in the diets containing RSBM decreased the weight of the pancreas by 0.7 and 4.2%, at 24 and 35 d, respectively.

Table 5.8 Pancreas weights (g/100 g of the body weight) of broilers at different ages.

RSBM[1] (%)	Protease (g/kg)	Age (days)		
		10 d	24 d	35 d
0	**0.1**	0.510	0.230	0.170
	0.2	0.513	0.220	0.147
	0.3	0.607	0.245	0.147
10	**0.1**	0.645	0.295	0.185
	0.2	0.590	0.315	0.200
	0.3	0.605	0.305	0.203
20	**0.1**	0.692	0.357	0.217
	0.2	0.733	0.340	0.208
	0.3	0.705	0.327	0.198
Pooled SEM		0.02	0.01	0.01
Main effects				
0		0.543^{c}	0.232^{c}	0.154^{b}
10		0.613^{b}	0.305^{b}	0.196^{ab}
20		0.710^{a}	0.341^{a}	0.208^{a}
	0.1	0.616	0.294	0.191
	0.2	0.612	0.292	0.185
	0.3	0.639	0.292	0.183
Sources of variation				
RSBM		***	***	**
Protease		NS	NS	NS
RSBM x protease		NS	NS	NS

[a,b,c] Means bearing uncommon superscript within a column are significantly different at ;**p<0.01; ***p<0.001; NS= non-significant ; [1]RSBM = raw soybean meal (SBM was replaced by RSBM at 0, 10 and 20%, equivalent to 0, 30 and 60 g/kg of diet, respectively); SEM= pooled standard error of means. One bird per cage, and 6 birds per treatment were killed to evaluate the pancreas at 10, 24 and 35 d of age.

The weight of the pancreas was not, however, significantly (p>0.05) affected by the interaction between the main factors (protease x RSBM) in the diets at any point of assessment.

5.3.5 Ileal nutrient digestibility

As shown in Table 5.9, increasing the amount of RSBM in the diets strongly and statistically decreased the AID of CP at 24 d. When an inclusion rate of RSBM increased, the AID value of indispensable AA, for example arginine, histidine, lysine, methionine and threonine were significantly decreased. The AID values of dispensable AA, for example serine and glycine were significantly decreased (p>0.05) when an inclusion rate of RSBM was increased in the diets. However, neither the indispensable nor dispensable AA were statistically different (p>0.05) when supplemented with an increased levels of protease in the period of 1-24 d.

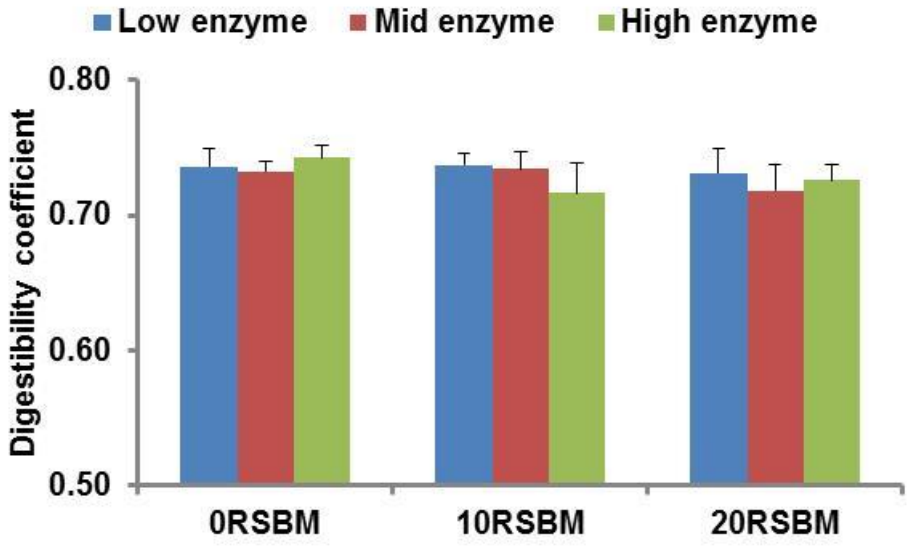


Figure 5.1 The ileal digestibility of gross energy at 24 days of age.

Low enzyme= 0.1 g/kg protease; mid enzyme= 0.2 g/kg protease; high enzyme=0.3 g/kg protease.

The AID value of methionine was 94.1%, which is the highest value, compared to the other indispensable AA; for example, threonine was the lowest (75.4%). Glycine was shown to be the lowest value of AID among the dispensable AA (74.8%) during the period from 1-24 d. The apparent ileal digestibility of the gross energy (GE) at 24 d is shown in Figure 5.1.

Neither the level of RSBM nor the protease had any significant impact ($p>0.05$) on the ileal digestibility values of GE. There were no significant effects ($p>0.05$) on the AID of the CP, GE or any AA due to interactions between the main factors (protease x RSBM) in the diets during the assessed period from 1 to 24 d. The digestibility of indispensable and dispensable AA, in general, followed the same trend as that of the CP.

Table 5.9 Effects of graded levels of RSBM and protease on apparent ileal digestibility of CP and AA at 24 d.

RSBM %	Protease g/kg	CP	Indispensable amino acids									Dispensable amino acids			
			Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val	Ala	Gly	Pro	Ser
0	**0.1**	0.800	0.899	0.843	0.833	0.831	0.889	0.954	0.844	0.784	0.818	0.828	0.780	0.808	0.805
	0.2	0.795	0.874	0.825	0.811	0.818	0.879	0.946	0.823	0.766	0.814	0.811	0.753	0.798	0.787
	0.3	0.802	0.883	0.833	0.827	0.829	0.882	0.953	0.835	0.772	0.828	0.804	0.763	0.805	0.794
10	**0.1**	0.782	0.869	0.89	0.797	0.800	0.865	0.936	0.812	0.758	0.800	0.798	0.744	0.784	0.774
	0.2	0.777	0.87	0.827	0.808	0.813	0.873	0.939	0.821	0.763	0.809	0.815	0.763	0.798	0.777
	0.3	0.763	0.859	0.800	0.774	0.784	0.855	0.941	0.795	0.744	0.759	0.784	0.724	0.768	0.751
20	**0.1**	0.763	0.874	0.812	0.786	0.794	0.860	0.939	0.810	0.742	0.776	0.790	0.750	0.788	0.763
	0.2	0.755	0.848	0.789	0.754	0.769	0.845	0.928	0.776	0.728	0.747	0.781	0.735	0.765	0.742
	0.3	0.755	0.852	0.795	0.760	0.774	0.836	0.930	0.783	0.725	0.747	0.773	0.721	0.772	0.739
Pooled SEM		0.006	0.003	0.005	0.006	0.006	0.4	0.002	0.005	0.006	0.007	0.006	0.006	0.005	0.006
Main effects															
0		0.799^{a}	0.886^{a}	0.834^{a}	0.823^{a}	0.826^{a}	0.883^{a}	0.951^{a}	0.834^{a}	0.774^{a}	0.820^{a}	0.815	0.766	0.804^{a}	0.795
10		0.774^{b}	0.869^{b}	0.815^{ab}	0.793^{ab}	0.799^{ab}	0.864^{ab}	0.939^{b}	0.810^{b}	0.755^{ab}	0.790^{ab}	0.799	0.744	0.784^{b}	0.768
20		0.758^{b}	0.858^{b}	0.799^{b}	0.767^{b}	0.779^{b}	0.847^{b}	0.932^{b}	0.790^{b}	0.732^{b}	0.757^{b}	0.781	0.735	0.775^{b}	0.748
	0.1	0.782	0.881	0.825	0.805	0.809	0.871	0.943	0.822	0.762	0.798	0.805	0.758	0.793	0.781
	0.2	0.777	0.867	0.814	0.791	0.800	0.866	0.937	0.807	0.752	0.790	0.802	70.50	0.787	0.769
	0.3	0.774	0.865	0.809	0.787	0.796	0.858	0.942	0.804	0.747	0.778	0.787	0.736	0.782	0.761
Sources of variation															
RSBM		***	***	***	***	***	***	***	***	***	***	*	0.06	*	***
Protease		NS	0.08	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*
RSBM x protease		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscript within a column are significantly different at NS= non-significant;***p<0.01; *p<0.001; RSBM = raw soybean meal (SBM was replaced by RSBM at 0, 10 and 20%, equivalent to 0, 30 and 60 g/kg of diet, respectively); SEM= pooled standard error of means;

5.3.6 Protein contents, activities of digestive enzymes and mucosal morphometry

Increasing the level of RSBM reduced ($p<0.05$) the concentration of pancreas tissue proteins, but the concentration was increased ($p<0.01$) when the diets were supplemented with microbial protease, particularly at 0.2 g/kg at 10 d. The activities of trypsin and lipase similarly tended ($p=0.06$) to improve at low (0.1 g/kg) levels of protease supplementation. Interaction between the main factors (protease x RSBM) had no effects ($p>0.05$) on the activity of any digestive enzymes, including trypsin, chymotrypsin, general proteolysis, lipase, maltase, sucrase, or alkaline phosphatase, at 10 d (the data are not shown).

The analysed tissue protein contents and activities of digestive enzymes at 24 d are shown in Table 5.10. Increasing the inclusion rate of RSBM negatively influenced the activities of chymotrypsin ($p<0.05$) and alkaline phosphatase ($p=0.05$), but had no effect ($p>0.05$) on the activities of trypsin, GP, lipase, maltase and sucrase. Although the concentration of the jejunal tissue protein was not ($p>0.05$) affected, that of the pancreatic tissue was increased ($p<0.05$) due to supplementation of protease. The activities of most pancreatic enzymes, including trypsin ($p<0.05$), chymotrypsin ($p<0.01$), GP ($p<0.05$) and lipase ($p=0.06$), were positively influenced by protease supplementation. Adding the protease, particularly at 0.1 g/kg or 0.2 g/kg, also influenced ($p<0.01$) the activity of alkaline-phosphatase ($p<0.01$). Except for alkaline- phosphatase ($p<0.05$), there was no significant effect of interaction between RSBM and protease on any tissue protein concentrations or any other enzymatic activities at 24 d.

Table 5.10 Effects of dietary RSBM and protease supplementation on tissue protein concentration, and pancreatic and jejunal enzyme activities of broiler chicks at 24 days of age.

RSBM[1] ,%	Protease g/kg	Tissue protein conc. (mg/g) ----------		Enzyme activities in nmole/mg protein/min						
				Pancreas				Jejunum		
		Pancreas	Jejunum	Trypsin	Chymotrypsin	GP	Lipase	Maltase	Sucrase	AP
0	**0.1**	37.5	31.2	4.3	4.0	5.1	2.3	0.94	0.43	0.70^{ab}
	0.2	36.4	30.4	4.3	4.4	5.3	2.5	1.09	0.38	0.82^{a}
	0.3	34.9	30.5	6.0	5.5	6.5	3.6	1.03	0.45	0.54^{b}
10	**0.1**	36.7	31.1	3.5	2.7	4.9	3.7	0.87	0.40	0.59^{b}
	0.2	38.0	31.0	3.7	3.4	5.1	1.9	0.97	0.40	0.67^{ab}
	0.3	35.9	31.2	5.5	4.5	5.6	3.0	0.92	0.46	0.62^{ab}
20	**0.1**	38.0	31.2	4.0	2.8	5.2	2.1	0.89	0.35	0.50^{b}
	0.2	35.8	31.1	5.1	4.1	5.7	2.7	0.89	0.46	0.65^{ab}
	0.3	36.5	30.4	5.0	4.0	5.5	2.8	0.98	0.49	0.60^{ab}
Pooled SEM		0.3	0.1	0.2	0.2	0.1	0.2	0.03	0.02	0.02
Main effects										
0		36.2	30.7	4.9	4.6^{a}	5.6	2.8	1.02	0.42	0.69
10		36.9	31.1	4.3	3.6^{b}	5.2	2.9	0.92	0.42	0.63
20		36.8	30.9	4.7	3.6^{b}	5.5	2.5	0.92	0.43	0.58
	0.1	37.4^{a}	31.1	4.0^{b}	3.2^{b}	5.1^{b}	2.7	0.90	0.39	0.60
	0.2	36.7^{ab}	30.8	4.4^{b}	4.0^{b}	5.4^{b}	2.4	0.98	0.41	0.71
	0.3	35.7^{b}	30.7	5.5^{a}	4.7^{a}	5.9^{a}	3.2	0.98	0.47	0.59
Sources of variation										
RSBM		NS	NS	NS	*	NS	NS	NS	NS	0.05
Protease		*	NS	*	**	*	0.06	NS	Ns	**
RSBM x protease		NS	NS	NS	NS	NS	NS	NS	NS	*

[a,b,c] Means bearing uncommon superscript within a column are significantly different at *p<0.05; **P<0.01; NS= non-significant; 1RSBM = raw soybean meal (SBM was replaced by RSBM at 0, 10 and 20 %, equivalent to 0, 30 and 60 g/kg of diet, respectively); SEM= pooled standard error of means; GP= general proteolysis; AP= alkaline phosphatase.

Table 5.11 Effects of dietary RSBM and protease concentration of protein and DNA concentration (mg/ g), and ratio of protein to DNA in pancreas at 24 and 35 days of age.

RSBM	Protease	Protein		DNA		Protein : DNA	
%	g/kg	24 d	35 d	24 d	35 d	24d	35 d
0	**0.1**	37.5	38.0	4.6	7.0	8.7	5.5
	0.2	36.4	38.1	5.5	8.4	6.6	4.7
	0.3	34.9	37.7	6.0	6.9	6.9	5.8
10	**0.1**	36.7	36.9	4.3	5.9	9.3	8.1
	0.2	38.0	37.0	5.1	6.6	7.2	6.3
	0.3	35.9	37.7	4.0	7.3	9.7	5.8
20	**0.1**	38.0	38.0	4.5	6.6	7.7	7.2
	0.2	35.8	37.5	6.7	7.6	7.1	5.0
	0.3	36.5	37.3	6.3	6.9	6.7	5.5
Pooled SEM		0.3	1.7	0.30	0.3	1.38	0.6
Main effects							
0		36.2	37.9[a]	5.3	7.1	7.5	5.3
10		36.9	37.3[b]	4.5	6.3	8.7	6.6
20		36.8	37.6[ab]	5.8	7.0	7.2	5.8
	0.1	37.4[a]	37.7	4.5	6.6	8.7	6.7
	0.2	36.7[ab]	37.5	5.7	7.5	6.9	5.3
	0.3	35.7[b]	37.6	5.5	6.4	7.6	5.7
Sources of variation							
RSBM		NS	*	NS	NS	NS	NS
Protease		*	NS	Ns	NS	NS	0.08
RSBM x protease		NS	NS	NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscript within a column are significantly different at *p<0.05; NS= nonsignificant; [1]RSBM = raw soybean meal (SBM was replaced by RSBM at 0, 10 and 20%, equivalent to 0, 30 and 60 g/kg of diet, respectively); SEM= pooled standard error of means.

The result of tissue protein and DNA contents, and ratio of tissue protein between the two are shown in Table 5.11. The pancreatic tissue protein contents at 35 d decreased ($p<0.05$) with increase in inclusion rate of RSBM. Increasing the level of RSBM or protease had no influences ($p>0.05$) on the DNA concentrations of the pancreas at 24 or 35 d of age. The ratio of protein to DNA at 35d tended ($p=0.08$) to increase at low (0.1 g/kg) level of protease supplementation, but this supplementation effect was absent at 24 d.

Although the data are not shown, neither increasing the level of RSBM nor of protease affected ($p>0.05$) mucosal morphometry of the jejunum at 10 d of age. The interactions between RSBM and protease significantly increased the height of the villi ($p<0.05$) and the depth of the mucosal ($p<0.01$) and tended ($p=0.06$) to influence the apparent surface area of the villus. The interaction between RSBM and protease tended (0.08) to be significant for crypt depth and the ratio of the villus height to the crypt depth at 10 d of age. Except for villus height or mucosal depth ($p=0.08$), increasing the level of RSBM or protease in the diets of the birds had no effects ($p>0.05$) on any of mucosal morphometry at 24 d of age.

5.4 DISCUSSION

5.4.1 Diets and the gross response

The values of TI, UA and NSI increased in line with increasing in contents of RSBM in the diets. These results agree with Zhang *et al.* (1996) who reported that the contents of protein solubility, TI and UA are correlated with the amounts of unheated soybeans in the diet. During the early periods (1-10 d and 1-24 d), increasing the level of RSBM in the diets of the birds had no influence on the FI, but the FI was decreased when considered over the entire trial period (1-35 d). This result partially agrees with the result of other researchers (Mogridge *et al*., 1996; ASA, 2004) who reported that birds fed on diets with RSBM experienced low FI. The reason for the reduction in feed consumption in the current study might be due to the negative impact of the ANF, particularly the TI in the RSBM.

The BWG and FCR also decreased with increasing inclusion rates of RSBM during the early periods (1-10 d). In general, this result agrees with other scholars (ASA, 2004; Palacios *et al*., 2004; Romero and Plumstead, 2013) who reported that the performance of non-ruminant animals is affected by the dietary TI. Increasing the level of RSBM in the diets however had no significant effects on the BWG of the birds during the period from 1-24 d or 1-35 d. This result agrees with Loeffler *et al.* (2012) who reported that broilers adapt to ANF in the diet with age advances. The stability in BWG in the current study might also be due to the reduction in the negative impact of TI in tested diets by supplemental microbial protease.

Increasing the level of microbial protease in the diets improved the BWG of the birds during the periods from 1-10 and 1-24 d, which also tended to improve during the period from 1 to 35 d. These results agree with other scholars (Yadav and Sah, 2005; Barletta, 2011; Jacela *et al*., 2009) who reported that microbial protease can break down both stored proteins and proteinaceous anti-nutrients in diets.

During the period from 1 to 24 d, the feed efficiency was improved in response to supplementation with microbial protease, but this response was weaker during the 1-10 d or 1-35 d. This result partially agrees with these researchers, Cowieson and Adeola (2005) and Rada *et al.* (2014) who reported that supplementation with microbial protease improved the performance of birds.

The meat yield of the birds was not affected by replacing the commercial SBM with the tested level of RSBM. Most of the measures, including the dressed percentage, breast, thighs and neck, were not affected by inclusion of protease, but the weight of drumsticks and wings were

increased. These results partially disagree with Rada *et al*. (2014) who reported that the meat parts of broilers were positively affected by the addition of microbial enzymes.

5.4.2 Visceral organ development

Increasing the inclusion rate of RSBM in the diets resulted in an increase in the weight of the pancreas compared to the birds fed an RSBM-free diet, and this was highly pronounced in young birds. The concentration of DNA or the ratio of tissue protein to DNA was not affected by increasing the levels of RSBM or protease in the diets of the broilers. However, protein: DNA ratio tended to increase in early life. The increase in weight of the pancreas is due to more to hypertrophy than hyperplasia.

The magnitude of change in the weight of the pancreas also decreased with age. These results agree with Mirghelenj *et al*. (2013) who reported that the relative weight of pancreas of chickens fed extruded full-fat soybean meal was higher than that of birds fed control diets at 21 d, but no effect was observed at 42 d. Similar results were reported by many other researchers (Pacheco-Dominguez, 2011; Beukovic *et al.,* 2012; Mirghelenj *et al*., 2013). However, in contrast Rocha *et al*. (2014) reported that the relative weight or histological structure of the pancreas of broilers fed on diet with TI was not related with the age of the birds. The reasons for the reduction in the weight of the pancreas as the birds grew might be linked to the use of exogenous microbial protease in the current study.

The small intestine was significantly heavier when levels of RSBM were increasingly added to the diets during the period from 1-10 d. This result partially agrees with other researchers (Mogridge *et al.,* 1996; Mayorga *et al*., 2011) who reported that birds fed on diets containing RSBM had heavier pancreas and duodenum.

Regardless of the increasing levels of RSBM in the diets, increasing the inclusion rate of protease significantly reduced the weight of the small intestines during the start phase. Similarly, Ao (2011) reported that enzyme supplementation can reduce the negative impacts of anti-nutrients. Increasing the inclusion rate of RSBM in the diets, however, had no significant influence on the weight of the other internal organs, such as the liver, heart, bursa and spleen, up to 24 d. The reason why these internal organs were not affected by the high concentration of TI in the current diets is unclear but the supplemental protease may play some role.

5.4.3 Ileal nutrient digestibility, tissue protein content, activities of digestive enzymes and mucosal morphometry

Although increasing the level of RSBM in the diets decreased the AID of CP, it was not significantly improved by increasing the level of supplemental protease. This result agrees with Marsman *et al.* (1997) who reported that protease and carbohydrase supplementation in diets had no significant influence on the AID of CP digestibility.The AID values of most of the indispensable and dispensable AA decreased with increasing level of RSBM in the diets. These results agree with those of de-Coca-Sinova *et al.* (2008) who reported that the ileal AA digestibilities were greater for diets with lesser dietary TI concentrations. The response of AID of CP and AA to protease supplementation in this study appears to be insignificant mainly because the data are being compared against a diet that contains a low level of protease. No negative control (no protease) was tested in this study due to animal welfare concerns. However, neither the digestibility of indispensable nor the dispensable AAs were statistically influenced by supplementation with increasing levels of protease.

The content of the pancreatic tissue protein was minimally increased at low and medium levels of protease supplementation. The reason for this change is not clear. The activities of chymotrypsin amidase and alkaline phosphatase decreased with increasing levels of RSBM in the diets. This may be in response to high levels of TI in the diets although activities of most of other enzymes were unaffected.

Most of the pancreatic digestive enzyme activities were improved due to the dietary supplementation with microbial protease. These results partially agree with those of Yuan *et al*. (2008) who reported that higher activities of amylase and trypsin were found when the diets were supplemented with enzymes.

There are no previous reports on the effects of TI on the mucosal morphometry. In the current study only the villus height and mucosal depth were affected by the interaction effects between the main factors (protease x RSBM) in the diets. Yuan *et al.* (2008) had previously observed a positive effect of enzymes on villus height and apparent surface area of the villus in birds.

5.5 CONCLUSION

Although some of the dietary treatment groups contained TI beyond the threshold levels for non-ruminant animals, supplementation with microbial protease enabled the birds to tolerate

up to 20% of the RSBM, replacing the commercial SBM without greatly compromising the productivity. Although RSBM reduced the growth, during the early periods (1-10 d), over time, all treatment groups achieved almost the same BWG. Pronounced pancreatic hypertrophy was identified in the broiler chickens fed diets containing high levels of RSBM, during the entire study period. However, the response of the birds suggested that the pancreas remained functional and produced enough enzymes to digest the nutrients in the diets containing RSBM. The results of this study also showed some positive effects of supplemental protease on endogenous protease. The results of this study suggest that there is hope for higher inclusion levels of RSBM in broiler diets, possibly along with higher doses of the test protease. A cost-benefit analysis may also beneficial when higher doses of the enzyme are used.

CHAPTER 6: EXTRA-DOSING OF MICROBIAL PROTEASE AND PHYTASE IN BROILER DIETS CONTAINING A MEDIUM LEVEL OF RAW, FULLFAT SOYBEAN MEAL

ABSTRACT

A 3 x 3 + 1 factorial study, with 3 levels of protease (0.0, 0.2 or 0.4 g/kg) and 3 levels of phytase (0.1, 0.2 or 0.3 g/kg), and a control diet was employed to assess the effects of extra-doing of protease and phytase in diets containing raw full-fat soybean meal (RSBM), replacing commercial SBM at 25%, on the performance of broilers. The control diet had no RSBM or protease. All treatment groups were replicated six times, with nine birds per replicate**.** The birds were housed in cages, in a climate-controlled room and fed diets (starter, grower and finisher) formulated to Aviagen standards for Ross 308 broiler. Content of trypsin inhibitor (TI) in diets was around 10193.4 TIU/kg. Birds in the control group consumed more feed ($p<0.05$) than others in the 1-10 d and 1-35 periods, but not in 1-24 d ($p>0.05$). The BWG of birds in the control group was higher ($p<0.05$) than that of other groups in the periods of 1-24 d and 1-35 d, but the FCR was not affected. Increasing levels of microbial protease improved ($p<0.05$) the FI and BWG of birds during the early period (1-10 d). During the 1-35 d period, increasing the inclusion rate of protease improved the BWG (2.2%) and FCR (2.8%) of birds compared with those fed protease-free diets, but this effect was not significantly ($p>0.05$) different. The FI ($p<0.05$) and BWG ($p<0.01$) from one to 24 d were improved by extra-dosing with phytase. The weight of the breast and thigh increased ($p<0.05$) in response to extra-dosing of phytase and protease to the diet. The BWG was improved at 1-10 d and 1-24 d when extra-dosing with protease ($p<0.05$) and phytase ($p<0.01$), respectively**.** The relative weight of the pancreas was significantly reduced with extra-dosing of protease ($p<0.001$) and phytase ($p<0.05$) to the diets. Extra-dosing with protease also reduced the weights of most internal organs. The apparent ileal digestibility (AID) of CP and amino acids (AA) were reduced by increasing levels of RSBM in diets. Extra-dosing of the diets with microbial protease increased the AID of a majority of AA. Increasing the protease supplementation improved the activities of some pancreatic enzymes, including trypsin (7.1%), general proteolytic activity (11.1%) and lipase (12.1%). Mucosal depth and apparent villus surface area were increased by 2.9 and 20%, respectively due to extra-dosing of phytase. It can be concluded that RSBM could replace the commercial SBM, up to 25% by RSBM in broiler diets, provided the diets are extra-dosed with right types of protease and phytase.

6.1 INTRODUCTION

Soybean meal (SBM) is the most extensively used protein source by the poultry industry. Although soybean is primarily grown for oil, the seed meal can also be fed to poultry as a full-fat meal. However, raw full-fat soybean meal (RSBM) contains anti-nutritional factors (ANFs) that negatively influence the utilisation of nutrients in poultry (Pettersson and Pontoppidan, 2013; Erdaw *et al.,* 2015c & 2016b). The best characterised ANFs in raw soybean seed are protease inhibitors, lectins and phytate (Pettersson and Pontoppidan, 2013). Protease inhibitors interfere with the biological activity of endogenous trypsin, thereby reducing the digestion of proteins (Dourado *et al*., 2011; Nahashon and Kilonzo-Nthenge, 2013). Various scholars (Mogridge *et al.,* 1996; Liu, 1997; Newkirk, 2010) have also observed that the feed intake (FI), body weight gain (BWG) and feed efficiency of birds fed diets containing RSBM were negatively affected by trypsin inhibitors.

Another important ANF in soybeans is phytate, which is primarily detected in the form of protein-phytate or protein-phytate-protein complexes, are resistant to digestion (Ravindran *et al.,* 2001; Chen *et al.,* 2013). Approximately 65-80% of the total phosphorus in soybean meal is trapped by phytic acid or phytate, and the activities of some endogenous enzymes in birds, such as phytase, is inadequate to digest these compounds (Lall, 1991; NRC, 1994).

Supplementing the diets with exogenous enzymes typically reduces the adverse effects of ANFs on non-ruminant animals (Munir and Maqsood, 2013). Microbial proteases are natural protein-digesting enzymes used in pig and poultry nutrition to break down stored proteins and proteinaceous anti-nutrients in various plant materials (Barletta, 2011; Ao, 2011). The adverse effects of the ANF found in RSBM can also be reduced through supplementation with exogenous enzymes (Ravindran and Son, 2011). For example, phytase hydrolyses phytic acid (Rostami and Giri, 2013) thereby improves protein and amino acid utilisation in birds (Biehl and Baker, 1997; Barletta, 2011). Guggenbuhl *et al*. (2012) also showed that supplementation with phytase increases the digestibility of CP and indispensable amino acids. Beyond the release of phosphorus, the body growth and carcass yield of birds are enhanced in diets supplemented with microbial phytase (Karimi *et al.,* 2013; Campasino *et al*., 2014). Barletta (2011) revealed that diets supplemented with phytase showed an economic benefit, as the digestibility of nutrients, such as energy, protein and amino acids, is improved. However, Selle and Ravindran (2007) indicated that there is no consensus between researchers concerning whether phytase enhances protein and energy utilisation in poultry diets.

Theoretically, different microbial enzymes target different anti-nutrients in diets and show additive effects through the increased release of nutrients from the diet (Adams, 2004; Barletta, 2011). Additionally, Cowieson and Adeola (2005) reported that combining microbial phytase with protease improved the gain-to-feed ratio of birds. The utilisation of essential nutrients has been shown to improve as a result of the synergistic effects of combined enzymes (phytase, carbohydrase and protease) on a wheat/canola-based diet (Simbaya *et al.*, 1996). Although several studies have been conducted examining the individual or combined effects of microbial phytase and protease, using normal broiler diets, information concerning the effects of exogenous feed enzymes on the physiological response of broilers fed diets containing RSBM is limited. Therefore, the objectives of this study were to evaluate the attributes of individual, as well as the combined (synergistic) effects of extra-dosing protease and phytase in diets containing 25% RSBM on the gross and physiological response of broilers.

6.2 MATERIALS AND METHODS

The experiment was conducted at the Centre for Animal Research and Teaching (CART) University of New England (UNE), and the study was approved by Animal Ethics Committee (Authority No: AEC15-044), prior to commencement.

6.2.1 Diets

A 3 x 3 + 1 factorial study was used to evaluate the performance of broiler chickens fed diets containing RSBM (SBM was replaced by RSBM at 25%, equivalent to 75 g/kg of diet) and supplemented with three levels of protease (Ronozyme® ProAct: 0, 0.2 and 0.4 g/kg, equivalent to 0, 15000 and ~ 30000 PROT/kg of diet, respectively) and three levels of phytase (Ronozyme® Hiphos: 1000, 2000 and 3000 FYT/kg, equivalent to 0.1, 0.2 and 0.3 g/kg of diet, respectively). The control diet contained only 0.1 g/kg of phytase and contained neither RSBM nor protease. Each dietary group was replicated six times, with nine birds per replicate. The birds were fed starter (0-10 d) grower (10-24 d) and finisher (24-35 d) diets (corn-soybean based) formulated to Aviagen standards (Tables 6.1, 6.2 and 6.3) for Ross 308 broiler.

Table 6.1 Ingredients and composition of basal and control starter (0-10 d) diets (as-fed basis).

RSBM (%)	**0**	**25**		
Proteas (g/kg)	**0.0**	**0.0**	**0.2**	**0.4**
Ingredients (g/kg)				
Corn (Rolled)	593.6	594.0	594.0	594.0
Soybean meal	300.0	225.0	225.0	225.0
Raw soybean meal	0.0	75.0	75.0	75.0
Meat meal	62.9	72.3	72.3	72.3
Canola Oil	16.0	7.9	7.9	7.9
Dical Phos	7.7	6.7	6.7	6.7
Limestone	6.2	5.3	5.3	5.3
Salt	3.0	3.0	3.0	3.0
L-lysine	2.7	2.7	2.7	2.7
DL-methionine	2.3	2.5	2.5	2.5
Premix 2 kg/mt[1]	2.0	2.0	2.0	2.0
L-threonine	2.0	2.0	2.0	2.0
Sodium bicarb	1.1	1.1	1.1	1.1
Choline Cl	0.5	0.5	0.5	0.5
Protease	0.0	0.0	0.2	0.4
Nutrients (g/kg)				
ME Poultry (MJ/kg)	12.29	12.29	12.29	12.29
Crude Protein	225.8	226.0	226.0	226.0
Crude fat	23.2	44.6	44.6	44.6
Arginine	14.4	14.4	14.4	14.4
Lysine	14.0	14.0	14.0	14.0
Methionine	5.7	5.7	5.7	5.7
Methionine+ cysteine	8.8	8.8	8.8	8.8
Threonine	9.9	9.9	9.9	9.9
Calcium	10.0	10.0	10.0	10.0
Phosphorus avail	5.0	5.0	5.0	5.0
Choline	1.4	1.2	1.2	1.2

[1]Premix (supplied Activity per ton feed): Cu (sulphate), 8 g; Fe (sulphate), 60 g; I (iodide), 1.0 g; Se (selenate), 0.3 g; Mn (Manganese), 80 g; Zn (sulphate and oxide), 60 g; Mo (Molybdenum), 1 g; Cobalt (Co), 0.3 g.Vitamin A (retinol), 12 MIU; Vitamin D_3 (cholecalciferol), 3.5 MIU; Vitamin E (tocopheryl acetate), 40 g, Vitamin K3 (menadione), 2 g; thiamine (Vitamin B1), 2 g; riboflavin (B_2), 6 g; niacin (B_2), 50 g; pantothenate, 11 g; pyridoxine, 20 g; folate (Vitamin B9), 1.5 mg; Biotin (vitamin H), 100 g; Vitamin B_{12} (cyanocobalamin), 20 mg; Vitamin B_6; Vitamin H, 1.5 g ; biotin, 100 mg; Antioxidant, 25 g. RSBM=raw soybean meal. The dietary treatments were basal diets containing 3 levels of protease (0.0, 0.2 or 0.4 g/kg of diets) and supplemented with 1000, 2000 or 3000 FYT/kg phytase, equivalent to 0.1, 0.2 or 0.3 g/kg of diet, respectively. In the basal diets, the SBM was replaced by RSBM at 25%, equivalent to 75 g/kg of diet. Except phytase (0.1 g/kg), control diet did not contain RSBM or protease.

Table 6.2 Ingredients and composition of control and basal grower (10-24 d) diets (as-fed basis).

RSBM (%)	**0**	**25**		
Protease (g/kg)	**0.0**	**0.0**	**0.2**	**0.2**
Ingredients (g/kg)				
Corn (Rolled)	563.9	572.9	572.0	572.0
Soybean meal	300.0	225.0	225.0	225.0
Raw soybean meal	0.0	75.0	75.0	75.0
Meat meal	60.0	60.0	60.0	60.0
Canola Oil	44.6	36.5	36.5	36.5
Dical Phos	9.0	9.0	9.0	9.0
Limestone	5.0	5.0	5.0	5.0
Ti0	5.0	5.0	5.0	5.0
Salt	2.3	2.3	3.5	3.5
L-lysine	2.4	2.4	2.4	2.4
DL-methionine	3.3	2.3	2.3	2.3
Premix 2 kg/mt[1]	2.0	2.0	2.0	2.0
L-threonine	0.9	0.9	0.9	0.9
Sodium bicarb	0.8	0.8	0.8	0.8
Choline Cl	0.8	0.8	0.8	0.8
Protease	0.0	0.0	0.2	0.4
Nutrients (g/kg)				
ME Poultry (MJ/kg)	12.59	12.59	12.59	12.59
Crude Protein	221.6	216.8	216.8	216.8
Crude fat	69.7	71.3	71.3	71.3
Arginine	14.2	13.9	13.9	13.9
Lysine	13.6	13.3	13.3	13.3
Methionine	6.6	6.6	6.6	6.6
Methionine + cystiene	9.6	9.6	9.6	9.6
Threonine	8.7	8.6	8.6	8.6
Calcium	9.6	9.4	9.4	9.4
Phosphorus avail	5.1	4.9	4.9	4.9
Choline	1.5	1.3	1.3	1.3

[1]Composition of the premix was as in Table 6.1; RSBM=raw soybean meal. The dietary treatments were basal diets containing 3 levels of protease (0.0, 0.2 or 0.4 g/kg of diets) and supplemented with 1000, 2000 or 3000 FYT/kg phytase, equivalent to 0.1, 0.2 or 0.3 g/kg of diet, respectively. In the basal diets, the SBM was replaced by RSBM at 25%, equivalent to 75 g/kg of diet. Except phytase (0.1 g/kg), control diet did not contain RSBM or protease.

Table 6.3 Ingredients and composition of control and basal grower (24-35 d) diets (as-fed basis).

RSBM (%)	**0**	**25**		
Protease (g/kg)	**0.0**	**0.0**	**0.2**	**0.4**
Ingredients (g/kg)				
Corn (Rolled)	636.6	637.6	637.6	637.6
Soybean meal	300	225.0	225.0	225.0
Raw soybean meal	0.0	75.0	75.0	75.0
Canola oil	35.6	27.5	27.5	27.5
Dical Phos	7.9	8.0	8.0	8.0
Meat meal	0	6.5	6.5	6.5
Limestone	10.2	9.4	9.4	9.4
DL-methionine	2.0	2.5	2.5	2.5
Salt	2.3	2.1	2.1	2.1
Premix 2kg/mt[1]	2.0	2.0	2.0	2.0
Sodium bicarb	1.9	1.0	1.0	1.0
L-lysine	0.4	1.0	1.0	1.0
Choline Cl	0.4	0.5	0.5	0.5
L-threonine	0.5	1.8	1.8	1.8
Protease	0.0	0.0	0.2	0.4
Nutrients (g/kg)				
ME Poultry (MJ/kg)	13.5	13.5	13.5	13.5
Crude Protein	197	196	196	196
Crude fat	53.8	56.6	56.6	56.6
Arginine	12.6	12.5	12.5	12.5
Lysine	10.5	10.9	10.9	10.9
Methionine	4.9	5.3	5.3	5.3
Methionine + cysteine	7.9	8.3	8.3	8.3
Threonine	7.6	8.8	8.8	8.8
Calcium	8.5	8.5	8.5	8.5
Phosphorus avail	4.2	4.3	4.3	4.3
Choline	1.4	1.2	1.2	1.2

[1]Composition of the premix was as in Table 6.1; RSBM=raw soybean meal. The dietary treatments were basal diets containing 3 levels of protease (0.0, 0.2 or 0.4 g/kg of diets) and supplemented with 1000, 2000 or 3000 FYT/kg phytase, equivalent to 0.1, 0.2 or 0.3 g/kg of diet, respectively. In the basal diets, the SBM was replaced by RSBM at 25%, equivalent to 75 g/kg of diet.
Except phytase (0.1 g/kg), control diet did not contain RSBM or protease.

6.2.2 Animal husbandry

A total of 540 day-old Ross 308 male broiler chicks, with an average body weight of 34.4±0.095 g, were obtained from a local commercial hatchery (Baiada Poultry Pty. Ltd., Tamworth, Australia). Nine chicks of average body weight were randomly allocated into 60 cages. The birds were housed from one to 35 days of age in a cage-rearing system under controlled climate. All other routine practices for animal management, and sampling procedures were performed as described in Section 5.2.2.

6.2.3 Gross response to the diets

The gross responses of birds, in terms of FI, BWG, FCR and meat yield, were determined according to the procedures described in Section 5.2.4.

6.2.4 Internal organ development and sampling

The procedures for sacrificing the birds, measuring the internal organs and collecting tissue samples were conducted using the same methods described in the previous chapter, Section 5.2.3.

6.2.5 Chemical analysis

Chemical analyses for DM, ash, GE, CP, AA and TiO_2, were performed using the same methods described in Section 5.2.5.

6.2.5.1 Determination of apparent ileal crude proteins and amino acid digestibility

Apparent ileal CP and AA digestibility (%) were calculated using the same method described in Section 5.2.5.1.

6.2.6 Tissue proteins and pancreatic enzyme activities

As described in Section 5.2.6, the same procedure (s) was also followed in the present study for processing the tissues, analysing the protein contents and examining the activities of pancreatic enzymes.

6.2.7 Aminopeptidase activity in the jejunum

The activity of aminopeptidase in jejunal tissue was determined using the method of Caviedes-Vidal and Karasov (2001). One millilitre of 2 mM L-alanine-p-nitroanilide (Sigma A9325) solution (49.14 mg L-alanine-p-nitroanilide/100 mL of 0.2 M sodium phosphate buffer, pH 7.0) was incubated at 41^0C for 5 min in a shaking water bath. Subsequently, a 10-µL aliquot of tissue homogenate was added to the pre-warmed substrate solution, followed by further incubation at 40^0C for 10 min. The reaction was terminated with one mL of ice-cold 2 M acetic acid solution, and the absorbance was read at 384 nm. A standard curve was prepared using concentrations of p-nitroaniline ranging from 0 to 0.1 mM and dissolved in 0.2 M sodium phosphate buffer at pH 7.0.

6.2.8 Mucosal morphometry of the jejunal tissues

To assess mucosal morphometry, all procedures, such as fixation, processing, Sectioning and staining of jejunal tissues, were performed using the method (s) described in Section 4.2.5.

6.2.9 Statistical analysis

General linear model (GLM) was used to analyse the data using Minitab software version 17 (Minitab, 2013). The data, particularly comparing control with test diets were analysed for significance using one-way ANOVA. The GLM was also used to evaluate the effects of extra-dosing protease and phytase enzymes, and interactions between the two factors. The differences were considered to be significant at $p < 0.05$, and significant differences between mean values were also separated using Duncan's test. The mean value of control diet was contrasted with the mean value of tested diets using pre-determined analysis. Analysis of pre-determined contrast was conducted to compare control group as well as protease-free test-diets with other treatment groups.

6.3 RESULTS

6.3.1 Gross response to the diets

The gross response of the birds, in terms of FI, BWG and FCR is shown in Table 6.4. Birds fed on the control diet, in general consumed more ($p<0.05$) feed during the 1-10 d and 1-35 d periods. The BWG was also significantly higher in the periods of 1-24 and 1-35 d for birds fed control diet as compared to those fed diets containing RSBM, but the entire FCR value was not affected. The FI and BWG of the birds were significantly improved ($p<0.05$) in diets extra-dosed with microbial protease during the early period (1-10 d). Although increasing the level of protease improved the BWG and FCR of birds, achieving approximately 2.7% more than that of birds fed on protease-free diets, there was no significant ($p>0.05$) effects of the supplement over the 1-35 d period.

The addition of an extra-dose of microbial phytase to the diets significantly improved ($p<0.01$) the FI and BWG during the 1-24 d period. Although no significant differences ($p>0.05$) were observed, extra-dosing the diet with microbial phytase increased the FI and BWG by 3.7 and 4.8% (during 1-10 d period) and 3.0 and 2.8% (during the 1-35 d period), respectively, compared to the other groups. Although the BWG was marginally ($p=0.06$) improved during the early period (1-10 d), the FI, BWG or FCR were statistically the same ($p>0.05$), in response to the interaction between the main factors (RSBM x protease) during the 1-35 d period. The meat and parts yields of experimental birds are shown in Table 6.5.

Table 6.4 Effects of protease and phytase extra-dosing in diets containing raw soybean on the FI, BWG (g/b) and FCR of broilers during 1-10, 1-24 or 1-35 days of age.

Protease g/kg	Phytase g/kg	Feed intake			Body weight gains			FCR		
		1-10	1-24	1-35	1-10	1-24	1-35	1-10	1-24	1-35
0.0	**0.1**	215.0	1416	3108	189.6	1083	2118	1.14	1.31	1.47
	0.2	222.1	1410	3116	180.1	1034	2152	1.19	1.36	1.45
	0.3	206.8	1448	3047	185.6	1142	2179	1.13	1.27	1.40
0.2	**0.1**	217.8	1422	3013	196.6	1080	2174	1.11	1.32	1.39
	0.2	226.2	1470	3215	194.5	1125	2229	1.16	1.31	1.44
	0.3	234.8	1604	3294	214.2	1214	2225	1.10	1.32	1.49
0.4	**0.1**	221.7	1437	3033	187.5	1087	2139	1.18	1.30	1.42
	0.2	225.9	1463	3076	198.2	1102	2243	1.13	1.33	1.37
	0.3	238.2	1534	3092	202.2	1137	2214	1.18	1.35	1.40
Pooled SEM		2.51	15.93	29.32	2.31	10.62	18.54	0.01	0.05	0.01
		Main effects								
0.0		214.6^{b}	1425	3090	185.1^{b}	1086	2149	1.17	1.31	1.44
0.2		226.3^{ab}	1499	3174	201.8^{a}	1140	2209	1.12	1.32	1.44
0.4		228.8^{a}	1478	3067	196.0^{ab}	1109	2196	1.17	1.33	1.40
0.1		218.2	1425^{b}	3051	191.2	1083^{b}	2144	1.14	1.31	1.43
0.2		224.7	1448^{ab}	3139	191.0	1087^{b}	2205	1.18	1.33	1.42
0.3		226.6	1528^{a}	3144	200.7	1164^{a}	2206	1.13	1.31	1.43
Sources of variation										
Protease		*	NS	NS	*	NS	NS	NS	NS	NS
Phytase		NS	*	NS	NS	**	NS	NS	NS	NS
Protease× phytase		NS	NS	NS	0.06	NS	NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different at NS= non-significant; * p<0.05; **p<0.01 ; 1control = normal diet without the raw soybean meal; SEM= pooled standard error of means; RSBM=raw soybean meal (SBM was replaced by RSBM at 25%, equivalent to 75 g/ kg of diet).
[2]Test-diets= diets containing 25% RSBM-equivalent to 75 g/kg of diet.

The birds in the control group had approximately 8.1% more breast weight (p<0.05) than other birds of test diets. Approximately 9.8 (thigh) and 10.4% (drumstick) more weights (p<0.05) were recorded as compared with the other birds. The weight of the thigh was increased as a result of the extra-dosing of microbial phytase in the diets.

Table 6.5 Effects of extra-dosing protease and phytase in diets containing raw soybean on the meat yield (g) of broilers at 35 d.

Protease g/kg	Phytase g/kg	Carcass yields				
		Dressed %	Breast	Thigh	Drumstick	Wing
0.0	**0.1**	80	427	216	204	170
	0.2	76	422	216	198	164
	0.3	79	429	223	204	172
0.2	**0.1**	76	408	209	193	165
	0.2	77	454	214	483	169
	0.3	81	477	226	216	180
0.4	**0.1**	78	405	213	199	177
	0.2	78	439	227	205	179
	0.3	80	454	227	213	179
Pooled SEM		1.01	6.12	2.53	30.82	1.73
		Main effects				
0.0		78	426^{b}	218	202	168.6^{b}
0.2		78	443^{a}	218	205	171.8^{ab}
0.4		79	433^{ab}	222	206	178.2^{a}
	0.1	78	413^{b}	214^{b}	199	171
	0.2	77	438^{ab}	219^{ab}	203	171
	0.3	80	450^{a}	225^{a}	210	177
Sources of variation						
Protease		NS	*	NS	NS	*
Phytase		NS	*	*	NS	NS
Protease x phytase		NS	NS	NS	NS	NS

^{a,b,c} Means bearing uncommon superscript within a column are significantly different; ^{1}control = normal diet without RSBM; SEM= pooled standard error of means; NS= non-significant; * p<0.05; RSBM=raw soybean meal (SBM was replaced by RSBM at 25%, equivalent to 75 g/ kg of diet). ^{2}Test-diets= diets containing 25% RSBM-equivalent to 75 g/kg of diet.

6.3.2 Development of internal organs

There were no significant (p>0.05) effects of RSBM or of the two enzymes supplements or their interactions on internal organ weights of birds at 10 day age (data not shown).

However, Extra-dosing of neither microbial protease nor phytase had significant effects (p>0.05) on the internal organs of the experimental birds during the 1-10 d period. However, at 24 d, the weight of duodenum, SI (entire) and heart were reduced (p<0.05) by protease supplementation (Table 6.6). Except for the spleen (p<0.05), increasing levels of phytase had no influence (p>0.05) on the weights of other internal organs at 24 d of age.

Table 6.6 Effects of the extra-dosing of microbial protease and phytase in diets containing raw soybean on the weight of the visceral organs (g/100 g of body weight) of broilers at 24 day of age.

Protease g/kg	Phytase g/kg	Duode-num	G+P	SI	Heart	Liver	Bursa	Spleen
0.0	**0.1**	1.05[a]	3.13	4.47	0.77	2.66	0.20	0.09
	0.2	0.93[ab]	3.14	3.91	0.85	2.71	0.19	0.07
	0.3	1.08[a]	2.89	4.66	0.73	2.81	0.19	0.10
0.2	**0.1**	1.04[a]	3.09	4.59	0.72	2.74	0.23	0.09
	0.2	0.90[ab]	3.14	3.78	0.69	2.69	0.20	0.07
	0.3	1.03[ab]	2.78	4.54	0.74	2.71	0.14	0.09
0.4	**0.1**	0.95[ab]	2.94	3.76	0.64	3.03	0.22	0.12
	0.2	0.97[ab]	3.14	3.29	0.77	2.86	0.21	0.08
	0.3	0.83[b]	3.00	3.69	0.70	2.88	0.21	0.08
Pooled SEM		0.03	0.10	0.09	0.01	0.04	0.00	0.00
Main effects								
0.0		1.02	3.05	4.35[a]	0.78[a]	2.73	0.19	0.09
0.2		0.99	3.00	4.30[a]	0.72[b]	2.71	0.19	0.08
0.4		0.92	3.03	3.58[b]	0.70[b]	2.92	0.21	0.09
	0.1	1.01	3.05	4.27	0.71	2.81	0.21	0.10[a]
	0.2	0.93	3.14	3.66	0.77	2.75	0.20	0.07[b]
	0.3	0.98	2.89	4.30	0.72	2.80	0.18	0.09[ab]
Sources of variation								
Protease		*	NS	*	*	0.05	NS	NS
Phytase		NS	NS	NS	NS	NS	NS	*
Protease × phytase		*	NS	NS	NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different at ; NS= non-significant; * p<0.05; [1]control = normal diet without RSBM; SEM= pooled standard error of means;; SI= small intestine (jejunum and ileum with their contents); gizzard and proventriculus (G+P) were weighed with contents; RSBM=raw soybean meal (SBM was replaced by RSBM .[2]Test-diets= diets containing 25% RSBM-equivalent to 75 g/kg of diet.

At 24 d of age, the weight of the duodenum was significantly influenced by the interaction between protease and phytase, but the interaction between the two factors was not different for the other internal organs.

Table 6.7 Effects of the extra-dosing of protease and phytase in diets containing raw soybean on the weight of the pancreas (g/100 g of the body weight) at 10, 24 or 35 d.

Protease g/kg	Phytase g/kg	10 d	24 d	35 d
0.0	**0.1**	0.76	0.37	0.22
	0.2	0.73	0.40	0.23
	0.3	0.66	0.34	0.25
0.2	**0.1**	0.70	0.37	0.24
	0.2	0.65	0.39	0.23
	0.3	0.66	0.32	0.23
0.4	**0.1**	0.74	0.25	0.22
	0.2	0.70	0.25	0.24
	0.3	0.68	0.25	0.23
Pooled SEM		0.01	0.01	0.01
		Main effects		
0.0		0.72	0.37[a]	0.24
0.2		0.67	0.36[a]	0.24
0.4		0.71	0.25[b]	0.23
	0.1	0.73	0.33[a]	0.23
	0.2	0.69	0.34[a]	0.24
	0.3	0.67	0.31[b]	0.24
Sources of variation				
Protease		NS	***	NS
Phytase		0.06	*	NS
Protease × phytase		NS	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different at* p<0.05; ***p<0.001; NS= non-significant; [1]control = normal diet without RSBM; SEM= pooled standard error of means; RSBM=raw soybean meal (SBM was replaced by RSBM. One birds per cage, and totally 6 birds per treatment were killed to evaluate the pancreas at 10, 24 and 35 d of age. [2]Test-diets= diets containing 25% RSBM-equivalent to 75 g/kg of diet.

The weight of the pancreas relative to body weight at different ages is shown in Table 6.7. The weight of the pancreas at 10 and 35 d of age was not affected (p>0.05) by the extra-dosing of microbial protease; however, due to extra-dosing of phytase, the weight of the pancreas was tended (p=0.06) to be reduced by approximately 8.2% less at 10 d of age. As compared with the control, the weight of the pancreas obtained from birds on the test diets were significantly and strongly higher at 10 d (p<0.01), 24 d (p<0.01) and 35 d (p<0.05) of age by approximately 33, 40 and 36%.

Table 6.8 The preliminary cost benefit analysis of the diets containing raw, full-fat soybean meal and extra-dosed with protease and phytase for broiler chickens (1-35 d).

RSBM, %	Protease g/kg	Phytase g/kg	$/ MT of the diet	Production costs ($/kg BWG)
25	**0.0**	**0.1**	396.40	0.582
		0.2	392.50	0.568
		0.3	390.60	0.547
	0.2	**0.1**	401.80	0.558
		0.2	397.90	0.573
		0.3	396.00	0.587
	0.4	**0.1**	407.20	0.577
		0.2	403.30	0.552
		0.3	401.40	0.562
Control[1] (0 %RSBM)	**0.0**	**0.1**	400.00	0.580
Pooled SEM				0.005
Significance				NS

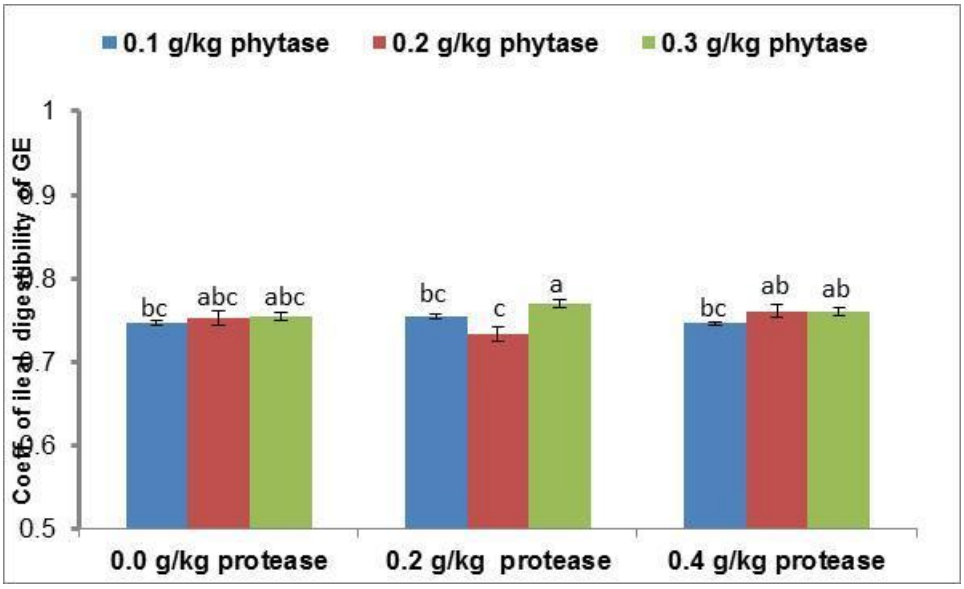
RSBM= raw soybean meal; [1]control= diets prepared without RSBM and protease; NS= non-significant.

The extra dosing of microbial protease ($p<0.001$) and phytase ($p<0.05$) as main factors, significantly reduced the weight of the pancreas at 24 d of age. No significant difference ($p>0.05$) in the weight of the pancreas as a result of the interaction effects between protease and phytase was observed during any of the periods assessed (1-10, 1-24 or 1-35 d).

As shown in Table 6.8, preliminary cost-benefit analysis showed that there was no statistical ($p>0.05$) difference when birds on the test diets were compared with those on the control. For example, birds on the treatment diets containing 25% of RSBM (replacing commercial SBM) supplemented with 0.3 g phytase/kg diet and without protease, and on the treatment supplemented with of 0.4 g protease/kg and 0.2 g phytase/ kg diet had around 6 and 5%, respectively, economic advantage compared to those allocated the control diet, but this was not significant ($p>0.05$). Diet costs were generally lower (not significantly) with RSBM in diets, but slightly increased as protease content went beyond 0.2 g/kg. The production cost of the best combined supplementation of microbial enzyme, for example 0.4 g protease x 0.2 g phytase, is cheaper than positive control diet (Table 6.8).

6.3.3 Ileal nutrient digestibility

The results of the AID for CP and AA are shown in Table 6.9. Birds fed the control diet had significantly higher AID of CP and all AA than birds fed the test diets containing 25% RSBM during the 1-24 d period. Methionine was the only AA, which the AID was not influenced ($p>0.05$) by presence of RSBM, protease or the phytase in diets. The greatest response to enzyme supplementation was observed for lysine.



[a,b,c] **Means bearing uncommon superscripts are significantly different.**

Figure 6. 1 Apparent ileal digestibility of gross energy (GE) on diets containing 25 % of raw soybean meal

Extra-dosing the test diets (but not the control diet) with protease improved the AID of CP and AA at 24 d of age. Extra-dosing of some of the diets with microbial phytase, improved the digestibility of threonine ($p<0.05$) and lysine ($p<0.01$). Phenylalanine and serine were the only AA, which the digestibility was influenced by the interaction between protease and phytase, but this result was not consistent.

As shown in Figure 6.1, the AID of gross energy was significantly improved ($p<0.001$) when the diets were extra-dosed with phytase. The AID was improved by only 1% in birds fed diets extra-dosed with protease and this response was not significant ($p>0.05$). The AID of the gross energy was also significantly improved ($p<0.01$) by interaction between protease and phytase.

Table 6.8 Effects of extra-dosing of protease and phytase on diets containing RSBM on GE, apparent ileal digestibility of CP and AAs for broilers at 24 d.

Protease g/kg	Phytase g/kg	Indispensable amino acids										Dispensable amino acids			
		CP	**His**	**Arg**	**Thr**	Val	**Lys**	**Met**	**Iso**	**Leu**	**Phe**	**Ser**	**Gly**	Pro	Ala
0.0	**0.1**	0.723	0.763	0.814	0.672	0.700	0.788	0.925	0.700	0.739	0.745^{bc}	0.665^{c}	0.646	0.719	0.737
	0.2	0.723	0.758	0.810	0.664	0.686	0.791	0.926	0.693	0.746	0.744^{c}	0.671^{c}	0.666	0.726	0.751
	0.3	0.724	0.762	0.815	0.681	0.705	0.795	0.913	0.706	0.735	0.746^{c}	0.678^{c}	0.657	0.715	0.732
0.2	**0.1**	0.731	0.756	0.814	0.657	0.706	0.786	0.920	0.688	0.733	0.741^{c}	0.661^{c}	0.672	0.723	0.747
	0.2	0.716	0.764	0.808	0.663	0.718	0.798	0.928	0.725	0.756	0.769^{b}	0.694^{b}	0.685	0.736	0.741
	0.3	0.706	0.787	0.823	0.709	0.732	0.821	0.921	0.676	0.722	0.712^{d}	0.641^{d}	0.664	0.710	0.735
0.4	**0.1**	0.748	0.781	0.833	0.698	0.740	0.823	0.924	0.757	0.791	0.787^{a}	0.718^{a}	0.693	0.747	0.777
	0.2	0.750	0.792	0.845	0.705	0.752	0.839	0.912	0.758	0.789	0.788^{a}	0.722^{a}	0.685	0.744	0.775
	0.3	0.737	0.790	0.838	0.710	0.752	0.839	0.915	0.760	0.792	0.794^{a}	0.724^{a}	0.671	0.739	0.775
Control[1]		0.779	0.807	0.854	0.734	0.774	0.854	0.908	0.784	0.803	0.807	0.756	0.751	0.779	0.801
Pooled SEM		0.004	0.006	0.003	0.005	0.005	0.004	0.002	0.006	0.005	0.005	0.006	0.005	0.004	0.004
		Main effects													
0		0.724^{b}	0.761^{b}	0.813^{b}	0.673^{b}	0.697^{b}	0.792^{c}	0.921	0.700^{b}	0.740^{b}	0.745	0.672	0.657^{b}	0.720^{b}	0.740^{b}
0.2		0.718^{b}	0.769^{b}	0.815^{b}	0.676^{b}	0.719^{b}	0.802^{b}	0.923	0.696^{b}	0.737^{b}	0.741	0.665	0.674^{b}	0.723^{b}	0.741^{b}
0.4		0.745^{a}	0.788^{a}	0.839^{a}	0.705^{a}	0.748^{a}	0.834^{a}	0.917	0.758^{a}	0.791^{a}	0.790	0.722	0.683^{a}	0.743^{a}	0.776^{a}
	0.1	0.734	0.768	0.821	0.678^{b}	0.717	0.801^{b}	0.923	0.718	0.757	0.760	0.684	0.672	0.731	0.756
	0.2	0.730	0.772	0.822	0.678^{b}	0.719	0.810^{b}	0.922	0.725	0.764	0.767	0.696	0.678	0.735	0.757
	0.3	0.723	0.778	0.825	0.698^{a}	0.728	0.816^{a}	0.916	0.716	0.751	0.753	0.684	0.664	0.722	0.747
Sources of variation															
Protease		***	***	***	**	***	***	NS	**	***	***	***	**	**	***
Phytase		NS	NS	NS	*	NS	**	NS	NS	NS	0.09	NS	NS	NS	NS
Protease x phytase		NS	NS	NS	NS	NS	NS	NS	0.08	NS	**	*	NS	NS	NS

a,b,c Means bearing uncommon superscripts within a column are significantly different at; 2. NS= not significant; 3. *P < 0.05; 4. **P < 0.001; 5. ***P < 0.001; 6. The 1control = normal diet without RSBM and protease; 7. SEM= pooled standard error of means; 8. CP= crude proteins; 9. RSBM replaced commercial SBM at 25 % o, in diets. Each treatment was replicated 6 times. 2Test-diets= diets containing 25% RSBM-equivalent to 75 g/kg of diet. I= test-diet+ no-protease; II= test-diet+ protease.

Table 6.9 Effects of protease and phytase extra-dosing in diets containing raw soybean on the tissue protein concentration and pancreatic enzyme activities in broilers at 10 or 24 d of age.

Protease g/kg	Phytase g/kg	Tissue protein conc. (mg/g)		Pancreatic enzyme activities (nmole/mg protein/min)						
				10 d				24 d		
		10 d	24 d	Trypsin	Chymotrypsin	GP	Lipase	Trypsin	Chymotrypsin	GP
0.0	**0.1**	34.0	34.2	6.2	6.0	5.2	3.0	6.3	6.0	4.0
	0.2	34.8	35.1	4.9	5.3	4.5	3.0	4.4	5.8	4.9
	0.3	34.1	34.4	4.6	5.5	4.6	2.6	6.0	6.2	4.8
0.2	**0.1**	34.3	34.4	5.2	6.3	5.0	2.3	5.8	6.0	3.9
	0.2	34.2	34.6	5.0	5.1	4.7	2.9	6.9	6.4	4.5
	0.3	34.6	35.5	5.1	6.7	5.4	2.4	4.8	5.2	3.8
0.4	**0.1**	34.4	35.0	6.2	5.7	5.7	2.9	4.8	6.2	4.8
	0.2	33.3	34.7	6.0	4.7	5.5	3.8	5.5	6.5	4.8
	0.3	33.4	34.7	4.8	6.2	5.2	3.3	6.0	7.4	4.2
Control[1]	**0.1**	34.3	34.2	4.7	5.8	4.9	3.1	6.0	6.9	5.3
Pooled SEM		0.2	0.2	0.2	0.2	0.1	0.1	0.3	0.2	0.2
Main effects										
0.0		34.3	34.6	5.2	5.6	4.8	2.9	5.4	6.0	4.6
0.2		34.4	34.8	5.1	6.0	5.1	2.5	5.8	5.8	4.1
0.4		33.7	34.8	5.6	5.6	5.4	3.3	5.4	6.6	4.6
	0.1	34.2	34.6	5.8	6.0	5.2	2.8	5.6	6.1	4.3
	0.2	34.1	34.8	5.3	5.1	4.9	3.2	5.5	6.2	4.7
	0.3	34.0	34.9	4.8	6.0	5.1	2.8	5.6	6.2	4.2
Sources of variation										
Protease		NS	NS	NS	NS	NS	NS	NS	NS	NS
phytase		NS	NS	NS	NS	NS	NS	NS	NS	NS
protease x phytase		NS	NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different at $p<0.05$ (NS= non-significant); 1control = normal diet without RSBM; conc. = concentration; SEM= pooled standard error of means; GP= general proteolysis; RSBM= raw soybean meal (SBM was replaced by RSBM at 25%, equivalent to 75 g/ kg of diet).

6.3.4 Tissue proteins and digestive enzyme activities

The pancreatic tissue protein contents and enzyme activities are shown in Table 6.10. Neither increasing levels of microbial protease nor phytase had significant effects ($p>0.05$) on tissue proteins or enzymatic activities at 10 d or 24 d of age. There was no significant ($p>0.05$) effects due to interactions between the main factors (protease x phytase).

Increasing protease supplementation increased the activities of some pancreatic enzymes, such as trypsin (7.1%), general proteolytic activities (11.1%) and lipase (12.1%) at 10 d of age. At 24 d, the pancreatic protein content increased by 5.8% and chymotrypsin activity also increased (9.1%) as the result of protease supplementation of diets.

6.3.5 Mucosal morphometry

The results of the mucosal morphometry of the jejunum are shown in Tables 6.11 and 6.12. Increasing the level of protease in diets had no significant effect ($p>0.05$) on any measured histological parameter, except for the villus height ($p<0.05$) and crypt depth ($p<0.05$), which were significantly increased due to extra-dosing of phytase during the 1-10 d period.

The extra-dosing of phytase increased the mucosal depth and apparent villus surface area by 2.9 and 20%, respectively, but was not statistically significant ($p>0.05$). The villus height was significantly ($p=0.05$) influenced by interactions between the main factors (protease x phytase) in diets during the 1-10 d period. Birds fed the control diets were not significantly different ($p>0.05$) in the mucosal morphometry from the birds on the test diets during the 1-10 d period.

Increasing the inclusion rate of protease in diets had no significant influence ($p>0.05$) on the measured histological parameters. However, the villus height ($p=0.07$), mucosa depth ($p=0.08$) and villus to crypt depth ratio ($p=0.08$) at 24 d tended to be affected. The interaction between the main factors (protease x phytase) also affected the villus height ($p=0.05$) and mucosal depth ($p<0.01$) at 24 d of age.

Table 6.10 Effects of the extra-dosing of microbial protease and phytase in diets containing raw soybean meal on the mucosal morphometry (µm) of the jejunum at 10 d of age.

Protease g/kg	Phytase g/kg	Muscle depth	Mucosal depth	Villus length	Crypt depth	Villus: Crypt	AVSA (mm^2)
0.0	**0.1**	219.3	1141.2	1006.3	134.9	7.5	0.04
	0.2	184.9	1216.9	1079.6	137.3	7.9	0.04
	0.3	211.8	1156.5	1022.2	134.2	7.7	0.05
0.2	**0.1**	184.0	1124.7	993.5	131.2	7.6	0.04
	0.2	236.7	1300.9	1164.4	136.5	8.6	0.06
	0.3	232.6	1198.1	1056.8	141.3	7.5	0.04
0.4	**0.1**	240.6	1127.8	994.3	133.5	7.5	0.04
	0.2	241.7	1182.4	1049.2	133.2	7.9	0.04
	0.3	195.3	1236.6	1101.3	135.2	8.2	0.05
Control[1]		216.4	1203.3	1070.2	133.1	8.1	0.04
Pooled SEM		7.22	15.53	12.31	0.83	0.12	0.02
Main effects							
0.0		206.5	1168.8	1033.5	135.4	7.7	0.04
0.2		215.6	1202.7	1066.7	136.0	7.9	0.05
0.4		230.6	1178.1	1044.3	133.7	7.8	0.04
	0.1	211.4	1131.6^{b}	998.5^{b}	133.1	7.5	0.04
	0.2	222.4	1230.2^{a}	1094.7^{a}	135.5	8.1	0.05
	0.3	215.7	1188.5^{ab}	1051.5^{ab}	137.0	7.7	0.05
Sources of variation							
Protease		NS	NS	NS	NS	NS	NS
Phytase		NS	*	*	NS	NS	NS
Protease x phytase		NS	0.05	NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different at NS= non-significant; *$p<0.05$; [1]control = normal diet without RSBM; SEM= pooled standard error of means; AVSA= apparent villus surface areas; villus: crypt= villus height to crypt depth ratio; RSBM=raw soybean meal (SBM was replaced by RSBM at 25%, equivalent to 75 g/ kg of diet).

Table 6.11 Effects of the extra-dosing of microbial protease and phytase in diets containing raw, full-fat soybean meal on the mucosal morphometry (µm) of jejunum at 24 d of age.

Protease g/kg	Phytase g/kg	Muscle thickness	Villus length	Crypt depth	Mucosal depth	Villus: Crypt	AVSA (mm^2)
0.0	**0.1**	242.7	1253.9[ab]	145.9	1399.7[ab]	8.8	0.06
	0.2	219.4	1224.7[ab]	136.6	1361.2[ab]	9.0	0.07
	0.3	263.9	1196.8[ab]	152.7	1349.5[ab]	8.0	0.05
0.2	**0.1**	256.1	1398.0[a]	142.7	1540.7[a]	9.9	0.07
	0.2	280.1	1447.1[ab]	130.2	1577.3[ab]	11.2	0.06
	0.3	260.4	1105.5[ab]	141.4	1246.9[ab]	7.9	0.05
0.4	**0.1**	257.5	1183.1[b]	134.4	1317.5[b]	8.9	0.05
	0.2	299.3	1234.4[ab]	141.0	1375.4[ab]	8.9	0.05
	0.3	256.8	1229.1[ab]	136.1	1365.1[ab]	9.1	0.05
Control[1]		270.5	1365.0[ab]	136.3	1501.3[ab]	7.2	0.06
Pooled SEM		10.1	23.4	2.3	2.3	0.3	0.01
Main effects							
0		240.0	1227.7	144.3	1372.0	8.7	0.06
0.2		261.9	1284.3	140.1	1424.4	9.3	0.06
0.4		270.0	1220.1	137.2	1357.3	9.0	0.05
	0.1	252.0	1296.2	141.7	1437.9	9.3	0.06
	0.2	263.5	1273.1	137.1	1410.1	9.4	0.06
	0.3	259.6	1178.0	141.5	1319.6	8.5	0.05
Sources of variation							
Protease levels		NS	NS	NS	NS	NS	NS
Phytase levels		NS	0.07	NS	0.08	0.08	NS
Protease x phytase		NS	0.05	NS	*	NS	NS

[a,b,c] Means bearing uncommon superscript within a column are significantly different at NS= not significant; * $p<0.05$; [1]control = normal diet without RSBM; villus: crypt= ratio of villus height to crypt depth; SEM= pooled standard error of means; AVSA= apparent villus surface areas; RSBM= raw soybean meal (SBM was replaced by RSBM at 25%, equivalent to 75 g/ kg of diet).

6.4 DISCUSSION

6.4.1 Gross response to the diets

The gross response of birds fed the control (RSBM-free) diet, especially BWG was better than that of the birds on RSBM-containing diets during the 1-24 and 1-35 d periods. This result is partially consistent with the results of previous studies (Liu *et al.,* 1998; Newkirk, 2010), demonstrating that birds fed diets containing RSBM performed poorly. Since the average TI content in diets containing 25% RSBM was approximately 10,193.4 TIU/kg, a value beyond

the threshold level for birds, there were significant differences in the gross responses of these birds over the entire period (1-35 d) compared with birds fed on RSBM-free (control) diets. This may be due to the effects of supplemental protease, a fact that has been highlighted by Barletta (2011) who reported that microbial proteases are protein-digesting microbial enzymes that can break down both the stored proteins as well as proteinaceous anti-nutrients in vegetable proteins. The statistical similarity in gross response of birds fed diets containing 25% RSBM compared with birds fed the control diets, might reflect a reduction in the negative impact of TI through the supplementation of protease and phytase.

The extra-dosing of microbial protease improved the FI and BWG of birds in the early period (1-10 d). The extra-dosing of microbial phytase also significantly improved the FI and BWG of birds during the 1-24 d period. The response to both enzymes is similar to those reported previously during the 1-24 d period (Erdaw *et al.*, 2016a). However, both enzymes did not significantly improve the FI and BWG over 1-35 d, but managed to support productivity close to the breed standard. It is still not clear how much impact the test protease has on raw soya proteins, but the test phytase appears to be effective in dealing with the phytate contents of RSBM. This is a confirmation of results previously obtained in an *in vitro* assay by this research group (Erdaw *et al.*, 2016b).

The FI, BWG or FCR of birds was not affected through the interaction effects between protease and phytase in diets. However, other studies (Cowieson and Adeola, 2005; Murugesan *et al*., 2014) have reported improvements in the performance of birds as a result of the combined effects of microbial phytase and protease, although such studies were on RSBM-free diets.

6.4.2 Development of internal organs, tissue proteins and digestive enzyme activities

During all periods assessed birds fed on the control diets had significantly smaller pancreas than those on RSBM-containing diets. This result is consistent with previous findings (Newkirk, 2010; Erdaw *et al.*, 2015b), revealing that birds fed diets containing RSBM experience enlargement of pancreas. The mechanism surrounding these changes is not clearly understood.

The extra-dosing of microbial protease in diets significantly reduced the weight of the pancreas, duodenum, SI, heart and the liver at 24 d of age. These results are partially consistent with those of other studies (Pettersson and Pontoppidan, 2013; Ravindran, 2013) who showed that the supplementation of feed enzymes reduced the impact of anti-nutritional factors in non-

ruminant animals. The reduction in the weight of some internal organs, including the pancreas in the current study might indicate that the negative impact of trypsin inhibitors is reduced in response to test protease assessed in the study.

Except on pancreas, the test phytase in diets did not appear to have any effect on any internal organ weights. These results partially agree with Wang *et al.* (2013) who reported that phytase supplementation improved development of internal organs.

6.4.3 Apparent ileal nutrient digestibility and enzyme activities

Neither the extra-dosing of microbial protease nor phytase had significant effects on tissue protein content or enzyme activities at 10 or 4 d of age. However, there was marginal response to protease supplements by particularly, pancreatic enzymes. Murugesan *et al.* (2014) reported that increased activities of ileal maltase, sucrase and aminopeptidase in response to a cocktail of protease and phytase.

The AID of CP and a majority of AA, including isoleucine, leucine, phenylalanine, threonine and lysine were higher in broilers fed diets extra-dosed with microbial protease during the 1-24 d period. These results are consistent with those of previous studies (Oxenboll *et al*., 2011; Pettersson and Pontoppidan, 2013; Olukosi *et al.,* 2014) who showed that supplementation of diets with protease alone reduces the impact of anti-nutritional factors; thus to improve digestibility of proteins and AA. These authors also proposed that when proteases are combined with other feed enzymes, such as amylase, feed efficiency could be improved. The improvement in the AID of CP and AA of this study might be due to subsequent increase in activities of pancreatic enzymes of broilers due to the contribution of microbial protease supplementation.

Most of the AID values for indispensable AA, such as threonine, valine and isoleucine, increased with the addition of phytase, but this effect was not significant. Ravindran *et al. (*2001) and Amerah *et al.* (2014) have similarly shown that increasing the level of supplemental phytase in the diet improved the digestibility of nitrogen and all AA. The AID of majority of indispensable AA was improved due to the interaction between protease and phytase. This result is partially consistent with previous findings (Ravindran and Bryden, 1999; Olukosi *et al.,* 2014) in studies with other enzyme combinations.

6.4.4 Mucosal morphometry

There were no significant differences between treatment groups in any of the measured histological morphometric parameters, reflecting the extra-dosing of microbial protease in the diets; however, notably, the villus height and crypt depth were increased with phytase extra-dosing during the 1-10 d period. In addition, the extra-dosing of phytase also increased most jejunal mucosal parameters, such as villus length, mucosal depth and villus to crypt depth ratio, during the 1-24 d period. Moreover, the interaction effects between extra-dosing of protease and phytase, as main factors in the diets, significantly influenced the mucosal depth during the 1-24 d period. These results are partially in agreement with the results of Nourmohammadi and Afzali (2013) who reported that birds fed diets supplemented with phytase showed significant increases in the crypt depth and villus width of jejunum mucosal tissues.

6.5 CONCLUSION

The results of the present study suggest that commercial SBM could be replaced with RSBM at up to ≤ 25% in broiler diets if the diets are supplemented with appropriate microbial protease and phase. In general, birds fed on the control diets consumed more feeds and gained more body weight than others on the tested diets. The weights of most of the internal organs were also reduced in diets extra-dosed with protease. These results therefore suggest that the negative impact of ANFs, including TI, in diets could be minimised through supplementation with protease. The results of this study also indicate that when compared with phytase, the efficacy periods of extra dosing protease were earlier in diets containing RSBM. Although not significant, the AID of most of the AA was increased in diets extra-dosed with protease or phytase, and some AAs were significantly affected through interactions between protease and phytase. The villus height, mucosal depth and villus height to crypt depth ratio were significantly affected by extra-dosing of phytase in diets during the 1-24 d period. The results of this study largely support the improvement recorded in *in vitro* nutrient digestibility using the enzyme cocktail (protease+ phytase) described in previous chapter of this project. However, there is a need for the assessment of wellbeing of broilers fed diets containing RSBM and more cost-benefit analysis of the response to the diets that were tested.

CHAPTER 7: PERFORMANCE AND DIGESTIBILITY OF BROILER CHICKENS FED DIETS CONTAINING GRADED LEVELS OF RAW, FULL-FAT SOYBEAN MEAL SUPPLEMENTED WITH MICROBIAL PROTEASE

ABSTRACT

In order to evaluate the performance and ileal nutrient digestibility of broilers, a 2 x 3 study was conducted, with two levels of protease (0 or 0.2 g/kg) and three levels of raw, full-fat soybean meal (RSBM) that replaced the commercial SBM at 0, 15, or 25%, and a commercial diet (prepared without RSBM and protease). Phytase (2000 FYT/kg) was uniformly added, and each of these dietary treatment was replicated six times, with eight birds per replicate. Sawdust was used as the bedding material on which birds were raised in climate-controlled rooms and offered corn-soybean-based starter (1-10 d), grower (11-24 d) and finisher (1-35 d) diets, formulated to the Aviagen standard for Ross 308 broiler. On day 19, all of the birds on the commercial diets were transferred to N-free diet (NFD) for the next 5 consecutive days. Consequently, on day 24, samples of ileal digesta were collected from all of the treatment groups, including birds on the NFD. All of the remaining birds, except those on NFD were allowed to continue to 35 d of age to evaluate the gross responses, in terms of FI, BWG and FCR. Neither RSBM nor protease supplementation influenced ($p>0.05$) the FI throughout the entire experimental period (1-35 d). Increasing the level of RSBM in the diets reduced the BWG during the 1-10-d ($p<0.01$), 1-24-d ($p<0.05$), and 1-35 period ($p<0.05$). However, protease supplementation enabled significant improvement ($p<0.05$) in BWG and feed efficiency in the 1-24-d period. Increasing the RSBM inclusion rate in diets increased the weights of the gizzard + proventriculus (G+P) ($p<0.001$), pancreas ($p<0.001$), small intestine (SI) ($p<0.001$), heart ($p<0.001$), and spleen ($p<0.01$) and tended ($p=0.9$) to reduce that of the bursa at 24 d of age. Protease supplementation had no effect ($p>0.05$) on any of the measured internal organs. Increasing the inclusion rate of RSBM in the diets significantly increased ($p<0.001$) the loss of undigested and unabsorbed ileal CP, whereas both apparent ileal digestibility (AID) and the corresponding standardized ileal digestibility (SID) of CP were reduced significantly ($p<0.01$). When diets were supplemented with increasing levels of RSBM, the average loss of undigested and unabsorbed ileal AA was significantly increased, but the AID and SID of AA, except that of methionine, were also significantly reduced. Due to protease supplementation, however, approximately 6.5% of the loss due to undigested and unabsorbed ileal CP was reduced, and consequently, the AID and SID of CP were significantly increased ($p<0.05$). When the diets were supplemented with protease, the loss of undigested and unabsorbed ileal AA was reduced by approximately 4.5% (indispensable) and 1.9% (dispensable). Protease supplementation increased the AID and SID of AA, by between 0.11 and 2.32%, and 0 and 1.5%, respectively, except for that of methionine, compared to no supplementation, but the differences were not statistically significant ($p>0.05$). The AID

($p<0.5$) and SID ($p<0.05$) values of lysine were significantly improved with protease supplementation. It can be concluded that although commercial SBM was replaced by RSBM, at up to 25% in diets, the gross response of broilers over 35 d was not statistically affected. These results might be due to the supplementation of protease and phytase in diets. The AID and SID of CP and AA were significantly reduced with increasing levels of RSBM, but these were also slightly improved by supplemental protease.

7.1 INTRODUCTION

It is well-established that soybean meals have good nutritional quality for non-ruminant animals, but the nutritive value of raw, full-fat soybeans is adversely affected by the presence of ANF (Liu *et al*., 1998; Newkirk, 2010; Erdaw *et al*., 2016). Certain antinutrtional factors (ANF) are also heat-stable, and the most well-known (best characterized) ANFs in soybeans are protease inhibitors, lectins and phytates (Pettersson and Pontoppidan, 2013).

Trypsin inhibitor (TI) can be found in different compound states (Maliar *et al.,* 2004) and are the most important ANF in soybeans that are able to tightly bind to the trypsin and block its protein digestion capabilities (Nahashon and Kilonzo-Nthenge, 2011). The presence of dietary TI in legumes, such as soybeans, causes a substantial reduction in the digestibilities (up to 50%) of proteins and amino acids (AA) and protein quality (up to 100%) in non-ruminant animals (Gilani *et al.*, 2012). Nitrogen retention can be negatively affected by TI and result in an increase in endogenous nitrogen excretion (Banaszkiewicz, 2011; Dourado *et al*., 2013). Although Barth *et al*. (1993) reported that RSBM in diets caused endogenous protein losses, Clarke and Wiseman (2005) reported that AA digestibility in both commercial SBM and raw, full-fat SBM did not correlate with the concentration of TI. In general, however, AA digestibility could be affected by many factors, such as the undigested and unabsorbed AA of dietary origin, and due to endogenous losses (Ravindran *et al*., 2004).

Phytate (phytic acid) is another component of ANF in soybeans that is mostly found in the form of protein-phytate or protein-phytate-protein complexes and is resistant to digestion by proteolytic enzymes. Phytate is usually antagonistic by interfering with protein digestion through formation of chelates with nutritionally essential mineral elements (Liener, 1994; Hurrell, 2003; Chen *et al*., 2013). Phytate also impedes nutrient digestion through electrostatic

mechanisms, which reduces the solubility of proteins; consequently, increasing the loss of endogenous nutrients (Cowieson and Ravindran, 2007).

Although heating is considered to be the best method to eliminate or reduce ANF, some of the ANF in soybeans, such as the Bowman-Birk inhibitor (BBI), phytates and oligosaccharides are not heat-labile. These ANF remain a problem for meals prepared from raw soybean and poorly processed soya meals. Clemente *et al.* (2008) also reported that a type of trypsin inhibitor (TI) called the BBI exhibits resistance to heat treatment, so supplementation of those corn-soya-based diets with microbial enzymes including protease and phytase is necessary (Dourado *et al.,* 2011).

The potential for further improvements in the nutritional value of soybeans using exogenous enzymes has therefore been suggested by many researchers (Zohair *et al.,* 2010; Ao, 2011). Bedford and Schulze (1998) also suggested that supplementation with microbial enzymes could reduce the negative impacts of ANF in the feedstuffs, and the bioavailability of nutrients could be increased (Martinez-Amezcua *et al.,* 2006), subsequently reduces nutrient excretion (Adeola and Cowieson, 2011). Romero and Plumstead (2013) also added that the digestibilities of amino acids were improved when broilers were fed diets supplemented with exogenous protease, which had a synergistic effect with other feed enzymes, such as carbohydrase, in the improvement of protein digestibility.

However, the performance and ileal nutrient digestibility of broilers fed a high concentration of dietary TI (in raw soybean) when supplemented with phytase and proteases have not yet been evaluated. Therefore, the objectives of this study were to evaluate the performance of birds fed diets containing varying levels of RSBM and supplemented with protease and to evaluate the apparent and standardized ileal digestibility of CP and AA.

7.2 MATERIALS AND METHODS

The experiment was conducted at the Animal House of the University of New England (UNE). It was approved by the University's Animal Ethics Committee (Authority No: AEC15-044) prior to commencement.

7.2.1 Experimental design and diets

A 2 x 3 factorial study, with 2 levels of protease (0 or 0.2 g/kg) and 3 levels of RSBM, which replaced commercial SBM at 0, 15, or 25%, equivalent to 0, 45 or 75 g/kg of diet, respectively,

and a commercial diet (without protease and RSBM) was used in this study. Diets were uniformly supplemented with microbial phytase (2000 FYT/kg), and each treatment was replicated six times with eight birds per replicate.

The birds were raised on litters (sawdust) in a climate-controlled room. The birds were offered with corn-soybean-based starter (0-10 d) and grower (10- 24 d) diets formulated to the Aviagen standard for Ross 308 broiler chicks. On day 19, the birds on the commercial grower diet were transferred to a nitrogen-free diet (NFD) and allowed to feed for the next 5 consecutive days.

7.2.2 Animal husbandry and sampling

A total of 336 day-old Ross 308 male broiler chicks (43.84±0.18 g), were obtained from a local commercial hatchery (Baiada Poultry Pty. Ltd., Tamworth, Australia). Eight chicks were weighed and allocated into each of the 42 multi-tiered pens (600 x 420 x 23 cm), which were randomized. Every pen was equipped with a feeder and two nipple drinkers. The feeders were scrubbed and cleaned before providing the diets. The drinkers were also checked from time to time to ensure that they worked properly and they were also cleaned whenever they were dirty.

The temperature of the room was set at 33^0C for the first two days, with relative humidity between 49 and 60%. This temperature was then gradually reduced to 24^0C at 19 days of age and this was maintained for the remaining study period. For the first 2 days, 24 h of light (20 lux) was provided. This was then reduced to 23 h for the next 6 consecutive days, followed by 20 h lights (10 lux) for the remaining days. Feed, in the form of crumble (starter) and pellet was provided ad libitum, and the birds had free access to water. Mortality was recorded whenever it occurred.

Table 7.1 Ingredients and composition of starter (0-10 d) diets (as-fed basis).

	Commercial diet	**RSBM (%)**					
		0		**15**		**25**	
Protease (g/kg)	**0.0**	**0.0**	**0.2**	**0**	**0.2**	**0**	**0.2**
Ingredients (g/kg)							

Corn (Rolled)	594.0	594.0	594.0	600.0	600.0	594.0	594.0
Soybean meal	300	300	300	255.0	255.0	225.0	225.0
Raw soybean meal	0.0	0.0	0.0	45.0	45.0	75.0	75.0
Meat meal	62.9	62.9	62.9	61.1	61.1	72.3	72.3
Canola Oil	16.0	16.0	16.0	9.4	9.4	7.9	7.9
Dical phosphate	7.7	7.7	7.7	9.0	9.0	6.7	6.7
Limestone	6.2	6.2	6.2	6.5	6.5	5.3	5.3
Salt	3.0	3.0	3.0	3.0	3.0	3.0	3.0
L-lysine	2.7	2.7	2.7	3.1	3.1	2.7	2.7
DL-methionine	2.3	2.3	2.3	2.1	2.1	2.5	2.5
Premix 2 kg/mt[1]	2.0	2.0	2.0	2.0	2.0	2.0	2.0
L-threonine	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Sodium bicarb	1.1	1.1	1.1	1.6	1.6	1.1	1.1
Choline Cl	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Phytase	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Protease	0.0	0.0	0.2	0.0	0.2	0.0	0.2
Nutrients (g/kg)							
ME Poultry (MJ/kg)	12.59	12.59	12.59	12.59	12.59	12.59	12.59
Crude Protein	225.8	225.8	225.8	225.8	225.8	226.0	226.0
Crude fat	42.1	42.1	42.1	39.8	39.8	44.6	44.6
Arginine	14.4	14.4	14.4	14.3	14.3	14.4	14.4
Lysine	14.0	14.0	14.0	14.0	14.0	14.0	14.0
Methionine	5.7	5.7	5.7	5.2	5.2	5.7	5.7
Methionine + cysteine	8.8	8.8	8.8	8.3	8.3	8.8	8.8
Threonine	9.9	9.9	9.9	9.4	9.4	9.9	9.9
Calcium	10.0	10.0	10.0	10.3	10.3	10.0	10.0
Phosphorus avail	5.0	5.0	5.0	5.1	5.1	5.0	5.0
Choline	1.4	1.4	1.4	1.4	1.4	1.2	1.2

[1]Composition of the premix are as shown in Table 6.1; RSBM= raw soybean meal (SBM was replaced by RSBM at 0, 15 and 25 %, equivalent to 0, 45 and 75 g/kg of diet, respectively).

Table 7.2 Ingredients and ccomposition of NFD, commercial and basal grower (10-24 d) diets (as-fed basis).

	NFD[2]	**Commercial diet**	**RSBM (%)**		
			0.0	**15**	**25**
Ingredients (g/kg)					
Corn (Rolled)	0.0	579.0	579.0	584.7	577.5

Corn starch	433	0.0	0.0	0.0	0.0
Dextrose	405	0.0	0.0	0.0	0.0
Soybean meal	0.0	300	300	255.0	225.0
Raw soybean meal	0.0	0.0	0.0	45.0	75.0
Canola Oil	50	45.6	45.6	40.5	40.3
Meat meal	0.0	39.0	39.0	37.1	46.0
Cellulose	50.0	0.0	0.0	0.0	0.0
Dical phosphate	25.7	9.5	9.5	12.0	10.0
TiO_2	5.0	5.0	5.0	5.0	5.0
Limestone	11.9	10	10	7.5	8.5
Salt	0.0	2.3	2.3	2.0	2.3
L-lysine	0.0	2.0	2.0	2.4	2.2
DL-methionine	0.0	3.3	3.3	3.8	3.7
UNE premix 2 kg/mt[1]	2.0	2.0	2.0	2.0	2.0
L-threonine	0.0	0.6	0.6	1.5	0.9
Sodium chloride	2.5	0.0	0.0	0.0	0.0
Sodium bicarb	1.9	1.1	1.1	1.o	0.8
Magnesium sulphate	1.2	0.0	0.0	0.0	0.0
Potassium Chloride	9.0	0.0	0.0	0.0	0.0
Choline Cl	3.5	1.0	1.0	0.5	0.8
Phytase	0.0	0.2	0.2	0.2	0.2
Nutrients (g/kg)					
ME Poultry (MJ/kg)	14.44	13.28	13.28	13.28	13.28
Crude Protein	0.0	210.0	210.0	210.7	208.6
Crude fat	0.0	68.7	68.7	67.7	73.6
Arginine	0.0	13.4	13.4	13.3	13.3
Lysine	0.0	12.7	12.7	12.7	12.8
Methionine	0.0	6.5	6.5	6.7	6.6
Methionine + cysteine	0.0	9.4	9.4	9.7	9.6
Threonine	0.0	8.2	8.2	8.9	8.3
Calcium	10.0	9.9	9.9	9.4	9.8
Phosphorus avail	4.8	4.4	4.4	4.8	4.6
Choline	1.5	1.5	1.5	1.3	1.2

[1]composition of the premix are as shown in Table 6.1. In addition to the NFD and commercial diets, the basal diets containing varying levels of RSBM (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diets) was supplemented with protease (0.0, 0.2 g/kg).

Table 7.3 Ingredients and composition of finisher (24-35 d) diets (as-fed basis).

RSBM (%)	**0**		**15**		**25**	
Protease enzyme (g/kg)	**0.0**	**0.2**	**0.0**	**0.2**	**0.0**	**0.2**
Ingredients (g/kg)						
Corn (Rolled)	603.0	603.0	611.4	611.4	613.9	613.9
Soybean meal	300.0	300.0	255.0	255.0	225.0	225.0

Raw soybean meal	0.0	0.0	45.0	45.0	75.0	75.0
Canola Oil	51.1	51.1	46.3	46.3	43.5	43.5
Meat meal	10.0	10.0	6.0	6.0	6.0	6.0
Dical phosphate	15.8	15.8	15.5	15.5	15.5	15.5
Limestone	11.0	11.0	9.8	9.8	10.0	10.0
Salt	2.2	2.2	2.1	2.1	2.1	2.1
L-lysine	0.7	0.7	1.0	1.0	1.0	1.0
DL-methionine	2.2	2.2	2.7	2.7	2.8	2.8
UNE premix 2 kg/mt[1]	2.0	2.0	2.0	2.0	2.0	2.0
L-threonine	0.5	0.5	1.8	1.8	1.8	1.8
Sodium bicarb	1.0	1.0	1.0	1.0	1.0	1.0
Choline Cl	0.5	0.5	0.5	0.5	0.5	0.5
Phytase	0.2	0.2	0.2	0.2	0.2	0.2
Protease	0.0	0.2	0.0	0.2	0.0	0.2
Nutrients (g/kg)						
ME Poultry (MJ/kg)	13.49	13.49	13.49	13.49	13.49	13.49
Crude Protein	192.1	192.1	192.9	192.9	190.5	190.5
Crude fat	68.8	68.8	70.7	70.7	71.9	71.9
Arginine	12.3	12.3	12.3	12.3	12.1	12.1
Lysine	10.5	10.5	10.7	10.7	10.6	10.6
Methionine	5.0	5.0	5.4	5.4	5.4	5.4
Methionine + cysteine	7.9	7.9	8.3	8.3	8.3	8.3
Threonine	7.4	7.4	8.7	8.7	8.6	8.6
Calcium	8.7	8.7	8.5	8.5	8.5	8.5
Phosphorus avail	4.2	4.2	4.3	4.3	4.2	4.2
Choline	1.4	1.4	1.3	1.3	1.2	1.2

[1]composition of the premix are as shown in Table 6.1. [1]RSBM= raw soybean meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively).

7.2.3 Data collection

On day 24, the ileal digesta were collected from birds in all treatment groups, including those on the NFD. The ileal digesta samples were pooled on ice and then stored in a freezer (-20^{0}C) until analysis for nutrient composition. Except those birds on NFD, samples of internal organs were also collected on day 24. However, all birds, except those allocated to the NFD, were allowed to continue in the study to 35 days of age, with the aimed of evaluating the performance to market weight.

On days 10, 24 and 35, the body weight of birds and feed leftover were recorded to calculate body weight gain (BWG) and feed intake (FI), from which the feed conversion ratio (FCR) was computed. All sample collection was completed between 0900 and 1200 hr.

7.2.4 Chemical analysis and calculation of nutrient digestibility

The N contents in samples of diets and litter were determined by the Dumas combustion technique (LECO) Official Method 990.03 (Kjeldahl) and Official Method 984.13 (A-D) (AOAC, 2006). Then the CP was calculated as N x 6.25,
Quality measuring parameters of diets, containning varying levels of RSBM, such as KOH, TI, UA and NSI were analyzed as described in previous Section 5.2.5.

The concentration of titanium (Ti) in the ileal digesta and diets was determined using the method described by Short *et al*. (1996). The values of the nutrient and the Ti marker were used to calculate the ileal digestibility as follows:

7.2.5 Analysis of apparent and standardized ileal AAs and protein digestibilities

Ileal amino acid (AA) outflow (IAAF) and ileal crude protein (CP) outflow (ICPF) (mg/g intake) for all treatments (including NFD) were determined against titanium (Ti) as follows:

$$\text{IAAF or ICPF} = \frac{\text{AA or CP in digesta (mg/g)} \times \text{Ti in diet (mg/g)}}{\text{Ti in digesta (mg/g)}} \quad (1)$$

The coefficient of apparent ileal digestibility (AID) and the coefficient of standardized ileal digestibility (SID) of CP and AA were calculated using the following equations:

$$\text{AID} = \frac{(\text{diet AA or CP intake - total IAAF or ICPF})}{\text{diet AA or CP intake}} \quad (2)$$

$$\text{SID} = \frac{(\text{diet AA or CP intake- [total IAAF or ICPF-EIAAF or ECPF]})}{\text{diet AA or CP intake}} \quad (3)$$

where, EIAAF is the endogenous ileal amino acid flow, and ECPF is the endogenous crude protein flow calculated using Eq. 1 from the ileal digesta of chicks fed NFD.

7.2.6 Statistical analysis

A general linear model (GLM) was used to analyse the data using Minitab software version 17 (Minitab, 2013). The data were analysed using a general linear model (GLM) to evaluate the RSBM and enzyme supplementation levels as the main factors as well as their interactions. The differences were considered to be significant at $p < 0.05$, and the significant differences between mean values were also separated using the Duncan's test.

7.3 RESULTS

7.3.1 Gross response to diets

Analysed values of quality measuring parameters, including TI, UA, KOH and NSI of the test diets (contained graded levels of RSB) were reported in Section 4.2.2. The results of the gross response of birds in terms of FI, BWG and FCR are indicated in Table 7.4. The results revealed that by increasing the level of RSBM, the FI was negatively influenced ($p<0.05$) in the 1-35 d periods and 1-35 d ($p<0.05$) and to be tended in the 1-10 d ($p=0.08$), but protease supplementation did not significantly ($p>0.05$) influence the FI. Increasing the inclusion rate of RSBM in the diets significantly reduced the BWG in the periods of 1-10 d ($p<0.01$) and 1-24 d ($p<0.05$) and 1-35 d ($p<0.05$) to reduce the BWG in the 1-35-d period. However, protease supplementation improved ($p<0.05$) both the BWG and the feed efficiency in the 1-24 d period. Increasing the level of RSBM in diets reduced the feed efficiency by 2.94%, whereas diet supplementation with microbial protease improved the feed efficiency by 3.30%. However, the differences were not statistically significant ($p>0.05$).

There were no significant ($p>0.05$) interaction effects between RSBM and protease on the FI, BWG or FCR of birds during any of the assessed periods (1-10 d, 1-24 d or 1-35 d), and there were no significant ($p>0.05$) differences in bird mortality.

Table 7.4 Effects of protease in diets with raw soybean on FI, BWG (g/b) and FCR during different feeding phases.

RSBM[1] %	Protease g/kg	Feed intake (d)			Body weight gain (d)			FCR (d)		
		1-10	1-24	1-35	1-10	1-24	1-35	1-10	1-24	1-35

0	**0.0**	315.7	1774	3608	290.4	1364	2626	1.09	1.28	1.36
	0.2	317.1	1804	3552	296.5	1470	2631	1.07	1.23	1.35
15	**0.0**	294.2	1724	3425	279.4	1335	2566	1.05	1.29	1.39
	0.2	306.1	1663	3322	274.9	1402	2537	1.12	1.22	1.34
25	**0.0**	294.3	1735	3426	266.3	1330	2444	1.12	1.31	1.43
	0.2	291.2	1708	3481	264.6	1376	2516	1.10	1.24	1.35
Pooled SEM		4.3	22.2	37.5	3.8	16.7	28.5	0.02	0.01	0.02
Main effects										
0		316.4	1789	3580[a]	293.5[a]	1417[a]	2629[a]	1.08	1.26	1.35
15		300.1	1693	3456[ab]	277.2[ab]	1367[b]	2550[ab]	1.08	1.24	1.36
25		292.7	1707	3378[b]	265.5[b]	1338[b]	2473[b]	1.11	1.27	1.39
	0.0	301.4	1744	3490	278.7	1343[b]	2539	1.087	1.28[a]	1.39
	0.2	304.8	1716	3459	278.7	1406[a]	2563	1.095	1.23[b]	1.35
Sources of variation										
RSBM[1]		0.08	NS	*	**	*	*	NS	NS	NS
Protease		NS	NS	NS	NS	*	NS	NS	*	NS
RSBM[1] x protease		NS	NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different at *p<0.05; **p<0.01; NS= non-significant; [1]RSBM= raw soybean meal meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively); SEM= pooled standard error of means.

7.3.2 Visceral organ weights

As shown in Table 7.5, increasing the RSBM inclusion rate in diets significantly increased the weights of the gizzard + proventriculus (p<0.001), pancreas (p<0.001), small intestine (SI) (jejunum + ileum + duodenum) (p<0.001), heart (p<0.001), and spleen (p<0.01). The weight of the bursa also tended (p=0.9) to be increased at 24 d of age. Protease supplementation significantly increased (p=0.05) the bursa weight, but had no significant effects (p>0.05) on any of the other measured internal organs. The RSBM x protease interaction had no significant effects (p>0.05) on any of the measured internal organs at 24 d of age.

Table 7.5 Effects of supplemental protease in diets containing graded levels of raw soybean meal on the weights of internal organs (g/ 100 g body weight) at 24 d.

RSBM[1] %	**Protease g/kg**	**G+P**	**Pancreas**	**SI**	**Heart**	**Liver**	**Bursa**	**Spleen**
0	**0.0**	2.74	0.21	4.04	0.58	2.49	0.19	0.08
	0.2	2.79	0.21	4.02	0.54	2.66	0.20	0.08
15	**0.0**	3.72	0.36	5.43	0.69	3.07	0.20	0.12

	0.2	3.58	0.35	5.77	0.74	2.76	0.28	0.10
25	**0.0**	3.40	0.37	4.97	0.66	2.58	0.18	0.09
	0.2	3.31	0.35	4.71	0.64	2.87	0.20	0.08
Pooled SEM		0.10	0.02	0.19	0.02	0.09	0.01	0.01
Main effects								
0		2.77[c]	0.21[b]	4.03[b]	0.56[c]	2.58	0.20	0.08
15		3.65[a]	0.35[a]	5.57[a]	0.71[a]	2.91	0.24	0.11
25		3.36[b]	0.36[a]	4.84[a]	0.65[b]	2.73	0.19	0.08
	0.0	3.29	0.31	4.81	0.64	2.7	0.19	0.09
	0.2	3.23	0.30	4.71	0.64	2.8	0.23	0.09
Sources of variation								
RSBM[1]		***	***	***	***	NS	0.09	NS
Protease		NS	NS	NS	NS	NS	0.05	NS
RSBM[1] x protease		NS	NS	NS	NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different at
NS= not significant; ***p<0.001; **p<0.01; SEM= pooled standard error of means; SI= small intestine (jejunum, ileum and duodenum) were weighed with the contents; G+P (gizzard and proventriculus) were weighed with the contents; [1]RSBM= raw soybean meal meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively).

Table 7.6 The analysed values of crude protein and amino acid composition (g/kg) of the study diets (as-is bases) used.

Protease (g/kg)	**NFD**	**0.0**			**0.2**		
		----------			----------		
RSBM[1] (%)	**0**	**0**	**15**	**25**	**0**	**15**	**25**
Item							
CP	<6.0	224	219	222	224	221	217

Indispensable amino acids							
His	<0.1	5.5	5.3	5.5	5.5	5.4	5.2
Arg	<0.4	14.3	14.2	14.4	14.1	14.4	13.0
Thr	<0.2	8.7	8.4	8.8	9.2	9.2	8.4
Lys	<0.3	12.8	12.3	13.4	12.8	13.3	11.9
Met	<0.1	5.1	4.7	5.2	5.8	5.3	5.0
Val	<0.3	10.7	10.2	10.7	10.5	10.4	10.0
Ile	<0.2	9.0	8.7	9.1	8.8	8.8	8.2
Leu	<0.5	18.0	17.6	18.1	17.9	18.0	17.9
Phe	<0.3	10.4	10.1	10.4	10.3	10.3	9.8
Dispensable amino acids							
Ser	<0.3	10.6	10.4	10.6	10.5	10.5	10.1
Gly	<0.3	11.4	12.4	11.4	10.6	11.1	10.4
Asp	<0.5	20.3	20.1	20.7	19.8	20.4	19.0
Glu	<1.0	37.2	37.0	37.8	36.8	37.6	36.2
Ala	<0.3	11.0	11.2	11.0	10.7	10.9	10.8
Pro	<0.4	13.6	14.0	13.6	13.3	13.5	13.2
Tyr	<0.1	4.9	5.0	5.2	5.0	5.1	4.4

NFD= nitrogen free diets; [1]RSBM= raw soybean meal meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively).

Table 7.7 Effects of protease supplementation of diets containing raw soybean meal on the ileal flow (g/kg of FI) of undigested crude protein and amino acids (mg/g) at 24 d (as-is basis).

RSBM[1]	Protease	CP	Indispensable amino acids									Dispensable amino acids			
%	g/kg		His	Arg	Thr	Lys	Met	Vali	Ile	Leu	Phe	Ser	Gly	Ala	Pro
0	**0.0**	38.50	0.87	1.40	1.95	1.75	0.24	2.05	1.65	2.88	1.67	2.11	2.82	1.96	2.63
	0.2	41.30	0.96	1.47	2.06	1.97	0.28	2.32	1.86	3.30	1.89	2.26	2.94	2.21	2.82
15	**0.0**	40.60	1.02	1.56	2.23	2.03	0.30	2.46	1.99	3.50	1.97	2.49	3.02	2.29	2.93
	0.2	43.60	1.01	1.56	2.22	2.09	0.26	2.47	2.02	3.52	2.03	2.49	3.03	2.34	2.99
25	**0.0**	55.60	1.09	1.70	2.35	2.15	0.30	2.68	2.20	3.90	2.16	2.65	3.24	2.51	3.16
	0.2	65.30	1.12	1.82	2.36	2.33	0.31	2.78	2.30	3.99	2.28	2.71	3.26	2.53	3.11
Pooled SEM		0.9	0.02	0.05	0.04	0.05	0.01	0.06	0.05	0.10	0.05	0.05	0.04	0.06	0.06
Main effects															
0		39.90^{b}	0.92^{b}	1.43^{b}	2.01^{b}	1.86^{b}	0.26	2.19^{c}	1.75^{c}	3.09^{c}	1.78^{c}	2.18^{b}	2.88^{b}	2.09^{b}	2.72^{b}
15		42.10^{b}	1.01^{b}	1.56^{a}	2.22^{a}	2.06^{a}	0.28	2.46^{b}	2.01^{b}	3.51^{b}	2.00^{b}	2.49^{a}	3.03^{b}	2.32^{ab}	2.96^{ab}
25		60.50^{a}	1.10^{a}	1.76^{a}	2.35^{a}	2.24^{a}	0.30	2.73^{a}	2.25^{a}	3.94^{a}	2.22^{a}	2.68^{a}	3.25^{a}	2.52^{a}	3.14^{a}
	0.0	45.90	0.99	1.55	2.17	2.00	0.27	2.40	1.96	3.43	1.95	2.42	3.03	2.27	2.93
	0.2	49.10	1.03	1.61	2.22	2.11	0.29	2.52	2.05	3.60	2.05	2.48	3.07	2.34	2.95
Sources of variation															
RSBM		***	**	*	**	**	NS	***	***	***	***	***	***	**	**
Protease		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
RSMB x protease		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscript within a column are significantly different; SEM= pooled standard error of means; NS= not significant; *p<0.05; **p<0.01; ***p<0.001; [1]RSBM= raw soybean meal meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively).

Table 7.8 Effects of protease supplementation on diets containing raw soybean meal on the coefficient of apparent ileal digestibility (AID) of crude proteins and amino acids of broilers at 24 d (as- is basis).

RSBM[1] %	Protease g/kg	CP	Indispensable amino acids									Dispensable amino acids			
			His	Arg	Thr	Lys	Met	Val	Ile	Leu	Phe	Ser	Gly	Ala	Pro
0	**0.0**	0.775^{b}	0.818	0.905	0.762	0.848	0.945	0.786	0.800	0.824	0.825	0.793	0.770^{a}	0.815	0.806
	0.2	0.812^{a}	0.841	0.894	0.772	0.859	0.948	0.802	0.809	0.832	0.832	0.794	0.748^{ab}	0.815	0.801
15	**0.0**	0.783^{ab}	0.815	0.888	0.755	0.838	0.946	0.762	0.771	0.801	0.807	0.761	0.712^{cd}	0.779	0.777
	0.2	0.772^{b}	0.816	0.891	0.754	0.848	0.952	0.774	0.782	0.810	0.808	0.771	0.737^{bc}	0.792	0.784
25	**0.0**	0.757^{b}	0.785	0.867	0.721	0.810	0.941	0.727	0.724	0.780	0.773	0.737	0.692^{d}	0.768	0.766
	0.2	0.776^{b}	0.799	0.880	0.744	0.837	0.943	0.743	0.750	0.784	0.789	0.745	0.706^{d}	0.771	0.765
Pooled SEM		0.004	0.04	0.004	0.04	0.004	0.02	0.006	0.007	0.006	0.005	0.005	0.006	0.005	0.005
Main effects															
0		0.794	0.830^{a}	0.899^{a}	0.767^{a}	0.854^{a}	0.946	0.794^{a}	0.805^{a}	0.828^{a}	0.828^{a}	0.793^{a}	0.759	0.815^{a}	0.804^{a}
15		0.778	0.816^{b}	0.889^{ab}	0.755^{ab}	0.843^{ab}	0.949	0.768^{b}	0.777^{b}	0.805^{ab}	0.807^{ab}	0.766^{b}	0.725	0.785^{b}	0.781^{b}
25		0.766	0.792^{b}	0.874b	0.733^{b}	0.823^{b}	0.942	0.735^{c}	0.737^{c}	0.782^{b}	0.781^{b}	0.741^{c}	0.699	0.769^{c}	0.765^{c}
	0.0	0.772	0.806	0.887	0.746	0.832^{b}	0.944	0.758	0.765	0.802	0.802	0.764	0.725	0.787	0.783
	0.2	0.786	0.819	0.888	0.757	0.848^{a}	0.948	0.773	0.781	0.808	0.809	0.770	0.730	0.793	0.783
Sources of variation															
RSBM		**	***	**	***	**	NS	***	***	***	***	***	***	***	***
Protease		*	0.08	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS
RSBM x protease		*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	**	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different; pooled standard error of means; NS= not significant; *p<0.05; **p<0.01; ***p<0.001; [1]RSBM= raw soybean meal meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively);

Table 7.9 Effects of protease in diets with raw soybean meal on the standardized ileal digestibility (SID) of crude protein and amino acids at 24 d (as-is basis).

RSBM	Protease	CP	Indispensable amino acids									Dispensable amino acids			
%	g/kg		His	Arg	Thr	Lys	Met	Val	Ile	Leu	Phe	Ser	Gly	Al	Pro
0	**0.0**	0.804[b]	0.873	0.932	0.851	0.887	0.966	0.847	0.853	0.864	0.867	0.860	0.828	0.870	0.853
	0.2	0.840[a]	0.894	0.921	0.858	0.896	0.968	0.860	0.861	0.871	0.872	0.859	0.811	0.872	0.848
15	**0.0**	0.811[ab]	0.868	0.916	0.837	0.875	0.963	0.821	0.824	0.840	0.848	0.827	0.780	0.837	0.826
	0.2	0.799[b]	0.869	0.918	0.839	0.884	0.971	0.832	0.833	0.848	0.848	0.836	0.800	0.848	0.831
25	**0.0**	0.785[b]	0.841	0.897	0.810	0.850	0.961	0.789	0.780	0.819	0.815	0.806	0.761	0.826	0.815
	0.2	0.803[b]	0.853	0.907	0.825	0.873	0.962	0.803	0.802	0.823	0.830	0.812	0.770	0.828	0.813
Pooled SEM		0.004	0.005	0.004	0.004	0.004	0.002	0.006	0.007	0.006	0.005	0.005	0.006	0.004	0.005
Main effects															
0		0.822	0.884[a]	0.927[a]	0.855[a]	0.891[a]	0.967	0.853[a]	0.857[a]	0.868[a]	0.869[a]	0.859[a]	0.819[a]	0.871[a]	0.851[a]
15		0.805	0.869[a]	0.917[b]	0.838[b]	0.879[b]	0.967	0.827[b]	0.829[b]	0.844[b]	0.848[b]	0.831[b]	0.790[b]	0.843[b]	0.829[b]
25		0.794	0.847[b]	0.902[c]	0.818[b]	0.861[c]	0.961	0.796[c]	0.791[c]	0.821[b]	0.822[c]	0.809[c]	0.765[c]	0.827[b]	0.814[b]
	0.0	0.800	0.861	0.915	0.833	0.871[b]	0.963	0.819	0.819	0.841	0.843	0.831	0.789	0.844	0.831
	0.2	0.814	0.872	0.915	0.841	0.884[a]	0.967	0.831	0.832	0.847	0.850	0.836	0.793	0.849	0.831
Sources of variation															
RSBM		**	***	***	***	**	NS	***	***	**	***	***	***	***	***
Protease		*	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS
RSBM x protease		*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different; SEM= pooled standard error of means; NS= non-significant; *p<0.05; **p<0.01; ***p<0.001; [1]RSBM= raw soybean meal meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively).

7.3.3 Ileal crude protein and amino acid digestibility

The ileal loss of undigested and unabsorbed CP and AA, and the AID and SID of CP and AA are shown in Tables 7.7, 7.8 and 7.9. The results revealed that undigested and unabsorbed ileal CP loss was significantly ($p<0.001$) increased when the diets were supplemented with increasing levels of RSBM. On average, the loss of undigested and unabsorbed ileal AA, except that of methionine at 24 d of age, was significantly increased when the diets were supplemented with RSBM.

At 24 d of age, increasing the RSBM inclusion rate significantly reduced ($p<0.01$) the values of AID and SID for CP, and it also reduced the value of AID and SID of indispensable AA, by 0.4 to 8.5% and 0.6 to 7.7%, respectively, with the lowest value for methionine and the highest for isoleucine. When diets were supplemented with increasing levels of RSBM, the AID and SID values of dispensable AA were also reduced by between 4.9 to 7.9% and 4.4 to 6.6%, respectively, among which the lowest was for proline and the highest was for alanine.

Under microbial protease supplementation, the loss of undigested and unabsorbed ileal CP was reduced by approximately 6.5%, but the difference was not significant ($p>0.05$). The AID and SID of CP were significantly ($p<0.05$) increased when the diets were supplemented with microbial protease, and they were also significantly influenced ($p<0.05$) by the interaction effects between protease and RSBM at 24 d of age.

Although statistically the same ($p>0.05$), the average loss of undigested and unabsorbed indispensable and dispensable ileal AA, respectively, were reduced by approximately 4.5 and 1.9% when the diets were supplemented with protease. However, supplementation with protease enabled an increase in the AID and SID values of indispensable AA, which respectively ranged between 2.32 and 0.11% and 0 and 1.5 % more than the non-supplemented diets, but the differences were not statistically significant ($p>0.05$). Although the differences were not significant ($p>0.05$), the average AID and SID values of dispensable AA at 24 d of age were greater by 0.78 and 0.56%, respectively when the diets were supplemented with microbial protease. As assessed on day 24, the AID ($p<0.5$) and SID ($p<0.05$) values of lysine were significantly increased due to the supplementation of diets with microbial protease.

7.4 DISCUSSION

7.4.1 The gross response and internal organ development

Since the concentration of TI in some of the dietary groups of this study was approximately 10193.4 TIU/g, which is beyond the threshold level for non-ruminant animals (Hong *et al.*, 2004), the gross response of birds in terms of FI and BWG statistically differed over the 1-35-d period. The current results are consistent with those of other researchers (Barletta, 2011; Pettersson and Pontoppidan, 2013) who reported that protease can break down both the stored proteins and the proteinaceous anti-nutrients and subsequently improve nutrient digestibility.

As assessed at 24 d of age, the weights of most of the internal organs of the birds, including the gizzard + proventriculus, pancreas, SI, heart and spleen, were increased by increasing the inclusion rate of RSBM in diets. These current findings partially agree with those of other researchers (Mogridge *et al.*, 1996; Mayorga *et al*., 2011) who reported that birds fed diets containing RSBM had heavier pancreas and duodenum. The reason for the increased weights of most of the internal organs in this study might be due to the adverse impacts of ANF (in the RSBM) on most of the digestive system besides the pancreas. However, these differences may be due to the level of RSBM in the diets.

7.4.2 Ileal digestibility of amino acids and crude protein

Increasing the level of RSBM in diets significantly increased the loss of undigested and unabsorbed ileal CP, whereas the AID and the corresponding SID were significantly reduced. These results are inconsistent with those of Clarke and Wiseman (2005) who reported that the AID and SID of AA did not correlate with TI levels, indicating that other factors also affect the amino acid digestibility of FFSB and SBM in broilers.

When the diets were supplemented with increasing levels of RSBM, the average loss of undigested and unabsorbed ileal AA significantly increased, and this result agrees with that of other researchers (de Coca-Sinova *et al.,* 2008) who reported that the apparent digestibility of N and AA in broilers varies among SBM samples with greater values corresponding to lesser TI. Additionally, Barth *et al.* (1993) explained that the ingestion of food containing trypsin inhibitors affects the nitrogen balance by increasing the outflow of amino acids from endogenous secreta rather than through the loss of dietary amino acids. The reason for an increased loss of CP and AA with increasing levels of RSBM in diets of this study might be due to the adverse effects of ANF, mainly TI on digestibility.

As assessed at 24 d of age, increasing the inclusion rate of RSBM in broiler diets significantly reduced the AID and corresponding SID values of most indispensable and dispensable AA, except methionine. These findings are supported by other researchers (Rocha *et al*., 2014) who

reported that the apparent digestibility of nutrients was reduced when raw soybean meal was used. Results of the current study generally agreed with Gilani *et al.* (2012) who reported that high concentrations of ANF in the diets from grain legumes are responsible for poor digestibility of protein. However, the results of the present study contradict with those of Frikha *et al*. (2012) who reported that the SID of CP and lysine in broilers were increased at day 21 when the KOH and TIA values of soybean in diets were increased.

However, when diets were supplemented with microbial protease, the loss of undigested and unabsorbed ileal CP was reduced, whereas the AID and SID of CP increased even though not significant due to protease supplementation. These findings partially agree with Romero and Plumstead (2013) who reported that the addition of protease in conjunction with carbohydrases further increased the AID of AA in young broilers fed maize–SBM diets. Guggenbuhl *et al*. (2012) added that the dietary protease supplementation increased the AID of AA in piglets fed on corn-soy-based diets. Furthermore, Murugesan *et al.* (2014) reported that interaction between protease and phytase supplementation significantly increased the AIP of CP and AA in broilers.

This study also showed that the AID and SID values for methionine were not affected by increasing the RSBM (TI) level in broilers diets, and this finding is supported by Kwong and Barnes (1963) who reported that feeding unheated soybeans does not selectively impair the availability or tissue utilisation of methionine. However, there is a metabolic block in the utilisation of cysteine for protein synthesis. The reasons for the lack of effect on methionine are unclear.

The AID and SID values of lysine were significantly increased due to protease supplementation, and this result partially agrees with Liu *et al*. (2013) who reported that protease improved the apparent digestibility of amino acids by an average of 9.16% in broilers fed a maize-sorghum-based diet. However, it explains why only the SID of lysine, among the indispensable AA was increased although following the trend of CP when diet was supplemented with protease.

7.5 CONCLUSION

This study showed the commercial SBM could be replaced (≤25%) by RSBM in broiler diets without pronouncedly compromising productivity if the diets are supplemented with the right protease and phytase. Although the TI contents in some of the dietary groups in the current

study were beyond the threshold levels for broilers, the FCR were statistically similar throughout the entire study period (1-35 d). Therefore, it is evident from the present study that the test microbial protease could reduce the adverse impacts of dietary ANF, particularly TI, on the productivity of broilers. The mechanisms of the test protease are unclear, but one area of action appears to be evaluation in the loss of undigested and unabsorbed ileal CP and total AA, and an increase in the AID and SID of CP and AA.

CHAPTER 8: WELLBEING AND PERFORMANCE OF BROILER CHICKENS FED DIETS CONTAINING RAW, FULL-FAT SOYBEAN MEAL SUPPLEMENTED WITH MICROBIAL PROTEASE

ABSTRACT

A 2 x 3 factorial study was conducted with two levels of protease (0 or 0.2 g/kg) and three levels of raw full-fat soybean meal (RSBM) replacing the commercial SBM at 0, 15, or 25% to examine the wellbeing of broiler chickens. Microbial phytase (2000 FYT/kg) was also uniformly added and each treatment was replicated six times, with eight birds per replicate. The birds were raised in climate-controlled rooms using sawdust as the bedding material and offered corn-soybean-based starter (1-10 d), grower (11-24 d) and finisher (25-35 d) diets formulated to the Aviagen standard for Ross 308 broiler. Over the 1-35 d period, there were no statistical ($p>0.05$) differences in body weight gain (BWG) and feed conversion ratio (FCR) values of broilers between the treatment groups. Neither RSBM nor protease supplementation had any influence ($p>0.05$) on mortality or footpad dermatitis in the birds. At 24 d, intestinal lesions, as an indicator of necrotic enteritis, were also not affected by the dietary treatments. On day 24, the weight, length, width and strength of the tibia bone were reduced when the RSBM inclusion rate in the diets was increased, but these differences were absent at 35 d of age. Neither RSBM nor protease supplementation significantly ($p>0.05$) affected the DM, Ca and P contents of in the tibia at 35 d of age. On days 24 ($p<0.05$) and 35 ($p<0.01$), the concentration of Ca in the litter was reduced by higher levels of RSBM, but the litter P content was not affected. The litter N content which was measured on days 24 ($p<0.05$) and 35 ($p<0.01$), was increased with the increase in the RSBM levels in the diets, but it was reduced (not significantly) with protease supplementation by 2.5 and 4.4% at days 24 and 35, respectively. At day 14, the litter pH in pens in which the diets were supplemented with protease tended ($p=0.08$) to increase, but the litter moisture content and pH were similar on days 24 and 35. Protease supplementation significantly ($p<0.05$) increased the uric acid concertation in the litter at 35 d of age. Increasing the levels of RSBM in the diets further increased the uric acid by up to 7.7%, but the difference was not significant ($p>0.05$). Increased protease supplementation positively influenced ($p<0.05$) the ammonia concentration in the litter at 35 d of age. The concentration of inositol in the blood plasma at 24 d of age was reduced by up to11.2 and 3.3% with increased levels of RSBM and protease supplementation in broiler diets, respectively compared to the other parameters, but the differences were not significant

($p>0.05$). Electrolytes, such as Cl^-, were increased in the plasma when broiler diets were supplemented with microbial protease. Overall, the results of this study indicate that there are no major health-related risks associated with the replacement of commercial SBM with RSBM (≤25%) in broiler diets provided that the diets are supplemented with the kind of microbial protease (2000 FYT/kg).

8.1 INTRODUCTION

Soybean meal (SBM) plays an important role in the feeding of non-ruminant animals worldwide, but it is sometimes inaccessible, costly and of varying quality to the end users. These are some of the major problems associated with the use of commercial SBM in the poultry industry, but recent findings have suggested that both commercial SBM and the dietary oil in broiler diets could be partially replaced by full-fat SBM. In contrast to meals that are heat-treated prior to preparation, the availability of nutrients in meals prepared from raw soybean seed (RSBM) could be adversely affected by the presence of antinutritional factors (ANF), such as trypsin inhibitors (TI), lectin and phytic acid. The existence of these ANFs in diets negatively affects the nutrient utilization and digestibility of soybean proteins, and it could be the cause of digestive and metabolic diseases in non-ruminant animals (Dawson *et al.*, 1988; Sun and Qui, 2005).

The best characterized ANFs in soybeans are protease inhibitors, lectins and phytates (Pettersson and Pontoppidan, 2013). Trypsin inhibitors can be found in different compound states (Maliar *et al.*, 2004) and are the most important ANF in soybeans because they are able to tightly bind to the trypsin and block its protein digestion capabilities. Nitrogen retention can be negatively affected by the activity of TI which results in increased metabolic nitrogen excretion (Banaszkiewicz, 2011; Dourado *et al*., 2011). A high TI content in the diets of birds can lead to major nitrogen losses and result in poor litter quality (Ruiz and de Belalcázar, 2005; Ruiz, 2012).

Phytate (phytic acid) is another ANF component in soybeans; it is mostly found in the form of protein-phytate or protein-phytate-protein complexes which are resistant to digestion by proteolytic enzymes. Phytate is further antagonistic as it forms a chelate with nutritionally essential mineral elements (Liener, 1994; Hurrell, 2003; Chen *et al*., 2013). It also impedes nutrient digestion through electrostatic mechanisms and reduces the solubility of proteins, consequently increasing the loss of endogenous nutrients (Cowieson and Ravindran, 2007). Phytic acid is the principal form of stored phosphorus in many plants; for example, it is six

times higher in soybeans than other crops (Pallauf and Rimbach, 1997). Since phytic acid is not heat-labile, it is essential to supplement corn-soybean-based diets with exogenous phytase (Dourado *et al*., 2011).

In addition to reducing performance, ANFs in diets sometimes cause different diseases in non-ruminant animals, including digestive and metabolic disease (Herkelman *et al.,* 1991), and Palliyeguru *et al*. (2011) reported that increasing the amount of non-toasted soybeans in broiler diets resulted in a linear increase in sub-clinical lesions along the intestinal tract. Furthermore, Youssef *et al.* (2011) reported that the high dietary protein could increase the incidence of footpad dermatitis in birds as a result of increased uric acid and secondary ammonia production in the excreta or litter.

Therefore, the potential to further improve the nutritional value of soybeans using exogenous enzymes has been explored by many researchers (Ghazi *et al.,* 2010; Ao, 2011, Erdaw *et al.*, 2016c & d). Bedford and Schulze (1998) also suggested that supplementation with microbial enzymes could reduce the negative impacts of ANF in the feedstuffs and increase the bioavailability of nutrients (Martinez-Amezcua *et al.,* 2006), thus reducing nutrient excretion (Adeola and Cowieson, 2011). Kamel *et al*. (2015) reported that supplementation with mono-component protease improved protein digestibility, and in a review, Nahm (2007) concluded that supplementation of broiler diets with exogenous enzymes could reduce N excretion by up to 40%.

Microbial phytase can also hydrolyse the phytate into inositol and inorganic phosphates. Inositol has an insulin mimetic effect, which is a benefit of microbial phytase, on a range of animal species (Cowieson *et al*., 2015), and the concentration of inositol has been shown to increase in the blood plasma of broilers with the addition of phytase. Supplementation of diets with microbial phytase results in the hydrolysation of the bonds of phytic acids to increase the availability of nutrients, such as minerals, amino acids and carbohydrates (Olukosi *et al.*, 2008; Slominski, 2011). Qian *et al.* (1996) reported that the values of the physical parameters and mineral composition of the tibiae of broilers were improved by phytase supplementation.

However, the performance and wellbeing of broilers fed diets containing high TI concentrations (in raw soybean) when supplemented with phytases and proteases have not yet been evaluated. Therefore, the objectives of this study were to evaluate the performance and wellbeing, in terms of mortality, litter quality, footpad dermatitis, intestinal lesions, litter

quality and mineralization, of birds fed diets containing varying levels of RSBM and supplemented with protease.

8.2 MATERIALS AND METHODS

The experiment was conducted at the Animal House of the University of New England (UNE), and it was approved by the Animal Ethics Committee (Authority No: AEC15-044) prior to commencement.

8.2.1 Diets and experiment

The test raw soybean seed was purchased from a local supplier in northern New South Wales, Australia. The seeds were cleaned and hammer-milled. The crude protein content was around 40%. On the other hand, commercial soybean meal (SBM) was obtained from Cargill (Australia) Pty Ltd in New South Wales.

A 2 x 3 factorial study was employed, with two levels of protease (0 or 0.2 g/kg) and three levels of RSBM, replacing commercial SBM at 0, 15, or 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively. Microbial phytase (2000 FYT/kg) was uniformly supplemented to each diet and every treatment was replicated six times, with eight birds per replicate.

The birds were raised in a climate-controlled room with sawdust as the bedding material, and they were offered corn-soybean-based diets (starter, grower and finisher) formulated to Aviagen standards for Ross 308 broilers. Additional litter material was added to each pen when the litter became wet or low.

Table 8.1 Ingredients and composition of starter (0-10 d) diets (as-fed basis).

	RSBM (%)					
	0		15		25	
Protease (g/kg)	**0.0**	**0.2**	**0**	**0.2**	**0**	**0.2**
Ingredients (g/kg)						
Corn (Rolled)	594.0	594.0	599.6	599.6	594.0	594.0
Soybean meal	300	300	255.0	255.0	225.0	225.0
Raw soybean meal	0.0	0.0	45.0	45.0	75.0	75.0
Meat meal	62.9	62.9	61.1	61.1	72.3	72.3
Canola Oil	16.0	16.0	9.4	9.4	7.9	7.9
Dical phosphate	7.7	7.7	9.0	9.0	6.7	6.7
Limestone	6.2	6.2	6.5	6.5	5.3	5.3
Salt	3.0	3.0	3.0	3.0	3.0	3.0
L-lysine	2.7	2.7	3.1	3.1	2.7	2.7
DL-methionine	2.3	2.3	2.1	2.1	2.5	2.5
Premix, 2 kg/mt[1]	2.0	2.0	2.0	2.0	2.0	2.0
L-threonine	2.0	2.0	2.0	2.0	2.0	2.0
Sodium bicarb	1.1	1.1	1.6	1.6	1.1	1.1
Choline Cl	0.5	0.5	0.5	0.5	0.5	0.5
Phytase	0.2	0.2	0.2	0.2	0.2	0.2
Protease	0.0	0.2	0.0	0.2	0.0	0.2
Nutrients (g/kg)						
ME Poultry (MJ/kg)	12.59	12.59	12.59	12.59	12.59	12.59
Crude Protein	225.8	225.8	225.8	225.8	226.0	226.0
Crude fat	42.1	42.1	39.8	39.8	44.6	44.6
Arginine	14.4	14.4	14.3	14.3	14.4	14.4
Lysine	14.0	14.0	14.0	14.0	14.0	14.0
Methionine	5.7	5.7	5.2	5.2	5.7	5.7
Methionine + cysteine	8.8	8.8	8.3	8.3	8.8	8.8
Threonine	9.9	9.9	9.4	9.4	9.9	9.9
Calcium	10.0	10.0	10.3	10.3	10.0	10.0
Phosphorus avail	5.0	5.0	5.1	5.1	5.0	5.0
Choline	1.4	1.4	1.4	1.4	1.2	1.2

[1]Composition of the premix are as shown in Table 6.1; RSBM= raw soybean meal (SBM was replaced y RSBM at 0, 15 and 25 %, equivalent to 0, 45 and 75 g/kg of diet, respectively).

Table 8.2 Ingredients and composition of grower (10-24 d) diets (as-fed basis

	RSBM (%)					
	0		15		25	
Protease enzyme (g/kg)	**0.0**	**0.2**	**0.0**	**0.2**	**0.0**	**0.2**
Ingredients (g/kg)						
Corn (Rolled)	578.6	578.6	584.7	584.7	577.5	577.5
Soybean meal	300	300	255.0	255.0	225.0	225.0
Raw soybean meal	0.0	0.0	45.0	45.0	75.0	75.0
Canola Oil	45.6	45.6	40.5	40.5	40.3	40.3
Meat meal	39	39	37.1	37.1	46	46
Dical phosphate	9.5	9.5	12.0	12.0	10.0	10.0
TiO_2	5.0	5.0	5.0	5.0	5.0	5.0
Limestone	10	10	7.5	7.5	8.5	8.5
Salt	2.3	2.3	2.0	2.0	2.3	2.3
L-lysine	2.0	2.0	2.4	2.4	2.2	2.2
DL-methionine	3.3	3.3	3.8	3.8	3.7	3.7
Premix, 2 kg/mt[1]	2.0	2.0	2.0	2.0	2.0	2.0
L-threonine	0.6	0.6	1.5	1.5	0.9	0.9
Sodium bicarb	1.1	1.1	1.0	1.0	0.8	0.8
Choline Cl	1.0	1.0	0.5	0.5	0.8	0.8
Phytase	0.2	0.2	0.2	0.2	0.2	0.2
Protease	0.0	0.2	0.0	0.2	0.0	0.2
Nutrients (g/kg)						
ME Poultry (MJ/kg)	13.28	13.28	13.28	13.28	13.28	13.28
Crude Protein	210.0	210.0	210.7	210.7	208.6	208.6
Crude fat	68.7	68.7	67.7	67.7	73.6	73.6
Arginine	13.4	13.4	13.3	13.3	13.3	13.3
Lysine	12.7	12.7	12.7	12.7	12.8	12.8
Methionine	6.5	6.5	6.7	6.7	6.6	6.6
Methionine + cysteine	9.4	9.4	9.7	9.7	9.6	9.6
Threonine	8.2	8.2	8.9	8.9	8.3	8.3
Calcium	9.9	9.9	9.4	9.4	9.8	9.8
Phosphorus avail	4.4	4.4	4.8	4.8	4.6	4.6
Choline	1.5	1.5	1.3	1.3	1.2	1.2

[1]Composition of the premix was shown in Table 6.1. RSBM=raw soybean meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively).

Table 8.3 Ingredients and composition of finisher (24-35 d) diets (as-fed basis).

RSBM (%)	**0**		**15**		**25**	
	---		---		---	
Protease enzyme (g/kg)	**0.0**	**0.2**	**0.0**	**0.2**	**0.0**	**0.2**
Ingredients (g/kg)						
Corn (Rolled)	603.0	603.0	611.4	611.4	613.9	613.9
Soybean meal	300.0	300.0	255.0	255.0	225.0	225.0
Raw soybean meal	0.0	0.0	45	45	75.0	75.0
Canola Oil	51.1	51.1	46.3	46.3	43.5	43.5
Meat meal	10.0	10.0	6.0	6.0	6.0	6.0
Dical Phosphate	15.8	15.8	15.5	15.5	15.5	15.5
Limestone	11.0	11.0	9.8	9.8	10.0	10.0
Salt	2.2	2.2	2.1	2.1	2.1	2.1
L-lysine	0.7	0.7	1.0	1.0	1.0	1.0
DL-methionine	2.2	2.2	2.7	2.7	2.8	2.8
Premix, 2 kg/mt[1]	2.0	2.0	2.0	2.0	2.0	2.0
L-threonine	0.5	0.5	1.8	1.8	1.8	1.8
Sodium bicarb	1.0	1.0	1.0	1.0	1.0	1.0
Choline Cl	0.5	0.5	0.5	0.5	0.5	0.5
Phytase	0.2	0.2	0.2	0.2	0.2	0.2
Protease	0.0	0.2	0.0	0.2	0.0	0.2
Nutrients (g/kg)						
ME Poultry (MJ/kg)	13.49	13.49	13.49	13.49	13.49	13.49
Crude Protein	192.1	192.1	192.9	192.9	190.5	190.5
Crude fat	68.8	68.8	70.7	70.7	71.9	71.9
Arginine	12.3	12.3	12.3	12.3	12.1	12.1
Lysine	10.5	10.5	10.7	10.7	10.6	10.6
Methionine	5.0	5.0	5.4	5.4	5.4	5.4
Methionine + cysteine	7.9	7.9	8.3	8.3	8.3	8.3
Threonine	7.4	7.4	8.7	8.7	8.6	8.6
Calcium	8.7	8.7	8.5	8.5	8.5	8.5
Phosphorus avail	4.2	4.2	4.3	4.3	4.2	4.2
Choline	1.4	1.4	1.3	1.3	1.2	1.2

[1]composition premix is as shown in Table 6.1. RSBM=raw soybean meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively).

8.2.2 Animal husbandry and sampling

A total of 288 day-old Ross male broiler chicks (average body weight of 43.84±0.18 g), were obtained from a local commercial hatchery (Baiada Poultry Pty. Ltd., Tamworth, Australia To maintain uniformity, the chicks were weighed and eight chicks were randomly allocated into each of the 36 pens (600 x 420 x 23 cm).

One medium-sized feeder (78 x 12 x 8 cm) was used per cage. The birds were housed from 1 to 35 days of age in pens, in climate-controlled rooms. Each pen was equipped with a feeder and two nipple drinkers. The feeders were scrubbed and cleaned before providing the diets. The drinkers were also checked from time to time to ensure that they worked properly and they were also cleaned regularly.

The temperature of the rooms was set at 33°C for the first two days with a relative humidity of between 49 and 60%. This temperature was then gradually reduced to 24°C at 19 days of age and this was maintained for the remaining study period. For the first 2 days, 24 h of light (20 lux) was provided. This was then reduced to 23 h for the next 6 consecutive days, followed by 20 h lights (10 lux) for the remaining days. Feed was provided *ad libitum* (crumble for starters), and pellets. The birds had free access to water, and were provided with starter (0-10 d), grower (10-24 d) and finisher (24-35 d) diets (corn-soybean based) formulated to Aviagen standards for Ross 308 broilers.

8.2.3 Gross response to the diets

On days 10, 24 and 35, the body weight of birds and feed leftover were recorded to calculate the body weight gain (BWG) and feed intake (FI) and thereby computing for the feed conversion ratio (FCR). Mortality was recorded whenever it occurred. All sample collection was completed between 0900 and 1200 h.

8.2.4 Chemical analysis

For dry matter (DM), or moisture content, sub-samples of litter or bone (in duplicate) were weighed into crucibles of known weight and placed in a forced air convection oven (Qualtex Universal Series 2000, Watson Victor Ltd, Perth, Australia) at 105 °C for 24 h. The weight of the sample was then recorded after being cooled for 50 minutes in a desiccator at room temperature.

Equation: DM (moisture) % = (weight of a sample after oven-drying/weight of sample before oven-drying) x 100.

After recording the DM weight of the samples (in duplicate), the crucible with the oven-dried sample was placed in a Carbolite CWF 1200 chamber furnace (Carbolite, Sheffield, UK) and ashed at an initial temperature of 350 °C for 1 h followed by ashing at higher temperature of 600 °C for 2 h to determine the ash content of the sample. The ash (residue) was weighed, recorded and the ash content was calculated as follows:

Ash % = (weight of ashed sample/weight of its oven-dried content (DM)) x 100.

8.2.5 Mineral and nitrogen analysis

Based on different digestion systems, nitric acid, hydrochloric acid and perchloric acid were added to analyse for mineral tibia bone ash. Diluted samples and the standard solution were separately put into a set of fresh tubes for spectroscopy (ICP) (Vista MPX, Melbourne, Australia). The Ca and P were measured at 616 and 231nm wavelength, respectively. Approximately 0.2 g was placed into a Schott bottle in a scrubbed fume cupboard. Two milliliters of a mixture of $HClO_4$ (70%) perchloric acid and H_2O_2 (30%) were added to each tube. Each tube was loosely covered with a lid and set overnight. Then one ml of H_2O_2 was added and tubes were tightly sealed and placed in an oven set at 80^{o}C for 30 minutes. The bottles were allowed to cool slightly and a further 1 mL H_2O_2 was added before they were capped tightly and digested for 1 hour at 80^{o}C. The solution was made to a volume of 25 mL by adding distilled water and filtered through Whatman No.1 filter paper for ICP analysis (Anderson and Henderson, 1986).

The nitrogen content of diets was determined by the Dumas combustion technique (LECO) Official Method 990.03, Kjeldahl and Official Method 984.13 (A-D) (AOAC, 2006a).

Measuring the quality of diets containing RSBM, in terms of contents of KOH, TI, UA and NSI has been previously described in Section 5.2.5.

8.2.6 Determination of tibia bone properties

On days 24 and 35, the tibiae (right and left) were excised as flesh-intact drumsticks from randomly sampled and humanely killed birds from each pen and labelled. All of the tibiae were then pooled by treatment and stored in a freezer (-20^{o}C) until processing.

Right tibia: The right tibia was used to evaluate the physical characteristics of the bones. After thawing at room temperature, the flesh, including the adhering tissues, was removed, cleaned and allowed to dry at room temperature.

The parameters for the physical characteristics of the tibiae including weight, length, width, strength and density were then measured. Width was measured at the middle of the bone; both facial sides of the middle point were measured with a digital calliper; and the average was taken.

The tibiotarsal index was calculated by dividing the weight of the tibia by its length, and the robusticity index was also calculated by dividing the length of the tibia by the cube root of its weight (Mutus *et al.,* 2006). Tibia strength was measured by the breaking method using an Instron testing machine (Instron Ltd., High Wycombe, UK) (constant crosshead speed of 10 mm/min and a distance between the supports of 50 mm). When measuring the breaking strength, the groove of each bone was placed upright (the same facial direction), and the force was always applied at the middle of each tibia.

The tibia volume was measured by submerging an individual bone into a water-filled cylindrical tube, and the displaced water was re-collected and measured (v) with a graduated cylinder. The density (D) was calculated by dividing the weight of the tibia by its volume (W/V).

***Left tibia*:** The concentration of DM, ash and minerals was measured using the left tibia bone of the broilers. A left tibia, as a flesh-intact drumstick, was immersed in boiling water (100 °C) for 10 min, and after cooling to room temperature, the flesh, including the adhering tissue and patella (cartilage) was removed by hand. The tibia was then cleaned and air-dried for 24 h at room temperature, and the DM and ash contents, and the mineral composition of the tibiae were then determined.

8.2.7 Intestinal lesions and footpad dermatitis in broilers

Determination of intestinal lesions: Intestinal lesions were scored following the same method described by Truscott and Al-Sheikhly (1977). On day 24, two birds per pen were randomly selected and killed by cervical dislocation. The jejunum and ileum were then incised and washed with normal saline; the mucosal surfaces were inspected; and any necrotic lesions were recorded (scored). A range of ranks were used to score the lesions as follows: score 0, no lesions; score 1, focal necrosis (1-10 mm in diameter); score 2, necrotic patches (1-2 cm in diameter); score 3, coalesced necrosis; and score 4, pseudo-membrane covering the entire epithelial surface.

***Footpad dermatitis*:** Footpad dermatitis in the birds was assessed (scored) following the procedure described by Hocking *et al*. (2008). On days 24 and 35, the footpads of 4 randomly selected birds per pen were assessed (scored) and recorded. The scoring value that was representative of the severity of the footpad problem was assigned as follows: score 1, a small, black necrotic area; score 2, a black area forming a scale; score 3, a pronounced scaled area; and score 4, more than half of the footpad covered by necrotic cells.

8.2.8 Determination of the total nitrogen, pH and moisture in the litter

On days 14, 24 and 35, litter samples were collected from 3 representative sites per pen, avoiding areas near and below the feeders and drinkers. These samples were then thoroughly mixed, pooled and labelled and then analysed for their N, pH and moisture contents.

Evaluation of the litter pH: Litter pH was determined following the same method described by Benabdeljelil and Ayachi (1996). Approximately 10 g of a subsample were vigorously agitated and suspended in Milli-Q water (in 1:2.5 ratio) and left to rest at room temperature for one h. The pH value of the litter suspended in water was then measured with a pH-meter.

8.2.9 Analysis of inositol concentration, electrolytes and metabolites in plasma

On day 24, blood samples from randomly selected male birds from each pen were collected from the vena jugularis into a 10-mL tube (potassium/EDTA-vacutainers) and cooled on ice. The plasma was obtained after centrifuging the blood at 3,000 rpm at 4 °C, and plasma inositol was determined following the method described by Leung *et al*. (2011). Briefly, one mL of a plasma sample was added to a test tube containing 2 mL of methanol and then centrifuged at 3,000 rpm for 10 min. Then, approximately 0.5 mL of the supernatant was transferred into a one-mL glass injector vial and steamed with nitrogen at 40^0C until the liquid was evaporated. Finally, the sample was dispensed overnight with 200 µL of pyridine and 100 µL of BSTFA, after which inositol measured using inositol as the inner standard with a 6890 GC mass spectrometer (Agilent Technologies, China). The pH and the electrolytes in the blood plasma were analysed using a Radiometer ABL800 FLEX blood-plasma analyser (Medical ApS -DK-2700, Copenhagen, Denmark).

8.2.10 Analysis of ammonia and uric acid in litter

To determine the ammonia and uric acid in the litter, a 300-mg dried litter sample was mixed with 10 mL of Milli-Q water and homogenized by homogenizer. The mixture was centrifuged at 3,000 x g for 10 min to obtain the supernatant. The supernatant was diluted 10 times with

Milli-Q water and this solution was used to determine both the ammonia and uric acid contents in the litter.

***Ammonia in litter*:** The content of ammonia in the litter was determined in accordance with instructions on ammonia assay kit (catalogue number AA0100, Sigma-Aldrich, Saint Louis, Missouri, USA). Approximately, 0.2 mL of the diluted litter sample was transferred to a test tube containing 2.0 mL of ammonia assay reagent, and the content was then thoroughly mixed and incubated for 5 min at 21 °C. After reading the one-time absorbance at 340 nm (initial) against the blank, which was similarly prepared with 2.0 mL of ammonia assay reagent mixed with 0.2 mL of water, the remaining aqueous content (samples) in each tube was added with 20 µL of L-glutamate dehydrogenase solution (catalogue number G2294) and further incubated at 21 °C for 5 min. The final absorbance was read at 340 nm, and the concentration of ammonia (mg/g) was calculated as follows. For each of the blank and test reagents, ΔA340 = A-initial– A-final; (ΔA340) test = ΔA340 (test) - ΔA340 (blank) = NH3 (mg/mL) of the original sample, where, A = Δ (ΔA340) test; TV = total assay volume in mL; V = sample volume in mL; MW (molecular weight) of ammonia = 17 g/mole or, equivalently, 17 µg/µmole; F = the dilution factor from the sample preparation; SV = millimolar extinction co-efficient for NADPH at 340 nm = 1/cm; D = light path (cm) = 1 cm.

***Uric acid in the litter*:** The uric acid concentration in the litter was measured using the method described by Kageyama (1971). Briefly, a uric acid stock standard (1 mg/mL) solution was prepared with approximately 100 mg of pure uric acid (Sigma Aldrich Co., LLC**,** U2625) + 80 mg of lithium carbonate + 15 mL of Milli-Q water, and this mixture was heated for 5 min at 60 °C. After cooling, 100 mL of Milli-Q water were added followed by several drops of chloroform, and the resulting solution was stored in a refrigerator to be used within a week. A uric acid working standard solution was prepared by diluting 10 mL of uric acid stock standard solution with 100 mL of Milli-Q water (10 mg/100 mL).

For each sample, four test tubes were labelled A, B, C and D, and 0.2 mL of a diluted litter solution was placed in tubes A and B, while 0.2 mL of the uric acid working standard was placed into tubes C and D. Then, 3.0 mL of the colour reagent (containing 10 g of ammonium phosphate dibasic + 0.3 mL of 85% phosphoric acid + 10 mL of methyl alcohol + 0.2 mL of acetyl acetone + 10 mg of catalase (Sigma Aldrich Co., LLC, C1345) + 2 mg of uricase (Sigma Aldrich Co., LLC**,** 94310) + 100 mL of Milli-Q water that were mixed and filtered) were added

to test tubes A and C. In test tubes B and D, 3.0 mL of the blank reagent (containing 10 g of ammonium phosphate dibasic + 0.3 mL of 85% phosphoric acid + 10 mL of methyl alcohol + 0.2 mL of acetyl acetone (Sigma Aldrich Co., LLC, 05581) + 10 mg of catalase + 100 mL of Milli-Q water, that were mixed and filtered) were added. After vigorous mixing, the tubes were placed in a water bath at 37 °C for 70 min. Samples were allowed to cool in running water, and the absorbance of tube A was measured against B and C against D at 410 nm. A-2 step calculation of uric acid concentration in litter was conducted as follows

(a) Uric acid/ aqueous (mg/100 mL aqueous) = (Absorbance of A/Absorbance of C)^{x}100.

(b) Uric acid/dry sample (mg/g) = ([mg uric acid/100mL aqueous]/sample in an absorbance (mg))^{x}1000.

8.2.11 Statistical analysis

The general linear model (GLM) was used to analyse the data in Minitab software version 17 (Minitab, 2013). The data from the feeding trial were analysed using a general linear model (GLM) to evaluate the effects of RSBM and enzyme supplementation as the main factors as well as their interactions. The differences were considered to be significant at $p < 0.05$, and the significant differences between the mean values were also separated using Duncan's test.

8.3 RESULTS

8.3.1 Gross response to the diets

When increasing replacement of commercial SBM by RSBM from 0 to 25%, in broiler diets the values ANF were approximately increased, including TI (from 1747 to 10193.4 TIU/g, NSI (from 155.3 to 222.9 g/kg) and UA (from 0.16 to 1.53 ΔpH) (data not presented). As shown in Table 8.4, the summary results of gross response to the diet tested are summarized. Feed intake and BWG from hatch to 35 d were reduced by up to 3.4 and 6.0%, respectively in response to increase in RSBM, which were significantly different. Feed conversion ratio was increased by 4 percentages points, but these differences were not significant. These values were improved (not significantly) by supplementation with protease. There was no significant difference in mortality of birds between the tested and control diets in the entire study periods (1-35 d).

8.3.2 Physical and chemical characteristics of the tibia

The results of the analysis of the chemical and physical characteristics of the tibiae of the broilers are shown in Tables 8.5 and 8.6.

Table 8.4 Effects of protease supplementation in diets containing graded levels of RSBM on the FI (kg/bird), BWG (kg) and FCR (1-35 d).

Main effects				
RSBM[1] (%)	**Protease (g/kg)**	FI	BWG	FCR
0		3.58[a]	2.63[a]	1.35
15		3.46[ab]	2.55[ab]	1.36
25		3.38[b]	2.47[b]	1.38
	0.0	3.49	2.54	1.39
	0.2	3.46	2.56	1.35
Sources of variation				
RSBM		*	*	NS
Protease		NS	NS	NS
RSBM x protease		NS	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different; NS= non-significant; *p<0.05; RSBM=raw soybean meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively).

Some of the physical parameters, including weight ($p<0.01$), length ($p<0.05$), tibiotarsal index (weight/length) ($p<0.05$), strength ($p<0.05$), were significantly reduced, and the width also tended ($p=0.07$) to be reduced by 24 d of age in response to increasing levels of RSBM in diets. However, the width of the bone increased significantly ($p<0.05$) when diets were supplemented with microbial protease.

Although increasing the level of RSBM in the diets reduced some of the physical parameters of the tibiae, such as weight (2.8%), tibiotarsal index (2.4%) and strength (8.2%), less than other parameters, there was no statistical effect ($p>0.05$) of the factor at 35 d of age. The weight (0.8%), width (0.4%), density, tibiotarsal index (0.8%), robusticity index (0.24%) and strength (6.8%) of tibia bone were increased when the diets were supplemented with protease, but there were no significant ($p>0.05$) differences between the treatment groups. Interaction between protease x RSBM had no significant ($p>0.05$) effects on any of the other measured physical parameters at 24 or 35 d of age, except tibia was tended ($p=0.09$) to be stronger.

Increasing the inclusion rate of RSBM in broiler diets reduced the tibia DM, weight of ash content and ash percentage by 9.1, 12.5, 1.1%, respectively, but there were no statistical ($p>0.05$) differences at 24 d of age. The ash content of the bone was significantly reduced ($p<0.05$), and the DM also tended ($p=0.06$) to be reduced at 24 d of age when the diets were supplemented with protease.

Table 8.5 Effects of protease in diets containing raw, full-fat SBM on the physical characteristics of the tibiae at 24 and 35 d of age.

RSBM[1] %	Protease g/kg	24 d							35 d						
		Wt, g	L, cm	W, mm	D, g/mL	TI Wt/L	R, $L/Wt^{1/3}$	Strength, N	WT, g	L, cm	W, mm	D, g/mL	TI Wt/L	R, $L/Wt^{1/3}$	Strength, N
0	**0.0**	7.1	7.7	7.1	1.1	0.92	4.0	267.0	12.0	9.55	9.01	1.09	1.25	4.18	392.9
	0.2	7.3	7.8	7.5	1.1	0.94	4.0	280.8	12.2	9.60	8.99	1.13	1.27	4.18	394.6
15	**0.0**	6.3	7.2	6.8	1.1	0.88	4.3	214.7	11.7	9.54	8.99	1.09	1.22	4.21	378.5
	0.2	6.4	7.0	7.4	1.1	0.92	4.2	212.2	12.0	9.60	8.94	1.12	1.25	4.21	403.5
25	**0.0**	6.3	7.7	6.8	1.1	0.81	4.2	261.7	11.9	9.51	8.96	1.12	1.25	4.17	338.1
	0.2	5.8	7.4	6.8	1.1	0.78	4.1	204.6	11.6	9.52	8.99	1.10	1.22	4.21	384.4
Pooled SEM		0.17	0.07	0.09	0.03	0.02	0.04	9.43	0.22	0.05	0.09	0.01	0.02	0.02	12.12
Main effects															
0		7.2^{a}	7.8^{a}	7.3	1.1	0.93^{a}	4.0	273.9^{a}	12.09	9.58	9.00	1.11	1.26	4.18	393.7
15		6.4^{bc}	7.1^{b}	7.1	1.1	0.90^{b}	4.3	213.5^{b}	11.83	9.57	8.96	1.11	1.23	4.21	391.0
25		6.0^{c}	7.5^{ab}	6.8	1.1	0.80^{c}	4.2	238.9^{ab}	11.75	9.51	8.98	1.11	1.23	4.19	361.3
	0.0	6.6	7.6	6.9^{b}	1.1	0.87	4.2	247.8	11.84	9.53	8.99	1.10	1.24	4.19	369.8
	0.2	6.5	7.4	7.3^{a}	1.1	0.88	4.1	236.0	11.94	9.57	8.98	1.12	1.25	4.20	394.2
Sources of variation															
RSBM		**	*	0.07	NS	*	NS	*	NS	NS	NS	NS	NS	NS	NS
Protease		NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
RSBM x protease		NS	NS	NS	NS	NS	NS	0.09	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different at non-significant; *p<0.001; **p<0.01; NS=non-significant; SEM= pooled standard error of means; Wt= weight; L= length; W= width; TI= tibiotarsal index (weight/length); D= density (wt/vol); R= robusticity; N= Newton; RSBM=raw soybean meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0,45 and 75 g/kg of diet, respectively).

Table 8.6 Effects of protease in diets containing graded levels of raw soybean on DM and mineralization of the tibiae of broilers at 24 and 35 d of age.

RSBM[1], %	Protease, g/kg	24 d				35 d			
		DM, g	Ash %	Ca, %	P, %	DM, g	Ash %	Ca, %	P, %
0	**0.0**	3.5	47.2	37.7	19.4	6.4	58.0	41.5	19.6
	0.2	3.2	47.8	37.3	19.5	6.7	63.2	42.3	20.0
15	**0.0**	3.3	48.5	37.6	19.6	6.4	50.0	41.9	19.5
	0.2	3.0	45.7	36.5	19.0	6.5	51.8	41.9	19.9
25	**0.0**	3.1	48.0	37.4	19.6	6.3	58.1	41.6	19.7
	0.2	2.8	45.7	36.5	19.1	6.3	57.1	42.2	20.1
Pooled SEM		0.08	0.6	0.3	0.2	0.1	1.7	0.3	0.2
Main effects									
0		3.3	47.4	37.5	19.5	6.6	60.6	41.9	19.8
15		3.1	47.1	37.1	19.3	6.4	50.9	41.9	19.7
25		3.0	46.9	37.0	19.3	6.3	57.6	41.9	19.9
	0.0	3.3	47.9	37.4	37.6	6.4	55.4	41.6	19.6
	0.2	3.0	46.3	36.9	36.8	6.5	57.4	42.2	20.0
Sources of variation									
RSBM		NS	NS	NS	NS	NS	0.06	NS	NS
Protease		0.06	NS	NS	NS	NS	NS	NS	NS
RSBM x protease		NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c,] Means bearing uncommon superscripts within a column are significantly different; SEM= pooled standard error of means; NS= non-significant; DM= dry matter Ca= calcium; P= phosphorus; RSBM=raw soybean meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively).

Increasing the RSBM inclusion rate in the diets had no significant ($p>0.05$) influence on the DM and mineralization of the tibia bone at 35 d of age. The DM and mineral composition values of the tibia were not significantly ($p>0.05$) influenced by the protease x RSBM interaction at 24 and 35 d of age.

8.3.3 Litter moisture, nitrogen, uric acid, ammonia and pH

The values of the total N, uric acid, ammonia, moisture and pH in the litter are shown in Table 8.7. Increasing the level of RSBM in the diets had no significant ($p>0.05$) effects on the pH values of the litter, but protease supplementation tended ($p=0.8$) to increase the pH at 14 d of age. Although protease supplementation increased the pH of litter by 5.7% (14 d), 1.3% (24 d) and 1.2% (35 d), there were no statistical ($p>0.05$) differences between the treatment groups. The pH tended ($p=0.09$) to be higher at 14 d of age due to the interaction effects between the protease and RSBM in the diets. Neither protease nor RSBM supplementation had significant effects ($p>0.05$) on the moisture content of the litter on any of the assessed days (14 d, 24 d, or 35 d).

Table 8.7 Effects of protease in diets with raw soybean on the pH values and moisture content of litter on different days.

RSBM[1]	Protease	Moisture (%)			pH		
%	g/kg	14 d	24 d	35 d	14 d	24 d	35 d
0	**0.0**	46.0	67.1	67.5	6.9	7.8	7.9
	0.2	48.1	66.6	69.3	6.7	7.9	8.4
15	**0.0**	42.4	71.5	70.3	6.6	7.5	8.6
	0.2	45.6	64.7	68.4	7.1	7.8	8.4
25	**0.0**	39.7	68.9	68.2	6.4	7.8	8.3
	0.2	43.1	67.4	67.1	7.2	7.8	8.3
Pooled SEM		0.10	1.0	1.4	0.10	0.05	0.08
Main effects							
0		47.0	66.8	68.5	6.8	7.8	8.1
15		44.1	68.1	69.4	6.9	7.7	8.5
25		41.6	68.1	67.6	6.8	7.8	8.3
	0.0	42.9	69.2	68.8	6.6	7.7	8.3
	0.2	45.6	66.2	68.2	7.0	7.8	8.4
Sources of variation							
RSBM[1]		NS	NS	NS	NS	NS	NS
Protease		NS	NS	NS	0.08	NS	NS
RSBM[1] x protease		NS	NS	NS	0.09	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different; SEM= pooled standard error of means; NS= non-RSBM= raw soybean meal; RSBM=raw soybean meal (SBM was replaced by RSBM at 0, 15 and 25 %, equivalent to 0, 45 and 75 g/kg of diet, respectively).

As shown in Table 8.8, total N was increased significantly in the litter at 24 d ($p<0.05$) and 35 d ($p<0.01$) in response to increasing levels of RSBM. The nitrogen in the litter was decreased by 2.5% (24 d) and 4.4% (35 d) when diets were supplemented with microbial protease, but the differences were not significant ($p>0.05$). Protease supplementation of the diets significantly reduced ($p<0.05$) the uric acid content in the litter at 35 d of age. The concentration of ammonia in the litter was increased ($p<0.05$) due to protease supplementation.

The results of the minerals excreted in the litter are shown in Table 8.9. As assessed at 24 d ($p<0.05$) and 35 d ($p<0.01$), the Ca content was significantly reduced in the litter due to increasing levels of RSBM. However, the concentration of P was not significantly ($p>0.05$) affected. Protease supplementation increased the ash content in the litter by 3.7% and 2.5% compared to the other treatments, but the differences were not statistically significant ($p>0.05$). At 35 d of age, protease supplementation of the diets significantly increased ($p<0.01$) the contents of Ca in the litter.

Table 8.8 Effects of protease in diets containing graded levels of raw soybean meal on total N, uric acid and ammonia contents at 24 or 35 d of age.

RSBM %	Protease g/kg	Total nitrogen (g/kg)		Uric acid (mg/g of dry sample)	Ammonia (mg/mL)
		24 d	35 d	35 d	35 d
0	**0.0**	29.6	31.8	219.1	0.34
	0.2	30.5	33.8	215.3	0.41
15	**0.0**	32.4	38.0	272.4	0.21
	0.2	30.5	34.1	215.1	0.55
25	**0.0**	34.7	38.5	246.6	0.22
	0.2	33.6	35.5	207.2	0.49
Pooled SEM		0.7	0.64	7.7	0.05
Main effects					
0		30.1[b]	32.8[b]	217.2	0.39
15		31.4[ab]	36.1[a]	243.7	0.44
25		34.1[a]	37.0[a]	226.9	0.38
	0.0	32.4	36.1	246.1[a]	0.25[b]
	0.2	31.6	34.5	212.5[b]	0.48[a]
Sources of variation					
RSBM		*	**	NS	NS
Protease		NS	NS	*	8
RSBM x protease		NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different; SEM= pooled standard error of means; NS= non-significant; RSBM= raw soybean meal; *p<0.05;**p<0.01; RSBM=raw soybean meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively).

Phosphorus was also increased by 3.6% compared to the other treatments, but the difference was not statistically significant (p>0.05). The assessment on day 24 showed that the concentration of Ca and P in the litter were increased by 4.9 and 4.2%, respectively, when the diets were supplemented with protease, but they were not significantly (p>0.05) affected by the protease x RSBM interaction at 24 or 35 d of age.

8.3.4 Effects of diets containing raw, full-fat soybean meal and supplemented with protease on intestinal lesions and footpad dermatitis

The intestinal lesion and footpad dermatitis results for broilers are shown in Tables 8.10 and 8.11. Increasing the dietary level of RSBM and supplementing with protease in diets reduced the footpad dermatitis scores by 15.5 and 5.5%, respectively, compared to the other treatments, but there were no statistical (p>0.05) differences at 35 d of age

Table 8.9 Effects of protease in diets containing raw, full-fat soybean on the mineral contents of the litter at 24 and 35 d of age.

RSBM %	Protease g/kg	24 d			35 d		
		Ash (g)	Ca (%)	P (%)	Ash (%)	Ca (%)	P (%)
0	**0.0**	19.2	4.2	2.5	19.3	3.9	2.8
	0.2	18.5	4.3	2.4	19.1	4.0	2.7
15	**0.0**	17.7	3.6	2.2	19.8	3.2	2.6
	0.2	19.5	3.7	2.3	20.8	3.7	2.7
25	**0.0**	18.2	3.8	2.4	19.5	3.3	2.8
	0.2	19.2	4.2	2.5	20.1	3.8	2.9
Pooled SEM							
Main factors							
0		18.8	4.3[a]	2.4	19.2	3.9[a]	2.8
15		18.6	3.7[b]	2.2	20.3	3.5[b]	2.7
25		18.7	4.0[ab]	2.5	19.8	3.6[b]	2.8
	0.0	18.4	3.9	2.3	19.5	3.5[b]	2.7
	0.2	19.1	4.1	2.4	20.0	3.8[a]	2.8
Sources of variation							
RSBM		NS	*	NS	NS	**	NS
Protease		NS	NS	NS	NS	**	NS
RSBM x protease		NS	NS	NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different at NS= non-significant; *p<0.05; **p<0.01; SEM= pooled standard error of means;; Ca= calcium; P= phosphorus; RSBM=raw soybean meal (SBM was replaced by RSBM at 0, 15 and 25 %, equivalent to 0, 45 and 75 g/kg of diet, respectively).

Increasing the level of RSBM and protease supplementation in the diets, increased (not significantly) subclinical intestinal lesions by 9.3 and 2.5%, respectively. There was no significant influence (p>0.05) on intestinal lesions due to interaction between RSBM and protease, at 24 d of age.

Table 8.10 Effects of protease in diets containing raw soybean meal on footpad dermatitis and intestinal lesion scores.

RSBM %	Protease g/kg	Footpad dermatitis		Intestinal lesion
		24 d	35 d	24 d
0	**0.0**	0.67	2.74	1.17
	0.2	0.33	2.68	1.17
15	**0.0**	0.50	2.57	1.20
	0.2	0.17	2.43	1.20
25	**0.0**	0.50	2.39	1.33
	0.2	1.00	2.19	1.25
Pooled SEM		0.02	0.08	0.07
Main factors				
0		0.50	2.71	1.17
15		0.33	2.50	1.20
25		0.75	2.29	1.29
	0.0	0.56	2.57	1.17
	0.2	0.50	2.43	1.20
Sources of variation				
RSBM		NS	NS	NS
Protease		NS	NS	NS
RSBM x protease		0.06	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different; SEM= pooled standard error of means; NS= non-significant; RSBM=raw soybean meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively).

8.3.5 Inositol and electrolytes in blood plasma

Although not statistically significant, the concentration of inositol in the blood plasma of broilers at 24 d of age was reduced by 11.2 and 3.3% with increasing levels of RSBM and supplemental protease in the diets, respectively. Compared to the other treatments, the values of the electrolytes Na^{2+} and Cl^{-}, in the blood plasma at 24 d of age were decreased by 2.0 and 7.7%, respectively, in response to rising levels of RSBM. However, when diets were supplemented with protease, Cl^{-} was increased by 11.7% compared to the birds on non-supplemented diets.

Table 8.11 Effects of protease in diets containing graded levels of raw soybean meal and phytase (2000 FYT/kg) on the plasma concentrations of inositol and electrolytes in broilers at 24 d of age.

RSBM (%)	Protease (g/kg)	Inositol	pH	Electrolytes (mmol/L)	
				Na^+	Cl^-
0	**0.0**	67.2	7.83	111	84.4
	0.2	66.6	7.81	108.3	81.5
15	**0.0**	58.4	7.80	113.4	61.54
	0.2	56.0	7.86	90.7	67.7
25	**0.0**	63.4	7.89	100.2	63.2
	0.2	60.2	7.84	113.8	90.2
Pooled SEM		2.72	0.02	5.39	5.35
Main effects					
0		66.9	7.82	109.8	83.1
15		57.2	7.83	101.0	64.9
25		61.8	7.86	107.6	76.7
	0.0	63.0	7.84	108.2	69.7
	0.2	60.9	7.84	103.8	78.9
Sources of variation					
RSBM		NS	NS	NS	NS
Protease		NS	NS	NS	NS
RSBM x protease		NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different; SEM= pooled standard error of means; NS= non-significant; RSBM=raw soybean meal (SBM was replaced by RSBM at 0, 15 and 25%, equivalent to 0, 45 and 75 g/kg of diet, respectively).

8.4 DISCUSSION

8.4.1 Diets and the performance of broilers

Although the concentration of ANF, for example TI in some of the dietary groups in this study was approximately 10193.4 TIU/g, which is beyond the threshold level for non-ruminant animals (Hong *et al.*, 2004), the FCR over the 1-35 d periods were not statistically affected. Results of BWG, FI and FCR were slightly improved due to the positive effect of protease in the diets or and/or adjustment of the birds, over time. The results are consistent with those of other researchers (Barletta, 2011; Pettersson and Pontoppidan, 2013) who reported that protease can break down both the stored proteins and the proteinaceous anti-nutrients and subsequently improve nutrient digestibility.

8.4.2 Intestinal lesions and footpad dermatitis in relation to the wellbeing and performance of broilers

Birds fed diets containing RSBM appeared to be as healthy as those on other diets, with no significant differences in mortality. Increasing the level of RSBM in diets reduced the footpad dermatitis scores by 15.5% compared to the other treatments, but the differences were not significant. This trend toward reduced footpad dermatitis scores with increased levels of RSBM in diets might be directly related to the lower body weights of birds on RSBM containing diets.

Supplementation of diets with microbial protease reduced the footpad dermatitis score by 5.5% compared to the other treatments, but there were no significance differences between the treatment groups. It is most likely that this reduction might be related to the significant reduction in the uric acid contents in the litter, and this result partially agrees with Youssef *et al.* (2011) who reported that litter quality plays an important role in the incidence of footpad dermatitis, which, in turn is influenced by diet composition.

Increasing the level of RSBM in diets increased the number of intestinal sub-clinical lesions by approximately 9.3%, but the difference was not significant. The results of this study partially agree with Palliyeguru *et al.* (2011) who reported that increasing the amount of non-toasted soya bean in diets resulted in a significant linear increase in sub-clinical necrotic enteritis along the intestinal tract of broiler chickens. This effect may be the one that has not been commonly reported for ANF in SBM and provides new information on the cause of reduced performance on the diets containing RSBM.

8.4.3 Total nitrogen, uric acid, ammonia, pH and moisture of the litter

Supplementation of diets with increasing levels of RSBM significantly increased the total N content in the litter. This result agrees with the findings of other researchers (Banaszkiewicz, 2011; Dourado *et al.,* 2011) who reported that the activity of TI in digestive systems can negatively affect the N retention and result in increased metabolic N excretion. Some dietary N (CP) also remains undigested, as previously observed in another study in this project. This, along with unabsorbed CP and AA, are then excreted on to the litter.

Protease supplementation reduced the N content of litter, but there were no significant differences among any of the assessed days (24, or 35 d). These partially agree with those of Oxenboll *et al*. (2011) who reported that protease supplementation reduced nitrogen excretion.

Rokade *et al*. (2014) also reported that protease supplementation can minimize nitrogen excretion and the pH values of litters without affecting bird performance.

As assessed on day 35, the uric acid content was significantly increased in the litter when the diets were supplemented with microbial protease. The opposite pattern was observed for ammonia. The reduction in total N content with an increase in uric acid observed in the current study with protease supplementation is indicative of increased digestion of proteins, some of which is then metabolized into uric acid and excreted. Nitrogen is generally excreted in urine by birds in the form of uric acid.

8.4.4 Physical characteristics and mineralization of the tibiae

When supplementing the diets with increasing the level of RSBM, most of physical parameters of the tibiae in broilers, such as weight, length, tibiotarsal index and strength, were significantly reduced at 24 d of age, but none of the differences were significant on the next assessment (35 d). The reduction of measuring values of some physical properties of tibia bone in this study might be related to lower body weight, recorded for birds fed on diets containing RSBM.

As assessed on day 35, the values of the physical parameters of the tibia, including weight, width, tibiotarsal index, robusticity index and strength improved due to supplementation of protease, but there were no significant differences between treatment groups. The improved values of some physical properties of tibia bone, under protease supplementation might be related to the higher body weights recorded for birds fed on diets supplemented with protease.

8.4.5 Inositol, electrolytes and metabolites in plasma

Increasing the levels of RSBM in diets reduced the concentration of inositol in the blood plasma of broilers by 11.2% at 24 d of age. The main reason why the concentration of inositol was reduced in this study might be due to the adverse effects of ANF, such as phytate and TI, on the physiological processes, such as metabolism, of broilers.

When diets were supplemented with increasing levels of RSBM, some of the electrolytes, including Na^{2+} and Cl^{-} were decreased in the blood plasma at 24 d of age. However, when diets were supplemented with protease, Cl^{-} was increased compared to the non-supplemented diets. The reason why these electrolytes greatly increased in the plasma with increasing levels of RSBM in the diets was not clear. Generally, increasing RSBM may be negatively affecting

availability of minerals in plasma, and the inverse might be true when supplementing protease in diets.

8.5 CONCLUSION

It is not common practice to include RSBM in diets, but the result obtained in this study showed that this could be done without major detrimental effects on productivity or welfare of broiler chickens. The birds themselves tend to adjust to the diets with time, but further supplementation with protease has a positive effect. Therefore, it is evident from this study that microbial protease have the capacity to reduce the adverse impacts of dietary ANF on the productivity and wellbeing of birds. This is demonstrated by reduction in excretion and uric acid contents and slight improvement in bone quality with protease supplementation. The effect of age is obvious from changes in bone quality. Raw, full-fat soybean meal in diets tended to reduce the quality of tibia bone in early life (24 d), but the effect waned when the birds were assessed at 35 d of age. Further studies are required into the protease-combinations that will yield the best results.

CHAPTER 9: GENERAL DISCUSSION

9.1 Introduction

Feed cost is by far the major variable cost in poultry production. Most of the ingredients used for poultry feeding are categorized as energy or protein source. In comparison to other plant feed ingredients, soybeans have the best nutritional composition for poultry. High price and, in some places, the unavailability of SBM, are some of the problems faced by end-users. In addition to regular SBM, poultry producers use full-fat SBM (ASA, 1997; Willis, 2003), but raw SBM is rarely used due to the presence of ANF.

Full-fat soybean meals have the potential to replace both commercial SBM and dietary oil for broilers. As reviewed in Chapter 2, meals prepared from raw soy seeds, have ANF such as TI, phytate and lectin, which negatively affect soybean utilisation by poultry. Although heating is considered the best option to eliminate or reduce most of the ANF from soybean seeds, either under- or over-heating during processing reduces the nutritional value of SBM. Concern for the health of consumers is also ever present as the residues of the solvents used for extracting the oil may pose health risks.

The poultry industry therefore needs to utilise biotechnological techniques, like genetic modification of feedstuffs, or feed additives, e.g. enzymes in order to improve the nutritive value of SBM and other ingredients to maximize feed quality. Primarily, information on the physical and chemical properties of feed ingredients provides the basic guide on how to intervene and utilise feed ingredients.

Supplementing the diets with exogenous feed enzymes in general can help to reduce the adverse effects of many ANF on non-ruminant animals (Khusheeba and Sajid, 2013). Exogenous proteases are protein-digesting enzymes that break down both stored proteins and proteinaceous anti-nutrients in feeds. Besides releasing phosphorus, supplementation with microbial phytase also increases the digestibility of CP and amino acids of plant-based proteins (Barletta, 2011; Guggenbuhl *et al*., 2012). Dosković *et al. (*2013) have argued and concluded that enzyme supplementation of poultry diets has nutritional, economic and environmental benefits.

This project also provided the opportunity to explore the role of noble enzyme in diets containing RSBM. Although raw soybean meal (RSBM) is limited by high levels of ANF, it is similar in many respects to commercial full-fat SBM. This project investigated the

concentration of ANFs, which include TI, UA, NSI and KOH in broiler diets containing varying levels of RSBM, and the role of noble enzymes in diets containing RSBM.

9.2 Physicochemical properties of full-fat RSBM, and in *vitro* nutrient digestibility

As Stein *et al*. (2008) have reported that full-fat soybean is known to have a relatively low CP content (36-42 %) and high EE (18-22%). The results of the current study confirmed that the average CP was 39.3%, but the EE was 15.3%, which is far below the values reported in literature. The reason for this low EE value might be due to the variety of soybean crop and its origin. Further analysis in the current study indicates that raw full-fat soybean has a good AA composition similar to value reported by Pahm and Stein (2007) in their research on commercial full-fat. In this study, as expected, the calculated AME value of full-fat soybean was higher than that of commercial SBM. These current results are supported by the reports of previous studies; for example, Woodworth *et al.* (2001) reported that the concentrations of DE and ME in full-fat SBM are greater than those extracted SBM. Full-fat SBM, cooked or raw, has a high oil content, a source of energy.

Although Kong *et al*. (2015) reported that the *in vitro* digestibility of SBM was not significantly affected by addition of an enzyme complex, containing xylanase, protease, and phytase, this is in contrast to the current results of *in vitro* DM and CP digestibility of RSBM, which was improved by an enzyme cocktail (phytase + protease) in excess of the response obtained with individual products.

9.3 Response of broilers to the diets

As the inclusion rate of RSBM was increased in diets, most of the analyzed values of ANF, such as TI, NSI and KOH showed a proportional increase. For example, the concentration of TI in the RSBM was 13098.0 TIU/g prior to mixing and ranged between 1730.5 and 10,484.4 TIU/g (after mixed) in the treatment diets, which is beyond the threshold level for poultry. However, due to supplementation with microbial protease and phytase (the best combination), the response, in terms of FI, BWG, and FCR, was statistically similar to those of birds on the control diets, when fed for 1-35 d. These findings are in line with those of previous researchers (Simbaya *et al.,* 1996; Ayoola *et al.,* 2015) who reported that exogenous enzymes are used to alleviate the adverse effects of ANF for non-ruminant animals. Arriving at the same values for the gross response of birds on the tested diets as those on control diets in this project is most

likely indicative of the effectiveness of protease and phytase at reducing the adverse effects of the key RSBM ANF on birds.

As previous researchers (Mogridge *et a*l., 1996; Erdaw *et al.*, 2016d) found, the consumption of raw soybeans increased the size of the pancreas and the weight of some other internal organs of broilers, for example, duodenum. This response may be due to organs struggling to develop in order to perform their functions. The pancreas is the source of most of the endogenous proteases in poultry and may respond to inhibition of these enzymes by TI.

9.4 Tissue protein content, digestive enzyme activities and mucosal morphometry of the jejunum

The mucosal morphometry of the jejunum was neither affected by increasing RSBM nor by protease supplementation, which contradicts with Rocha *et al.* (2014) who reported that the intestinal villi of broilers were shorter in birds fed 15% raw full-fat soybeans. The lack of response in the current study may be due to the effect of the test enzymes, particularly phytase, which was included in all diets.

Tissue protein content and the activities of digestive enzymes of birds were influenced by the supplementation of protease in the diets. Although a pronounced pancreatic hypertrophy was identified in the broiler chickens fed diets containing high levels of RSBM, during the entire study period, on average, values of digestive, particularly pancreatic enzyme activities were improved when diets were supplemented with protease. The functionality of digestive enzymes, reported in this thesis are in line with Yuan *et al.* (2008) who reported that the increments in the activities of amylase and trypsin due to supplementation of enzyme complex. This response may be due to the effect of exogenous enzyme on ANF, including TI. There has only been limited research on effect of TI on the basic anatomy and function of the digestive system in poultry.

9.5 Ileal nutrient digestibility

Although slight improvements were observed when diets were supplemented with protease, the AID of CP and AA was strongly reduced by the presences of RSBM. These findings are supported by Rada *et al.* (2015) who reported that the AID of AA except methionine was reduced when diets contained raw in full-fat soybean. On the other hand, Clarke and Wiseman

(2005) found that AID did not correlate with TIA levels, indicating that other factors also affect the digestibility of AA in full-fat soybean meal and SBM. The reduced values of AID and SID of CP and AA at 24 days of age were also reflected in the gross response of broiler chicks, for example the BWG at the same age. However, this reduction in performance generally did not persist to 35 d. It is likely that young birds were more affected by the ANF.

The SID of CP and AA decreased markedly in response to increasing level of RSBM, but they were improved by supplementation with microbial protease. The current results are in line with those of Yua *et al.* (2016) who reported that the AID and SID of CP and total AA of non-ruminant animals, for example piglets, increased when the diets were supplemented with microbial protease. Similarly, Yu *et al*. (2007) have shown that supplementation with either protease by itself or a cocktail of protease and carbohydrase to a maize–soybean meal diet improved chicken growth. There are no previous reports on the effects of the test protease on nutrient digestibility in diets containing RSBM.

9.6 The effect of trypsin inhibitors on the wellbeing and health of broilers

Although the concentration of ANFs, particularly TI in some diets of the current study was beyond the threshold levels for birds, there was no significant difference in mortality recorded in birds. In addition, increasing the level of RSBM did not significantly elevate intestinal lesions of broilers. These results partially contradict with Palliyeguru *et al.* (2011) who reported that increasing the amount of non-toasted soya bean in diets resulted in a significant linear increase in sub-clinical necrotic enteritis along the intestinal tract of broiler chickens. The most likelihood of getting reduced health risk, on birds in association to ANF, particularly TI in diets might be contributed by supplemental protease, which could break down the stored and proteinaceous anti-nutrients in diets.

Youssef *et al.* (2011) have reported that litter quality plays an important role in the incidence of footpad dermatitis, which in turn is influenced by diet composition, this did occur in footpad dermatitis of birds. However, in this current study, even if the N and uric acid concentrations in litter were significantly increased when diets were supplemented with increasing RSBM and microbial protease, respectively, there was no significant effect on footpad dermatitis. The reason for increasing uric acid when supplanting protease in this study is not clear.

9.7 Conclusion and recommendations

The physico-chemical properties of RSBM, for instance the contents of ANF, including TI were different from those of the commercial SBM, and the gross energy content was higher for RSBM. Although the analysed value of CP was found to be lower for samples of RSNM, the total AA composition of RSBM and commercial SBM were almost equivalent. The concentrations of ANFs in seeds and diets still remained quite high, in spite of the heat treatment that was applied, or steam pelleting. This signified that an inadequacy in heating intensity and/or duration. However, *in vitro* testing showed that use of an enzyme cocktail (phytase + protease) was effective, and led to an increase in nutrients and phytate digestibility.

In the test diets containing RSBM, the AA composition of both the cold-pelleted and the mash diets appear to be better than those of the steam-pelleted diets, but ANFs, for example TI, were still above the threshold levels. The subsequent impact of these ANF was reflected in the reduced performance of the birds, especially during the early phase of production. Steam-pelleting alone might not be sufficient to reduce the negative impact of ANF in RSBM on birds.

Generally, it is not common practice to include RSBM in diets, but when microbial protease was supplemented and over the longer production cycle, birds tolerated up to 20% of the RSBM, replacing the commercial SBM without greatly compromising their productivity. Although there was a pronounced increase in the weight of the pancreas in birds fed diets containing high levels of RSBM, most of the pancreatic digestive enzyme activities were substantially improved due to the dietary supplementation with microbial protease. The response of the birds, in general, suggested that the pancreas remained functional and produced enough enzymes to digest the nutrients in the diets containing RSBM. The supplemental enzymes also aided some of this digestion.

When the enzymes are dosed at levels higher than currently recommended, commercial SBM could be replaced at up to 25% by RSBM without major impact on performance, welfare and feed costs. This was mainly true at the best combination of the protease and phytase. The AID of CP and AA was also significantly reduced because of increasing RSBM in diets, but slight improvements were found when diets were extra-dosed with protease or phytase, which might be an indicator of a positive effect of the enzymes on ANFs, including TI, in diets.

It is evident from this study that both microbial protease and phytase have the capacity to reduce the adverse impact of dietary ANF on the productivity and wellbeing of birds. This is demonstrated by reduction in excretion of N and uric acid contents and improvement in bone quality with protease supplementation. There were no obvious pattern of mortality, footpad

dermatitis and intestinal lesions in response to RSBM. The effect of age is also obvious from changes in bone quality and other gross responses. Raw, full-fat soybean meal in diets tended to reduce the quality of tibia bone in early life (24 d), but the effect waned when the birds were assessed at 35 d of age.

Commercial SBM can partially be replaced (≤25%) by RSBM for broilers provided appropriate combination of microbial protease and phytase as those tested in this project are supplemented in diets. There is a need for further studies into the following areas:

- The mechanisms of action of the test enzymes, especially with regards to their effect on raw soy proteins.
- Assessment of how raw soy protein is digested by the test enzymes, both *in vitro* and *in vivo.*
- In-depth assessment of the effect of RSBM at molecular, cellular and tissue levels.

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