CHAPTER 1
GENERAL INTRODUCTION

There has been an increasing interest in the identification of alternative fuels following the rapid rise in price of crude oil. Therefore, in recent years, bio-fuels have been one of the options considered. The United States and the European Union have promoted the use of bio-fuels, since this source of energy is renewable, reasonably clean and results in lower greenhouse gas emissions (Swiatkiewicz and Koreleski, 2008). Generally, bio-fuels, and ethanol in particular, are produced from grains and other crops through a fermentation process, during which approximately equal amounts of ethanol, CO_2 and distillers' dried grains with solubles (DDGS) are formed.

Distillers' dried grains with solubles, from the fermentation of cereal grains, have been used as a feed ingredient for farm animals for a long time, having first been incorporated into animal diets in the late 1930s (Lumpkins *et al.*, 2005). Early use of DDGS was justified by the presence of an "Unidentified Growth Factor" that improved the performance of poultry and livestock. Although DDGS were primarily fed to ruminants because of their high fibre content and nutrient variability, this feed ingredient has been recommended for inclusion in poultry diet since it is considered to be a good source of energy and protein for poultry. In general, all nutrients in the main grain are concentrated approximately three folds in DDGS, except starch, which is converted to ethanol and carbon dioxide during fermentation. Therefore, maize DDGS generally contain 27 % crude protein, 10 % oil and 0.8 % phosphorus, and can be regarded as a suitable feed ingredient for poultry (Babcock *et al.*, 2008).

Depending on the parent grain, drying practices, rate of solubilisation, and fermentation process, considerable variation in nutrient composition of DDGS has been documented (Cromwell *et al.*, 1993; Belyea *et al.*, 2004; Youssef *et al.*, 2008). Such variation is particularly problematic for diet formulation, in which an accurate estimate of nutrient specification of the ingredient must be provided. The quality of protein and in particular amino acid content of DDGS may also restrict DDGS inclusion at high levels in poultry diets (Fastinger *et al.*, 2006; Kim *et al.*, 2008; Kim *et al.*, 2010; Adeola and Zhai, 2012).

Fibre content is regarded as the key issue limiting the incorporation of DDGS in poultry diet. The carbohydrate contents in DDGS are lower than in cereal grains, most of it, non-starch polysaccharides (NSP). In most studies 40-50% of carbohydrates in DDGS are unaccounted for, being NSP, which have been overlooked (Choct and Peterson, 2009).

Nevertheless, it has been shown that DDGS can be included in broiler diets at rates up to 15% without adverse effect on performance (Thacker and Widyaratne, 2007). Despite considerable data in literature on the inclusion of DDGS in broiler diets, the actual reasons and mechanisms underlying the impaired growth of broilers, particularly at a high level of inclusion, are still unclear. It is unknown what impacts this by-product may have on the digestive function of poultry, and few researchers have conducted investigations to determine the effect of different exogenous enzymes at high levels of DDGS in the diet.

Undoubtedly, the extensive rearing system in the poultry industry, coupled with the strong pressure of genetic selections for the growth traits, may have compromised the immune system. This negative impact on the immune system may further be exacerbated by the ban of in-feed antibiotics in poultry diets. From that point of view, understanding of the nutritional impacts of diets and feed ingredients on the immune response, in particular disease development is an absolute necessity. There is a dearth of research on the effect of DDGS on health of broiler chickens, but the results for the health promoting or compromising influence of DDGS are contradictory. Such evaluation of DDGS is linked to the investigation of the microbial profile in the gastrointestinal tract (GIT) of poultry, an area on which there is limited information in the literature.

The energy economy of poultry diet has also been the subject of much debate and discussion as the price of feed, and therefore energy, has steadily increased over the past decade. Although metabolisable energy (ME) is predominantly used for the prediction of the energy value of poultry diets and ingredients, net energy (NE) provides a more precise estimate of energy content because it accounts for the energy losses as heat (Noblet, 2010a). For the fibrous ingredients, the drawback of the ME system is that it gives an overestimation of energy content. Due to the presence of high amounts of fibre in DDGS (Choct and Petersen, 2009), it is worth investigating whether the NE value of a diet containing DDGS may be able to better explain the utilisation of energy by broiler chickens.

Currently most DDGS available in Australia are imported, but the volume of local production is expected to rise with the commissioning of a second ethanol plant in 2008, and several new

plants that are planned to begin production in the near future. However, the nutritional value of DDGS produced in Australia, which is predominantly from sorghum, has not been extensively evaluated, especially for inclusion in poultry diet.

All considered, the general aim of the current dissertation is to better understand the influence of this ingredient on nutrient utilisation and gut health, along with the possible impact of enzyme supplementation as the key strategy to improve the nutritive value of DDGS for poultry with an emphasis on high dietary inclusion of this by-product in broiler diets.

Therefore, the objectives of this research were to:

- Investigate the nutritional composition of Australian DDGS from different batches and possible variability in composition;
- Identify the impact of microbial enzyme supplements on the nutritive value of sorghum DDGS in broiler chicken diets;
- Examine the influence of DDGS and microbial enzymes on gut health, general immune response and development of enteric disease, in particular necrotic enteritis;
- Characterise the microbial profile of healthy and challenged broilers fed DDGS, and
- Evaluate the energy utilisation of broiler chickens on diets containing DDGS with special focus on heat production and net energy value of the diets using different techniques.

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

There has been an increasing concern over the non-renewable nature of fossil fuel resources and environmental issues surrounding their use, particularly the high emission of greenhouse gases. This concern has been intensified by the increasing cost of fossil fuels, which has increased demand for renewable energy sources such as ethanol. Consequences of turning grains to fuels are two fold; firstly it pushes up grain price, and secondly, massively increases the availability of the by-products distillers' dried grains with solubles (DDGS) and glycerine (Westcott, 2007). The number of ethanol bio refineries is set to increase all around the world, including Australia. In 2010, it was shown that ethanol production increased to over 87 billion litres. Table 2.1 shows the global ethanol production from 2008 to 2011.

At present, cereal grains, maize in particular, are mainly used to produce ethanol; therefore, the pressure on maize supply is greater than ever, resulting in price rises. Moreover, the increasing amount of by-products obtained from ethanol production appears to be a serious issue for the environment and also has economic ramifications. In fact, the increased demand for maize caused an unexpected increase in maize price from $2.25 per bushel in 2005 to $7.00 in 2008 (Leibtag, 2008). Therefore, animal nutritionists have to address this problem by finding ways to efficiently utilise these by-products in feed. Distillers' dried grains with solubles, as the main by-product of ethanol production, have been considered to be a valuable feed ingredient available for livestock. However, its application to poultry nutrition has faced some limitations which hinder nutritionists from using large amounts of DDGS in diets. The presence of non-starch polysaccharides (NSP), variability, bulk density and flowability are the main concerns about high inclusions of DDGS. Understanding the need to use DDGS and the effects on bird performance are the main ways by which to enhance the utilisation of DDGS. In the current review, an attempt has been made firstly to introduce and justify the necessity of investigating DDGS as an alternative feed ingredient in poultry. This is followed by an explanation of the present limitations to the use of DDGS and the implication for poultry performance and nutrient utilisation.

Table 2.1 World ethanol fuel production (billion litres) in different countries or regions from 2007 to 2011

Country	2007	2008	2009	2010	2011
USA	24.55	34.96	41.39	49.21	52.62
Brazil	19.00	24.50	24.90	26.20	21.10
Europe	2.16	2.78	3.94	4.58	4.42
China	1.84	1.90	2.05	2.05	2.10
Canada	0.80	0.90	1.10	1.35	1.75
Asia (except China)	0.50	0.59	1.99	0.92	1.27
South America (except Brazil)	0.28	0.30	0.31	0.76	0.75
Mexico and Central America	Na	na	na	1.38	0.15
Australia	0.10	0.10	0.22	0.25	0.33
Africa	Na	na	na	0.17	0.14
Other	0.31	0.48	0.93	0.25	na
WORLD	**49.54**	**66.51**	**76.84**	**87.11**	**84.63**

Source: Alternative fuels and advance vehicles data centre; available at http://www.afdc.energy.gov/afdc/. Data were converted from million gallons to billion litres.

2.2 ALTERNATIVES TO CONVENTIONAL FEED INGREDIENTS

It is evident that poultry industries have a key role to play in terms of providing food resources for human consumption. Therefore they have considerable influence on the sustainability of the ecological system. Economic sustainability has been one of the most important criteria of sustainability, and is mainly associated with profitability (Preston, 1992). Cereal grains, particularly maize and soybean meal constitute the main ingredients of poultry diets, but they may not be readily available in many countries. The production and availability of conventional feed ingredients may be restricted due to various reasons. Climate vagaries and lack of arable land may be limiting factors to growing cereals in some countries. Under such circumstances, some countries do have to import large amounts of cereals, and this may constitute a disastrous financial situation particularly for developing countries. Besides, insufficient foreign exchange, especially for poorer, developing countries, makes the importation difficult (Farrell, 2005).

Currently, due to human population growth, demand for grains and additional food required for this population is rising. Thus, this demand may cause an increase in price of cereal grains needed for animal feeding. Apart from that, the cost of conventional feed ingredients has

recently increased further because of a rise in oil price. This affects poultry production, processing and transportation expenditure as well. Following the increase in oil price, it is predicted that maize and other common cereals will increasingly be used for fuel production. In addition, the European Union's decision to ban animal by-products in poultry diets has pushed up demand for conventional protein sources such as soybean meal (Farrell, 2005).

Regardless of price, there has been competition for land to grow crops, which has led to a decline in world grain production over the past few years (www.fao.org). In contrast, the production of other non-conventional feed ingredients has been more stable than that of conventional feedstuffs. All these constraints have triggered many nutritionists to source feasible alternatives for conventional ingredient resources. In replacing conventional feed ingredients, energy and protein are the most limiting factors that nutritionists need to deal with when formulating diets, since conventional sources, such as maize and soybean meal, mainly contribute energy and protein in poultry diets. Thus, alternative feedstuffs may fall into different categories depending on nutrient composition and the main purpose of their inclusion.

2.2.1 Energy sources

A variety of by-products of cereals and fruits have been used as sources of energy depending upon availability, price and nutritive value. It was shown by Farrell (1994) that about 10 to 20% of rice by-product can be incorporated in starter and finisher broiler diets without any detrimental effects on performance. Moreover, broken rice, which is normally not suitable for human consumption, can serve the same purpose since its AME content is as high as 13.5 MJ/kg (Farrell, 2005). Deoiled salseed meal is a non-conventional feed ingredient which is obtained as a by-product from oil production from sal fruits. In terms of nutritive value, this by-product resembles maize, but its rate of inclusion is highly limited due to its high tannin content (Mahmood *et al.*, 2008). Another substitute for cereals, molasses, may be used at up to 40% of the diet. The main issue with molasses is its high mineral content, which is known to cause diarrhoea in animals (Leeson and Summers, 2001). Tapioca meal is also considered a good source of energy and its usage has been reported at rates of up to 40% in poultry diet (Wizna *et al.*, 2009). Although cyanogenic substances in some varieties may be an issue, this can be overcome by drying practices.

Generally, under unsuitable climatic conditions where conventional cereals cannot be grown, millet appears to be a good substitute for poultry diets. Pearl millet and many of the small millets such as *kodon* and *sawan* can be included in the diet at rates of up to 20% (Hidalgo *et al.*, 2004). The main problem with millet is its susceptibility to rust, which reduces yield levels. Nevertheless, millet has a similar amino acid profile when compared to maize. Davis *et al.* (2003) reported AME in millet to be similar to maize. They also showed that an inclusion rate of 50% of a new rust-resistant hybrid of millet had no adverse effect on performance.

2.2.2 Protein sources

Many by-products and non-conventional feed ingredients can readily replace a portion of the protein in a complete diet. It is obvious that quality of protein, amino acid profile, cost effectiveness and presence of antinutritional factors are among the key factors to consider when using these ingredients in poultry diets. Only a few available alternative protein sources are briefly mentioned in this section. Although cottonseed meal (CSM) is used in limited amounts in poultry diets, its inclusion is considered to be remarkably economical. However, gossypol and high fibre content may restrict inclusion rates of CSM. Perez-Maldonado *et al.* (2001) found that rates of about 20% CSM can be included in diets when diets are formulated based on amino acid digestibility and gossypol is removed by neutralization. Another concern has been that CSM in poultry diets can cause discoloration of egg yolk and albumen which limits its usage in the diet.

Coconut and palm kernel meal are other alternative protein sources which contain 18-20% protein, but also have considerably large amounts of mannans and galactomannans (Farrell, 2005). Mustard cake, sesame meal, ramtil cake, linseed meal, cotton cake, cluster bean and maize gluten meal are other non-conventional protein sources which can contribute to protein for poultry. Some animal protein sources such as poultry by-product meal, feather meal, hatchery by-product, liver residue and blood meals are also being used in poultry diet for the same purpose.

2.3 DISTILLERS' DRIED GRAINS WITH SOLUBLES

2.3.1 Background and production

Generally, there are two conventional ways to produce ethanol. Based on different initial treatments of the cereal grains, the process will be either wet milling or dry milling (Figure 2.1). In wet milling plants, the volume of ethanol produced is less than from plants using dry mills. In addition, more capital and equipment are needed for the establishment of wet mill plants. Although most ethanol has been produced by wet mills, the dry process has increased significantly in the past decade. During maize processing, maize kernel is crushed to flour with no separation of the component parts of the grain. In the next step, water is added to form a slurry followed by addition of enzymes to convert starch to dextrose. Before the fermentation process, ammonia is added to regulate pH and as a substrate for yeast. During the fermentation process, carbon dioxide (CO_2) and ethanol are produced from sugar. The main by-product formed in the dry milling ethanol process is distillers dried grains with solubles (DDGS) (Rausch, 2005).

The wet milling process

Essentially, the wet milling process consists of starch production. In this process, after delivering the grains, maize for example, the kernel is separated into the germ, fibre, protein and starch. The maize is then exposed to a weak sulphuric acid solution, which separates grain into its components. In this step, bran and germ are removed from kernel. This separation enhances the quality of starch and consequent co-products. Following this step, the remaining gluten and starch are screened and then transferred to a centrifuge, to separate residues. After centrifuging, a valuable feed ingredient, named gluten protein, is formed, which contains 60 % protein. The remaining starch is also marketed after washing and drying (Rausch, 2005).

Generally, four main co-products are produced through the wet milling process. Condensed maize fermented extractive with about 25 % protein is usually used as a protein source in dairy and beef diets. This material contains minerals and B-vitamins and it may be used as a pellet binder in diets. The second main product is gluten germ meal, which is a valuable feed ingredient for poultry since it has a reasonable amino acid balance. In addition, two forms of maize gluten meal are derived in this process. One of them consists of fibre and bran, which may be available with condensed maize extractive. Its proximate analysis shows 21 %

protein, 2.5 % fat and 8 % fibre. The other form of maize gluten meal is a good source of protein, with an analysis of 60 % CP. This by-product provides high levels of methionine, xanthophylls and rumen bypass protein.

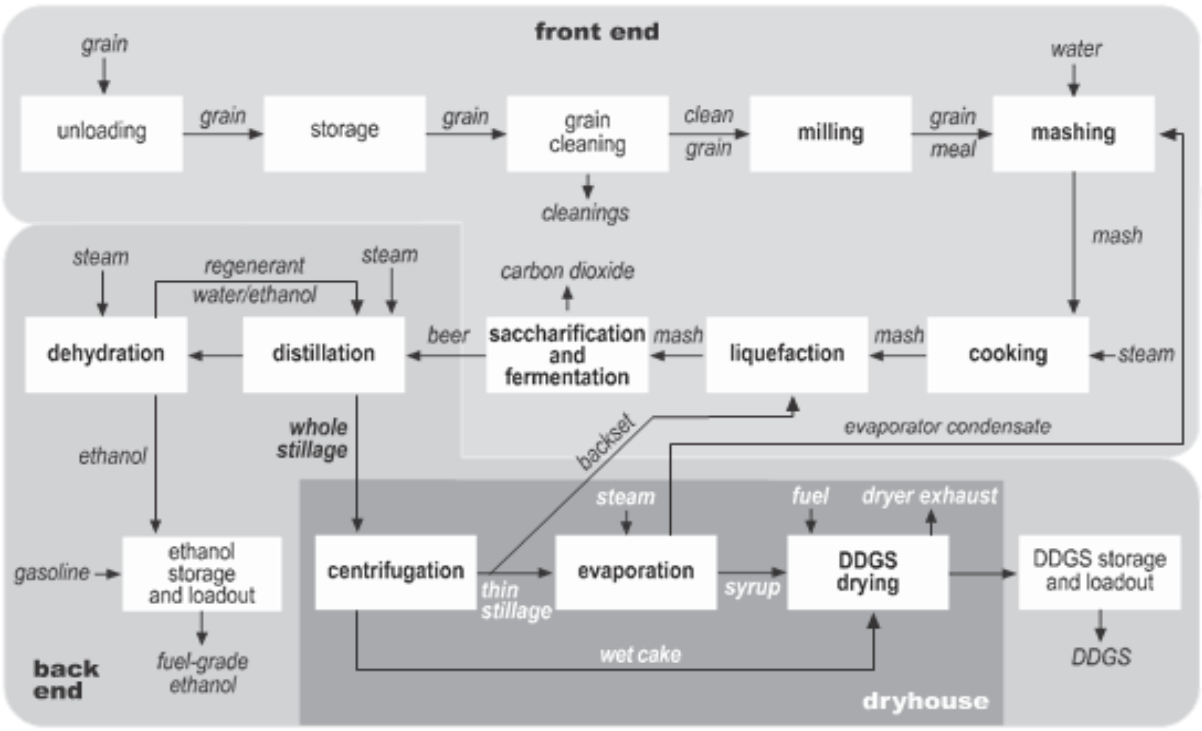

Figure 2.1 Dry milling processes (Monceaux and Kuehner, 2009)

The dry milling process

The dry milling process has been more popular than wet milling because of its emphasis on maximum yield of ethanol and by-products. This process comprises five basic steps: grinding, cooking, liquefaction, hydrolyzation and finally fermentation. Initially, maize kernel is ground by hammer mill then mixed with fresh water to make a slurry. In the next step, the slurry is subjected to alpha-amylase treatment which hydrolyses starch to dextrin, a mash-like product. In order to remove the contaminating lactic acid bacteria, the mash is cooked, then cooled to 32.2 $^{\circ}$C (Monceaux and Kuehner, 2009). After cooking, the mixture goes through a fermentation process in which glucoamylase and yeast are added to facilitate the conversion of dextrin to dextrose. Yeast, *Saccharomyces cerevisiae*, plays a key role in the conversion of dextrose to carbon dioxide and ethanol. Usually, 40-60 hours are needed for completion of the fermentation process. Ethanol is separated in a distillation area where by-

products including fibre, fat and protein can be collected. These solid materials need to be centrifuged to separate the liquid from the solid part. The liquid part is then evaporated to condense and form condensed solubles. After combination of the solid part and solubles, the mixture is dried to obtain the final by-product, named distillers' dried grains with solubles.

2.3.2 Nutrient composition of DDGS

As previously mentioned, cereal grains are used in the fermentation process to make alcohol. Among all cereal grains, maize is the most predominantly used grain in this process. A considerable number of studies have been conducted over the past two decades to assess the composition of maize DDGS. Wu and Sexson (1984) investigated the composition of different fractions of residues from maize alcohol distillation and reported 34.1, 12.7, 5.9 and 1.2 % protein, lipid, ash and phosphorus, respectively, in maize DDGS. They also found that off-screen residue in the ethanol process contains more protein than on-screen residue as a result of yeast cells, which have around 50 % protein. In another study conducted by Belyea *et al.* (2004) on samples produced in a period of five years, contained 31.3 % protein, 11.9 % fat, 10.2 % crude fibre, 5.1 % starch and 4.6 % ash.

Due to unavailability of maize in some areas, other cereal grains may be used for this purpose. Wheat alcohol distillation was also studied by Wu *et al.* (1984) who observed protein contents of 29 and 57 % for distillers' grains and centrifuged solid, respectively. They also found lower fat contents in the wheat fermentation products compared to those from maize, which may lead to a more desirable taste and better storage capability compared to similar residue from maize. Similarly, a wheat DDGS sample evaluated by Thacker and Widyaratne (2007) contained 35.67 % protein, 5.38 % fat, 4.61 % ash, 0.18 % calcium and 0.19 % phosphorus. Similar results were found by Nyachoti *et al.* (2005).

Most of the nutrients in maize can be found in DDGS, with the exception of starch, which is converted to ethanol. During the fermentation process, alcohol and carbon dioxide are formed from grain, therefore the concentration of nutrients in DDGS becomes three times higher than the amount of nutrient in the parent grains (Spiehs *et al.*, 2002). Based on available data from NRC (1994), DDGS contains about 27 % crude protein, 10 % oil and 0.8 % phosphorus. The composition of DDGS may be affected by the production method, type of main grains, drying practices, temperature and duration of drying. Numerous studies have been conducted to

investigate nutrient characteristics of DDGS. Spiehs *et al.* (2002) evaluated the nutrient content and variability of DDGS from ten ethanol plants in Minnesota and South Dakota. They found considerable variation between the ethanol plants. In that study, mean values and coefficients of variation were reported as follows: DM 88.9 %, 1.7 %; CP 30.2 %, 6.4 %; fat 10. 9%, 7.8 %; crude fibre 8.8 %, 8.7 %; Arg 1.2 %, 9.1 %; His 0.76 %, 7.8 %; Met 0.55 %, 13. 6%; and Ca 0.06 %, 57.2 %.

The physical, chemical and nutrient composition of nine DDGS samples from different beverages and alcohol production were evaluated by Cromwell *et al.* (1993). They reported protein content from 23.4 to 28.7 %; fat, from 2.9 to 12.8 %; ADF, from 10.3 to 18.1 %; NDF, from 28.8 to 40.3 %, and ash, from 3.4 to 7.3 %. The lightness (here referred to colour lightless) of DDGS ranged from very light to very dark and odour from normal to smoky. In that study, nutritional properties of samples were markedly related to lightness, indicating better nutritional value in light-coloured DDGS. It was also shown that some indispensable amino acids, such as lysine, arginine, and cystine were highly related to colour, which illustrates the sensitivity of these amino acids to overheating during the drying process. For instance, the lysine concentration of DDGS varied from 0.43 to 0.89 % between samples.

As is the situation with any feed ingredient in poultry, there is an emphasis on accurate metabolisable energy information for feed formulation. In this regard, metabolisable energy has usually been determined by the precision-fed rooster method (Lumpkins and Batal, 2005; Kim *et al.*, 2010). Recently, Kim *et al.* (2010) compared the TME_n content of processed DDGS samples that had undergone enzymatic milling to samples had been conventionally milled; they found the TME_n content to be 15.3 and 13.81 MJ/kg, respectively. Previously, Kim *et al.* (2008) had reported 14.87 MJ/kg for TME_n of conventional DDGS, while the figure for high-protein DDGS was 14.83 MJ/kg of DM. In another study conducted by Batal and Dale (2006), TME_n evaluation of seventeen samples from different plants showed a wide range from 10.42 to 13.35 MJ/kg, with a mean of 11.80 MJ/kg. Pederson *et al.* (2007) also showed a higher average energy content (22.73 MJ/kg) of ten DDGS samples compared to maize as their parental grain. It seems that the high energy content of DDGS is associated with a high level of fat in the ingredient (Swiatkiewicz and Koreleski, 2008). In a study on five DDGS samples conducted by Fastinger *et al.* (2006), gross energy contents were found to range from 20.29 to 20.80 MJ/kg; TME_n was even more variable, ranging from 10.4 to 12.75 MJ/kg. Parson *et al.* (2006) found the mean value for TME_n content of 20 samples to be 11.98 MJ/kg, which was less than the figure (12.96 MJ/kg) reported by NRC (1994). In a

review of published data on ME, Waldroup *et al.* (2007) recommended using the figure of 11.93 MJ/kg for DDGS. Nevertheless, depending on different methods and sample composition energy content and related TME_n may differ. Adeola and Ileleji (2009) showed different results for metabolisable energy in DDGS by using semi-purified and practical diets.

Generally, the measurement of TME_n for such variable samples is tedious and difficult; therefore some nutritionists have predicted TME_n of DDGS from chemical composition. In this regard, Batal and Dale (2006) found the fat content as the best criterion of TME_n followed by fibre, protein and ash. They also used several prediction equations from correlation of fat, fibre, protein and ash content to estimate TME_n of DDGS samples but they obtained low R^2, suggesting that these equations may not be very reliable.

The protein content of DDGS has been reported in several experiments to vary from 23 to 32 % (Cromwell *et al.*, 1993; Belyea *et al.*, 2004; Pedersen *et al.*, 2006; Fathi and Afifi, 2008). Several factors regulate the protein content of DDGS, as described previously. Distillers' soluble and wet grains are the two main components, and DDGS are formed by combining them. In this regard, substantial variations were shown by Belyea *et al.* (2004) in the composition of solubles from different batches. They postulated that the variation in protein content of DDGS may be influenced by the proportions of the included components. More recently, Kinsley *et al.* (2010) observed variable nutritive values of DDGS when different ratios of wet distillers' grains and solubles were blended together. Additionally, yeast protein and its amino acid concentrations may also have an effect on protein content of DDGS, since yeast protein constitutes approximately 50 % of the protein in DDGS (Belyea *et al.*, 2004). Furthermore, the drying process can have a crucial influence, not only on variability of nutrients, but also on concentrations and availability of amino acids in different samples (Martinez-Amezcua *et al.*, 2007; Bandegan *et al.*, 2009).

Recently, Bandegan *et al.* (2009) reported average Lys and Met concentrations of five different samples of wheat DDGS to be 0.74 and 0.61 %, respectively. More variable data for Lys content were observed by Fastinger *et al.* (2006), ranging from 0.48 to 0.76 % in five DDGS samples from different sources. Amongst the most limiting amino acids, Lys and Met appear to be most variable in DDGS, the reason being that the heating of wet distillers' grains may adversely affect the availability of heat-sensitive amino acids. Excessive heat during the drying process may accelerate reactions between reducing sugars and Lys (the Maillard reaction), which leads to unavailability of Lys in DDGS (Batal and Dale, 2006). In poultry,

the bonds formed between Lys and carbohydrates through Maillard reaction are almost impossible to break and as a consequence, this form of Lys cannot be absorbed (Fastinger *et al.*, 2006). Therefore, overheating during DDGS production is mainly responsible for losses in nutritional value of final by-products. It has also been shown that subsequent by-products resulting from excess heat appeared to be darker compared to those samples treated by less severe drying practices (Cromwell *et al.*, 1993; Kim *et al.*, 2008). Thus, the colour of DDGS can be used as a general measure of nutritive value and amino acid content of DDGS samples. Cromwell *et al.* (1993) observed the lowest Lys concentration in the darkest DDGS sample, amounting to 0.62 % and the highest amount of 0.86 % in the lightest sample. They also obtained a correlation as high as 0.67 between Hunterlab L score and Lys content for the same samples. Similar results were reported by Fastinger *et al.* (2006), indicating 30 and 15 % reduction in apparent and true amino acid digestibility, respectively, in a dark DDGS sample compared to four other light-coloured ones. These results agreed with values previously reported by Batal and Dale (2006) who showed that the amount of digestible Lys in light DDGS samples to be threefold that of the dark one. In contrast, Pahm *et al.* (2009) did not find any correlation between colour score and standardized digestible Lys, but a high correlation ($r^2 = 0.9$) between bioavailable Lys and Hunterlab L scores in DDGS. Similarly, by comparing two maize-derived DDGS samples, dark and light, Adedokun *et al.* (2009) observed low Lys digestibility in dark sample fed to turkey poults.

2.3.3 Limitations to use of biofuel by-products

The impact of biofuel by-products in animal feeding is generally more positive than negative. However, there are limitations to the use of these by-products for poultry feeding, some of which would have become obvious from the discussion of animal response to their inclusion. Depending upon variability, flowability, bulk density, handling and processing, there are some potential constraints to the use of DDGS regardless of effect on performance.

As previously described, substantial variations in chemical and physical properties of DDGS have been documented. It has also been shown that processing issues, including drying process, different main grains and rate of solubles may contribute to nutrient variations of this feed ingredient. High fibre content of DDGS may be another issue that limits its high inclusion in the diets. These constraints are discussed in greater detail in the following sub-sections.

2.3.3.1 Availability and cost

Feed accounts for the major cost of poultry production throughout the world. Therefore, the use of non-conventional feed ingredients to reduce feed costs is of great interests. Currently, most biofuel plants are located in a limited number of countries around the world, mostly in the USA and Brazil, and there is uncertainty over the number of plants being built in other parts of the world and also the extent of material being imported into developing countries which have limited budgets to construct costly biofuel plants. In this regard, the location of biofuel plants may be crucial to the ability to supply and distribute biofuel by-products to the feed industry, as transportation cost will be added to the final price and, hence, adversely affect the ability of the livestock producer to use such product. Furthermore, feed prices and any fluctuations will negatively influence the use of by-products in animal feeds, a factor which will be more noticeable in regions that have to import by-products. Besides, the demand from other markets may affect the availability of by-products for animal feeding. As a prime example, glycerol is an acceptable energy source for both ruminant and non-ruminant animals, but high demand for pharmaceutical use, polymer industry and the food industry may restrict its availability for animal feed (Iji and Barekatain, 2011).

2.3.3.2 Fibre content

Generally, during the fermentation process, most of the components present in cereal grains become concentrated in distillers' by-products except for starch, which is converted to ethanol. Therefore, the starch content of the final by-product is much lower than that of the original grain. A large proportion of the remaining carbohydrates are regarded as fibre, also called non-starch polysaccharides (NSP). The fibre content of by-products varies according to processing methods. High-protein DDGS, for instance, are produced when the germ is removed from the grain. Such material, therefore, contains less fibre and more protein compared to the conventional DDGS (Jacela *et al.*, 2010b). Nevertheless, neutral detergent fibre (NDF), acid detergent fibre (ADF) and total dietary fibre are approximately three times higher than those in the parent grain. While in ruminant animals these fibre factions can be readily digested due to high fibrolytic activities of the rumen microbes, poultry are unable to break down NSP because of the absence of such activities in their digestive tract. In addition, the presence of NSP in the non-ruminant GIT has been shown to contribute to poor

performance by creating a viscous environment and subsequent impediment to nutrient digestibility. However, there are limited data on the nature of NSP in DDGS. Choct and Petersen (2009) analysed six DDGS samples from different ethanol plants across the Midwest, USA and reported an average of 40 % total carbohydrates, most of which was identified as insoluble NSP with less that 10 % soluble NSP. Regarding the composition of NSP, they identified the main sugars to be glucose and xylose followed by arabinose, galactose and mannose in descending order of concentration. This would explain the possible adverse effect of arabinoxylans in birds fed DDGS-rich diets.

2.3.3.3 Bulk and storage

Storage and space allocation will be a primary issue for new ingredients introduced to the feed mills as there is always limited space designated for ingredients other than the common ones being used by feed manufacturers. A wide range of factors, including physical characteristics, flowability and raw material turnover will determine bin space allocation. Notably, not only can space allocation for the bulky ingredients such as DDGS be problematic (bulk density at around 480.6 kg/m^3), but also these ingredients take considerably more time and cost to be delivered and stored compared to the conventional ingredients and meals. Producers, therefore, sometimes are reluctant to accept biofuel by-products, in particular, DDGS owing to inconvenience and cost of handling. There are limited reports related to bulk density and particle size distribution of DDGS. In an experiment conducted by Knott *et al.* (2003), 16 samples from different ethanol plants in Minnesota and South Dakota were obtained to investigate average particle size and bulk density of DDGS. They reported considerable variation in average particle size of those 16 samples to be 1282 microns (SD=305, CV=24 %) and an average bulk density 572.4 kg/m^3. In the same experiment, there was a positive correlation between bulk density and moisture content, indicating that, bulk density tends to decrease as the moisture content of DDGS decreases.

The ability of the solid particles and powders to flow during unloading from storage containment or delivery vehicles is defined as flowability (Babcock *et al.*, 2008). Flowability of biofuel by-products, in particular, DDGS is regarded as an issue for transportation and storage, which can be affected by a wide range of factors involving cooling and drying practices, particle size and the amount of residual sugars (Shurson, 2005). It also seems that

humidity greater than 60 % has an adverse effect on flowability of DDGS due to a tendency of this by-product to absorb moisture (Babcock *et al.*, 2008).

Shipment concerns arising from moisture content and bulk density can also be an issue for the other by-products, such as wet dried grain with solubles (WDGS) with high moisture content (65 to 70 %). It has been voiced by some feed manufacturers that bagging of WDGS is sometimes a problem as the material ends up compacting (Babcock *et al.*, 2008).

2.3.3.4 Nutrient imbalance and variability

Variation and deficiency in some nutrients are regarded as the most important issues limiting the usage of biofuel by-products in poultry feeding. Generally, nutrient variability by-products results from several steps inherent in biofuel or ethanol production. These include different temperatures and drying practices, the enzymes used, and, in the case of DDGS, the rate of soluble incorporated. Additionally, live yeast added during the fermentation process may interact with the factors mentioned above to substantially influence the nutritive value of the final by-products. These discrepancies predominantly are reflected in protein, metabolisable energy and mineral contents of final products. Numerous studies have been conducted to investigate nutrient characteristics of DDGS. The study by Spiehs *et al*. (2002), which examined the nutrient variability of DDGS from ten ethanol plants in Minnesota and South Dakota, has been highlighted. In another study conducted by Batal and Dale (2006), TME_n evaluation of seventeen samples from different plants showed a wide range from 10.4 to 13.3 MJ/kg with a mean of 11.8 MJ/kg. Pedersen *et al*. (2007) also showed a higher average gross energy content (22.7 MJ/kg) of ten maize-derived DDGS samples compared to maize. The high energy content of DDGS is associated with a high level of fat in the residue (Swiatkiewicz and Koreleski, 2008).

Nutritionists regard nutrient imbalances as a major concern when biofuel by-products are incorporated in animal diets. Depending on the species of animal, different nutrients may be more important. Vander Pol *et al.* (2006) postulated that incorporation of high-fat WDGS in the diet containing more than 8 % fat in cattle may lead to a reduction in feed intake. These nutrient imbalances need to be considered for a variety of nutrients, such as minerals and amino acids. Batal and Dale (2003) reported a severe deficiency of sodium in laying hens using NRC values for DDGS, indicating the necessity for analysing DDGS samples prior to

incorporation into the diet. There is also a possibility of an increase in phosphorus and sulphur in ethanol by-products, which may occur when a greater rate of solubles is added to distillers' grain. Large amounts of sulphur (more than 0.4%) can cause polioencephalomalacia in cattle (Babcock *et al.*, 2008).

Finally, the presence of mycotoxins in biofuel by-products can be a potential threat for animal health and performance if the product is not monitored for contamination. It is noteworthy that the process of fermentation and production of biofuels is usually incapable of destroying mycotoxins; therefore, they will be found in by-products if the source grains were contaminated. Schaafsma *et al.* (2009) reported the mean concentration of deoxynivalenol, a toxin from *Fusarium graminearum*, in condensed distillers' solubles to be 7.11 mg/kg, which was four times higher than that in maize as main grain (1.80 mg/kg). In the same study, the concentration of deoxynivalenol was found to be 5.24 mg/kg. Nevertheless, care should be taken by regular monitoring and measurement of mycotoxins in grain and by-products to keep the concentration of mycotoxins close to the recommended level for the original grain; otherwise these anti-nutrients will be present in the final by-products, posing a risk to animal and possibly human health.

2.4 INFLUENCES OF DIETARY INCLUSION OF DDGS IN POULTRY NUTRITION

2.4.1 Growth response

Most of the by-products of the biofuel industries would naturally not be ideal ingredients for non-ruminant animals, particularly poultry, in view of their chemical composition and sometimes, physical properties. However, the quality of DDGS has continued to improve, with improvement in fermentation, distillation and drying processes. As a result, it has become a regular part of most pig and poultry diets in many parts of the world (Hastad *et al.*, 2003; Swiatkiewicz and Koreleski, 2008). These authors recommended that DDGS could be safely included in starter diets for broilers and turkeys at 5-8 % and 12-15 %, respectively in grower-finisher diets.

The results of experiments in which DDGS is investigated are, however, contradictory. In one case, the positive impact of DDGS on broiler performance was reported by Olukosi *et al.* (2010) who found improvement in all growth parameters including feed conversion ratio when 10 % DDGS was incorporated in maize-soybean meal diet. Recently, Loar *et al.* (2012) reported that feeding DDGS in both starter and grower phases decreased BWG and feed consumption and impaired FCR of broilers. In the same experiment, dressing percentage and breast meat yield also declined when DDGS was introduced into the feed. Similar observations were made in another experiment in the same lab (Loar *et al.*, 2010). In contrast, Min *et al.* (2011a) found no adverse effect on performance of broiler chickens with inclusion of DDGS at rates of up to 20 %. In another study, 12 % of maize DDGS did not cause any differences in terms of feed intake and feed conversion efficiency (Abdel-Raheem *et al.*, 2011). Skiba *et al*. (2009) reported a similar reduction in FCE in broiler chickens with only 10 % DDGS in the diet, but this was attributed mainly to feed particle selection and wastage rather than reduction in nutritive value. The relative economic efficiency of broiler chickens on 6 % DDGS was found to be approximately equal to that of chicks on a maize control diet (Shalash *et al.*, 2009). The summary of performance results of a number of studies on broiler chickens in which DDGS has been used is presented in Table 2.2.

The results of inclusion of DDGS in trials on other non-ruminant animals are somewhat similar. In a trial on weaned pigs, Avelar *et al*. (2010) reported a reduction in body weight gain when wheat DDGS was included in diets at between 5 and 20 %. This contrasts with the findings of Jones *et al*. (2010) who did not observe any negative effects of maize DDGS in the diet of weaner pigs in terms of feed consumption, weight gain or feed efficiency. Sorghum DDGS, on the other hand, reduced FCE. Feoli *et al*. (2008), however, observed a reduction in weight gain as a result of a reduction in DM, protein and energy digestibility in finishing pigs on diets containing 40 % maize DDGS. In laying hens, wheat and barley DDGS can be incorporated at up to 20 % in the diet without adverse impact on performance (Nasi, 1990). Similar results were reported by Lumpkin *et al.* (2005) at up to 15 % inclusion of DDGS in laying hen diets. In turkey, it has also been shown that 20 % DDGS resulted to similar weight gain and FCR in combination with poultry by-product meal (Noll and Brannon, 2006).

Table 2.2 Summary of selected studies reporting the performance of broilers fed different levels of DDGS

Authors	Source of DDGS	Feed intake	BWG	FCR	Inclusion rate	Age (day)
Loar *et al.* (2012)	Maize	Decreased	Decreased	Increased	14-28 %	42
Lumpkins *et al.* (2004)	Maize	Not affected	Decreased	Increased	18 %	42
Lumpkins *et al.* (2004)	Maize	Not affected	Not affected	Not affected	15 %	42
Vilarino *et al.* (2007)	Wheat	Not affected	Not affected	Not affected	10-20 %	37
Thacker and Widyarante (2007)	Wheat	Not affected	Not affected	Tended to increase	20 %	21
Wang *et al.* (2007a)	Maize	Decreased	Decreased	Increased	30 %	35
Wang *et al.* (2007b)	Maize	Not affected	Not affected	Not affected	15 %	42
Youssef *et al.* (2008)	Maize-wheat	Not affected	Not affected	Increased	10-15 %	35
Olukosi *et al.* (2010)	Maize	Increased	Increased	Decreased	10 %	42
Shim *et al.* (2011)	Maize	Increased	Increased	Increased	12 %	18

In a trial to determine the optimal level of inclusion of DDGS in diets for pigs, Linneen *et al*. (2008) observed a reduction in daily gain and feed consumption as DDGS levels rose in the diet, particularly beyond 10 %. There was, however, a linear improvement in these measurements with the inclusion of white grease at between 0 % and 6 %, along with the DDGS. The feed efficiency of the pigs was increased due to inclusion of DDGS in the diet. Min *et al*. (2008) have established that DDGS can be used at up to 30 % in broiler chicken diets without detrimental effects on feed intake; however, they showed that feed efficiency

was reduced, a result that was linked to the reduced pellet quality. Further supplementation with glycerine, at 5 %, had no effect.

2.4.2 Nutrient digestibility and digestive function

Along with bird performance, the utilisation of DDGS has also been investigated in several studies. Recently, Adeola and Zhai (2012) evaluated the metabolisable energy in the diets of 6-week-old broilers. They found that incorporation of 30 and 60 % of each of maize distillers grain (DDG) and DDGS linearly reduced ileal digestibility of DM and energy and also metabolizability of N and N-corrected energy. The higher fibre content in DDGS is usually recognized as the major culprit for such reduction, which not only lowers digestible energy but also adversely affects the digestibility of other nutrients. Considering total tract digestibility, Min *et al.* (2011b) demonstrated an increase in N and GE in the excreta content when 30 % DDGS was in the basal diet. However, AME and AME_n were not affected by inclusion of DDGS at a high level.

It appears that the effect of DDGS on nutrient digestibility in poultry is highly dependent on its level of inclusion in the diets. In some reports on laying hens, inclusion of 5, 10 or 15 % of maize DDGS showed no negative impacts on energy utilisation, nutrient digestibility or the balance of N, Zn and Ca (Swiatkiewicz and Koreleski, 2007a;b). However, the same authors found that when the level of inclusion rose to 20 %, the metabolisable energy value of the diet and crude fat digestibility were significantly compromised. As previously mentioned, this negative impact is not surprising due to high fibre content, poor quality of protein and also unavailability of certain amino acids. Such reduction has also been shown for the retention of N and P in broilers with increasing levels of wheat-DDGS during the starter period from 0 to 25 %, which was accompanied by an increase in the excreta content of N and P (Leytem *et al.*, 2008).

Distillers' dried grains with solubles contains considerably large amounts of protein compared to cereal grain; therefore, the amino acid profile and digestibility of amino acids are fundamentally important particularly when it is included at high levels in poultry diet. Evaluation of feed protein in poultry can be appropriately performed by pre-caecal digestibility of amino acids (Ravindran *et al.*, 1999). Using a linear regression approach, Kluth and Rodehutscord (2010) reported an adverse impact of 10 and 20 % DDGS on

digestibility of amino acids in the diets of broilers. A similar reduction in amino acid and DM utilisation has been reported in broilers fed maize-wheat DDGS (Abdel-Raheem *et al.*, 2011). Regardless of the quality of protein itself, a rapid passage rate through the GIT may also be responsible for the low amino acid digestibility and the digestibility of other nutrients. Shires *et al.* (1987) showed a slower passage rate for soybean meal than for canola meal in broiler chickens and White Leghorn hens and attributed these results to the higher fibre content of canola meal. Therefore, as DDGS has a high fibre fraction, the same influence may be reflected in a faster passage rate through the GIT of broilers, hence an impairment in nutrient digestibility. This poor amino acid digestibility may even be exacerbated by over-drying and improper processing technologies.

As investigated by Amezcua and Parsons (2007), digestibility of Lys in DDGS was reduced to 13 % when a heating practice was applied by autoclaving for 80 min or dried in an oven at 55 ºC for 60 min. The variation in digestibility of DDGS in broilers is documented in several studies, most of which are focused on diets containing maize- or wheat-based DDGS (Stein *et al.*, 2006; Pahm *et al.*, 2008).

Digestibility as an estimate of availability of amino acids has been widely used in the assessment of DDGS. Batal and Dale (2006) reported 70, 87 and 74 % digestibility of Lys, Met and Cys, respectively. Their results showed satisfactory digestibility of amino acids for poultry even though the values were slightly less than for maize as the parent grain. Similarly, apparent Lys digestibility of 5 DDGS samples evaluated by Fastinger *et al.* (2006) showed variable digestibility, ranging from 38.6 to 69.5 %. In the same experiment, the corresponding true digestibility for Lys varied from 78.3 to 82.4 % after adjusting for endogenous losses. These results concur with values reported by Palm *et al.* (2009) who observed great variability in digestibility of Lys against other indispensable amino acids. It is noteworthy that values compiled by NRC (1994) are different from those of present-day processed DDGS because those values were obtained mainly from DDGS of beverage origin. More recently, Bandegan *et al.* (2009) observed the lowest digestibility for Lys (35.6 %) and the highest for Phe (79.2 %). They also reported higher values of digestible amino acids evaluated for wheat compared to wheat DDGS samples. In another study, results of eight high-protein DDGS samples analysed by Jung and Batal (2009) showed Lys digestibility ranging from 67.5 to 85.6 %.

2.4.3 Microbial growth, gut health and enteric diseases

The ecology of the intestine of poultry is greatly affected by the composition and structure of feed consumed. Investigations on a number of Finnish poultry farms by Apajalahti *et al.* (2001) demonstrated that diet is the strongest determinant of the caecal bacterial community. This becomes crucial when certain pathogenic bacteria are stimulated and are provided the appropriate conditions to multiply, a situation which can cause enteric disease. In this regard, diet component appears to be fundamentally important in the outbreak of enteric disease, particularly necrotic enteritis (NE). Therefore, prior to explaining the impact of DDGS, the influence of dietary factors on gut health and development of enteric disease is briefly discussed.

Results of several studies indicate that soluble NSP in diets containing large amounts of barley, wheat or rye may predispose broilers to NE (Branton *et al.*, 1987; Riddell and Kong, 1992; Branton *et al.*, 1997). For instance, it has been shown that a higher ratio of wheat and barley to maize is well correlated with the incidence of NE (Kaldhusdal and Skjerve, 1996). Several mechanisms have been proposed for this effect. Among those mechanisms, a highly viscous environment in the gut may increase the substrate supply for the growth of microbes by restraining nutrient utilisation and absorption accompanied by a prolonged feed passage rate (Salih *et al.*, 1991) and enhanced mucus production (Langhout *et al.*, 1999). This could result in the proliferation of anaerobic bacteria, particularly *Clostridium perfringens*, in the small intestine (Choct *et al.*, 2006).

A high protein content of the diet is regarded as another predisposing factor to NE, and has been documented in literature, particularly for protein of animal origin (Truscott and Al-Sheikhly, 1977; Kaldhusdal and Skjerve, 1996; Drew *et al.*, 2004; Dahiya *et al.*, 2005; Dahiya *et al.*, 2007b). For instance, feeding fishmeal has been shown to result in an increase in proliferation of *C. perfringens* in the intestine of broilers when compared with a diet containing soy protein (Drew *et al.*, 2004). Drew *et al.* (2004) linked the more vigorous growth of C. *perfringens* to the higher content of glycine and methionine in the diet containing high fishmeal. In an investigation by Dahiya *et al.* (2007a), it was found that with an increase in the concentration of glycine, there was an increased number of *C. perfringens* in the ileum of broilers. It has been shown by Fuchs and Bonde (1957) that aspartic acid, cystine, histidine, leucine, phenylalanine, tryptophan, tyrosine, valine, arginine, glutamic acid and threonine are eleven amino acids essential for the growth of *C. perfringens* strains. They

also showed a synergy between glycine and lysine in promotion of the growth of some strains despite being non-essential. However, the mechanisms involved are still not fully understood. The foregoing indicates the importance of feed ingredient evaluation for any health-related impact on poultry production.

Despite the abundance of literature on the performance of broilers fed DDGS, very few attempts have been made to characterise the gut microbial profile of birds fed such diets. Recently, Loar *et al*. (2012) found that ileal or caecal counts of *C. perfringens,* measured either by plate count or qPCR, were not influenced by rising levels of DDGS at inclusion rates of up to 24 % in the diet of broiler chickens. The same results had been reported by Loar *et al.* (2010) under similar conditions, but in 28-day-old broilers fed up to 30 % DDGS. However, when evaluated by qPCR, a marginal linear trend was revealed towards greater colonisation of *C. perfringens* in the caecal content (Loar *et al.*, 2010). In another experiment they concluded that in the older birds, the gut, and therefore the microflora, are given time to become accustomed to the DDGS; hence the trend in *C. perfringens* changes was absent (Loar *et al.*, 2012). Loar *et al.* (2012) also found that at 14 or 28 % DDGS inclusion rate, *Escherichia coli* colonisation in the ileum of broilers was reduced, while no effect on caecal numbers of *Listeria monocytogenes* was noticed.

In a study conducted with pigs, Yang *et al.* (2010) compared 3 different types of DDGS, from wheat, maize and wheat-maize. They reported greater colonisation of *Entrobacteriaceae* and *Lactobacillus* in the faecal samples of pigs fed maize DDGS than those given diets containing wheat- or wheat-maize DDGS. However, in that experiment there was no control diet (without DDGS) which made any conclusion drawn for the effect of DDGS *per se* untenable. Recently, Tran *et al.* (2012) found a significant influence of maize DDGS with respect to putative *Lactobacillus reuteri* in the faeces of weanling pigs. In the same experiment, lactose and DDGS were shown to positively influence the maintenance of some *Lactobacillus* species, indicating that these beneficial bacteria may result in enhanced growth parameters in pigs.

There is a paucity of data regarding the influence of DDGS on broiler health under disease challenge, particularly enteric disease; therefore the discussion is mainly limited to the studies conducted on pigs and other animals. Reports indicate that incorporation of DDGS in grow-finish diets of pigs may alleviate the severity of ileitis (Whitney *et al.*, 2006a). In a study conducted on pigs, a reduction was observed in the severity of ileitis-related lesions in

the ileum and colon. This has been linked to the presence of insoluble fibre in DDGS, which may impede the proliferation of pathogenic organisms, while the soluble fibre acts in the opposite way (Whitney *et al.*, 2006a). In another work, Weber *et al.* (2008) demonstrated that inclusion of DDGS upregulated expression of some cytokines in the ileum, thereby suggesting a possible alteration in the immune function of pigs. However, neither metabolites nor cytokines were altered by feeding DDGS to pigs 4 h after the injection of-*E. coli* lipopolysaccharide, suggesting that DDGS may act in a localized manner to modulate inflammation (Weber and Kerr, 2011). In channel catfish, feeding 20 to 40 % DDGS resulted in an increase in serum total immunoglobulins with an increase in antibody titres after *Edwardsiella ictaluri* challenge (Lim *et al.*, 2009). In two experiments conducted by Tran *et al.* (2012) with weanling pigs, feeding maize DDGS at up to 30 % did not alter serum immunoglobulin (IgA and IgG).

These findings may explain a localized intestinal immune response that correspondingly affects the ability of animals to promote resistance to enteric disease or even predisposes them to certain diseases, depending upon the nature of the disease, experimental conditions, as well as the presence of other preventive or predisposing factors. In addition, it has been shown that yeast protein can account for approximately 20 and 3.9 % of total protein and dry matter content in DDGS, respectively (Han and Liu, 2010). In this regard, the presence of β-glucans in the yeast may have an indirect impact on immune function, as it has been shown that yeast β-glucans can directly increase secretion of several interleukins, including IL-1β, IL-8, 4, 12 and 6 (Sonck *et al.*, 2010). Hence, yeast protein in DDGS may play a role in modulating cellular and humoral immunity in animals.

There is only one report on the effect of DDGS in infected broilers in which no preventive effect of DDGS was observed. In that work, Perez *et al.* (2011) showed that feeding 0, 10 or 20 % DDGS failed to show any amelioration or prevention of *Eimeria acervulina* infection in broiler chickens. However, the population of bacteria associated with caecal mucosa was changed by increasing levels of DDGS in the diet shown by low intertreatment Cs value in the caeca. Although such changes may be interpreted as increased stability of the gut microflora (Bhandari *et al.*, 2008; Opapeju *et al.*, 2009), no association was found with *Eimeria* infection in the birds (Perez *et al.*, 2011). Nevertheless, such mechanisms and the interrelationship of microbial bacteria and development of enteric disease are largely unknown when DDGS is included in poultry diets.

The effect of exogenous enzymes with DDGS on broiler health has not been documented. However, as fewer in-feed antibiotics are allowed in animal diets, interest in exploring the health-promoting impact of exogenous enzymes has increased. In general, depolymerisation of cell wall polysaccharides by enzymes added to the diets lessens the negative effect on nutrient utilisation and absorption caused by cell wall encapsulation of nutrients. Such depolymerisation is thought to increase the utilisation of nutrients, thereby reducing the availability of substrate for microbial growth (Bedford and Cowieson, 2012). As a result of NSPase activities, a wide range of NSP oligomers may be produced varying in sugar component and molecular size (Jia *et al.*, 2009b). Such products include gluco-, galacto-, manno-, or xylo-oligomers (Desilva *et al.*, 1983) and have somewhat similar prebiotic effects which can promote the growth of beneficial bacteria such as *Bifidobacterium* and *Lactobaccillus spp.* in the gut (Jia *et al.*, 2009b). These products may also restrain the growth of some pathogenic bacteria (Gibson and Roberfroid, 1995). Moreover, microbes may be attracted by some other oligomers like mannanoligomers, which may result in detachment of microbes from intestinal binding sites via competitive exclusion (Adlerberth *et al.*, 2000). All these effects, plus the mucosa itself, may reduce the risk of pathogens and thereby the development of disease (Spring *et al.*, 2000).

2.5 SCOPE FOR INCREASED USE OF DDGS BY IMPROVING ITS NUTRITIVE VALUE

As the quantity of by-products produced by biofuels industry is increasing rapidly, attempts have been made to exploit them to their maximum potential in livestock diets. Depending upon different biofuel processes, species and the type of by-product, there have been a wide range of applications to enhance the nutritive value of by-products, to facilitate their wider inclusion in animal diets, especially for swine and poultry. In this section, existing applications employed to improve the nutritional quality of DDGS are discussed.

2.5.1 Further processing and management

It has been widely accepted that any alteration in the processing of biofuel, and in particular ethanol production, will lead to changes in the finished by-products (Belyea *et al.*, 2004;

Gibson *et al.*, 2005). Therefore, depending upon the species that by-products are fed to, a number of new technologies may be taken into consideration prior to and after the completion of by-product production. In modern bioethanol plants enzymatic milling (EM) is a new procedure in which hemicellulases and protease are used. These enzymes facilitate separation of non-fermentable fibre and germ prior to fermentation, and this leads to an increase in fat and protein content of DDGS, which makes it a more desirable ingredient for non-ruminant animals (Kim *et al.*, 2010). Further processing can be applied to the recovered germ and the pericarp and endosperm fibre to produce maize germ oil and phytosterols, respectively. In this regard, a combined separation method (Elusieve process) was developed by Srinivasan *et al*. (2005) in which sieving and elutriation were applied to separate fibre from DDGS. In the described process, an upward stream of air was created by a blower followed by sieving to separate particles based on density, size and the physical form of DDGS components. Srinivasan *et al*. (2005) also evaluated the nutrient characteristics of different fractions (lighter and heavier) obtained from the Elusieve method showing 13–41 % and 4–127 % increase in protein and fat content of the heavier fraction, respectively. Fibre content of the lighter fraction of DDGS was also reduced.

As discussed previously, the presence of NSP in DDGS could impede nutrient digestibility and therefore performance in non-ruminants, in particular broiler chickens. There are some reports on the beneficial role of extrusion in enhancement of nutrient digestibility through physical disruption of the cell wall, and hence breakdown of NSP to the smaller fractions (Camire, 1991; Oryschak *et al.*, 2010a; Oryschak *et al.*, 2010b). Oryschak *et al*. (2010b) found a 10–34 % increase in apparent ileal digestibility of amino acids in both extruded maize and wheat DDGS. High temperature and pressure through the extrusion process are believed to act as effective eliminators of microbial contamination and render cell content more accessible and susceptible to enzymatic hydrolysis (Oryschak *et al.*, 2010b). Camire (1991) ascribed the improvement in protein digestibility as a result of extrusion to denaturation of protein via heat and pressure applied through the extruder, which presumably increases the exposure of peptide bonds to enzymatic digestion. However, Al-Marzooqi and Wiseman (2009) recommended mild conditions of extrusion in order to avoid the adverse effect of high temperature on amino acid availability.

Pelleting as a further process on DDGS may also improve flowability, a constraint that was highlighted in Section 2.3.3.3. It has been demonstrated that any level of agglomeration

during the pelleting process can improve flowability of DDGS, but this must be undertaken at the ethanol plant, and this would probably increase the cost of production and hence the final price of DDGS (Behnke, 2007).

2.5.2 Effect of microbial enzymes on the nutritive value of DDGS

Among the key approaches to improving nutrient utilisation in poultry diets, the application of various exogenous enzymes has drawn enormous attention over the past decade. It has been consistently shown that exogenous enzymes enhance animal performance and nutrient utilization by animals, in particular non-ruminants. The application of exogenous enzymes in non-ruminant animals has been recently reviewed (Adeola and Cowieson, 2011). In this regard, diets containing high concentrations of NSP have shown considerable response to exogenous NSP-degrading enzymes. In addition, the concentration of NSP in DDGS is relatively high due to the fermentation process (Choct and Petersen, 2009) which removes most of the starch and almost triples the amount of NSP compared to that in the original grain. Specifically, this is a constraint to the use of this by-product by poultry, which are unable to digest NSP. Therefore, there may be a potential for the use of exogenous enzymes in the diets containing biofuel by-products.

For poultry, there are a few reports concerning the use of xylanase to break down NSP, to improve the growth performance of birds on DDGS-rich diets. Recently, Liu *et al.* (2011) demonstrated that the addition of xylanase to the diet of broilers containing maize DDGS could increase hemicellulose and DM digestibility by 5 and 20 %, respectively. Furthermore, Olukosi *et al.* (2010) observed additivity in the effects of xylanase, amylase, protease and phytase for weight gain of broiler chickens fed diets with and without DDGS. In that experiment, such sub-additive impact was only detected for apparent DM, N and P retention in the diet containing DDGS but not the basal maize-soybean meal diet. Supplementing *E. coli* phytase at 1000 FTU/kg in broiler diets containing 30 % DDGS resulted in improvements in apparent P digestibility and tibia ash mineralisation, but no significant enhancement in AME_n (Martinez-Amezcua *et al.*, 2006).

Conversely, some other studies have shown inconsistent results when enzymes are supplemented to broiler diets containing DDGS. In this regard, Min *et al.* (2011b) found no influence of an enzyme combination on performance when a high level of DDGS (30 %) was

incorporated in the diet of broilers. The same authors found that the enzyme supplementation had not revealed any positive influence on AME or GE digestibility in broilers fed DDGS. These observations were in close agreement with a previous study conducted in the same lab (Min *et al.*, 2009a). Similarly, in another investigation, Shalash *et al.* (2009) reported the failure of an enzyme preparation to improve performance of broilers fed maize DDGS. It appears that the improvement by addition of enzyme to the diets containing maize DDGS is limited because the maize or DDGS of maize origin contains a relatively low amount of soluble NSP on which exogenous NSP-hydrolysing enzymes can act. In addition, most of readily accessible carbohydrate components in the original grain are converted to ethanol, leaving mostly insoluble carbohydrates in the final by-product.

Exogenous multi-enzyme cocktails appear to improve amino acid digestibility of diets containing DDGS when fed to broiler chickens. Oryschak *et al.* (2010a) found between 6 and 19 % improvement in lysine, tryptophan, methionine, isoleucine, histidine and phenylalanine digestibility when a multi-enzyme complex was included in a diet with 15 % triticale DDGS and fed to broiler chickens. The effect of enzymes on the biodiesel by-product, rapeseed press cake, is also positive. Significant improvement in FCR (1.9 - 1.84) was found by Jozefiak *et al.* (2010) in broiler chickens on such diets.

The influence of enzymes, however, is also inconsistent for other non-ruminant animals on diets containing DDGS. In a series of experiments conducted by Jacela *et al*. (2010a) no effect of microbial enzymes was observed when finishing pig diets contained DDGS. This is not surprising because enzymes given the same name can vary in their substrate affinity and activity. In addition, variation in carbohydrate composition, difference in age and animal species may be logical explanations for the inconsistencies in response to exogenous enzymes with DDGS in the diets.

There are conflicting reports on the effectiveness of microbial enzymes in diets containing DDGS for pigs. Feoli *et al*. (2008) reported an improvement in nutrient digestibility and growth as a result of supplementation with microbial enzymes possessing β-glucanase, protease, amylase and xylanase activities. In contrast, Jones *et al*. (2008) did not observe any positive effect of microbial enzymes on diets containing sorghum DDGS when fed to pigs. Pigs on the diets with sorghum DDGS were also less efficient than those on maize DDGS (Jones *et al*., 2008).

2.6 HOW THE CURRENT STUDY ADDRESSES GAPS IN THE RESEARCH

As well-documented in the literature, the qualitative variation of DDGS is regarded as a major hindrance for the inclusion of this by-product in poultry diets. This highlights a need to analyse DDGS samples prior to their inclusion in the diets. In this regard, little is known about the variability between batches from the same ethanol plant, particularly for the DDGS originating from sorghum. Hence, the aim of this research is to comprehensively investigate the nutrient characteristics of sorghum DDGS. This will be presented in Chapter 3.

From the literature, it is also evident that broiler chicken performance in terms of feed intake, body weight and FCR varies when DDGS is incorporated in the diets. Such variation has recently been extensively documented in the literature. Besides, most research has focussed on DDGS from maize and wheat with apparently less work on the DDGS from sorghum. Moreover, in spite of the fact that a considerable number of studies has been conducted on the influence of DDGS in poultry, broiler chickens in particular, little is known about the impact of a higher rate of inclusion of this by-product, or the application of individual or combined enzymes to enhance the nutritive value of DDGS for broilers. It is also not clear to what degree carbohydrases can break down the NSP content of DDGS, an area that requires investigation. Therefore, the interactive effect of exogenous enzymes, xylanase and mono-component protease, with diets containing large amounts of DDGS has been evaluated in the first two feeding trials (Chapters 4 and 5) of the current doctoral thesis, with an emphasis on the existing gaps and shortcomings of research into digestive enzyme activities, degradation of NSP, gut development and performance of birds.

As previously mentioned, there is continuous need to understand the impact of dietary components, and hence different feed ingredients on bird health and the development of different diseases. From an economic standpoint for poultry production, this is vitally important. Very few attempts have been made to explore the role of DDGS on gut health and the possible alteration in the gut microbial profile of broiler chickens. Nor is there much in the literature concerning how birds react to DDGS in terms of growth performance, immune response and gut microflora under enteric disease challenge, particularly for commonly occurring diseases such as coccidiosis and necrotic enteritis. This mechanism and response,

along with enzyme supplementation, will be addressed in a challenge disease experiment presented in Chapter 6.

There is a need to assess the energy economy of diets containing DDGS possibly extended to net energy in response to current drive to develop NE system to use in Australia. Besides, there is a dearth of data in literature to show what energy system can better explain the contribution of DDGS in supplying the desired energy specification, net energy in particular, in the diet of broiler chickens. Such gaps in the literature will be covered in the final experiment (Chapter 7). The possible impact on heat production, net energy for production as well as carcass analyses for the retention of protein and fat will also be evaluated using comparative slaughter technique and indirect calorimetry.

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CHAPTER 3

NUTRITIONAL CHARACTERISTICS AND VARIABILITY OF SORGHUM DISTILLERS' DRIED GRAINS WITH SOLUBLES FROM DIFFERENT PRODUCTION BATCHES

Abstract

Six samples of sorghum distillers dried grains with solubles (DDGS) were obtained from an ethanol plant to characterise their nutrient composition and variability between different production batches. Samples were analysed for protein, ether extract, amino acids, minerals, phytate, starch, resistant starch, non-starch polysaccharides and free sugar contents. Mean values of DM of different DDGS batches varied from 890.5 to 931 g/kg while CP content revealed a higher coefficient of variation (CV) compared to DM. Fat content was found to be widely variable with 20.0 g/kg difference between batches 5 and 3 as two extremes for lipids. Total starch content varied from 59.7 g/kg in batch 3 to 72.7 g/kg in batch 6, with a CV of 7.31 %. The total carbohydrate content averaged around 262.2 g/kg of the DDGS samples, which was dominated by an insoluble fraction followed by free sugars. Only 27.6 g/kg soluble NSP was detected. Xylose and arabinose were found to be the main sugars of the soluble and free sugar component. Among the amino acids, lysine, methionine and tyrosine, in a decreasing order, were found to be most variable. The analyses revealed a negligible amount of phytate phosphorous with an average of 1.66 g/kg DM. In terms of CV, the current analyses did not exhibit high variability for most of the nutrients for the samples sourced from the same ethanol plant.

3.1 INTRODUCTION

Generally, in the process of fermentation, cereal grains are used to produce alcohol. Distillers' dried grains with solubles (DDGS) are the main by-product of this process which has been increasingly available as a feed ingredient due to the attractiveness of ethanol as an

alternative to fossil fuels. As previously discussed (Chapter 2), it is commonly expected that nutrients escaping from the fermentation process are concentrated approximately 3-fold over the parent grain in the final by-product. Thus, DDGS is an acceptable source of protein, amino acids, phosphorus and micro-nutrients for poultry feed. However, the variation in the composition of DDGS is regarded as a drawback hindering its high inclusion in poultry diet. The literature on the composition and the variability of DDGS is abundant (Belyea *et al.*, 2004; Belyea *et al.*, 2006; Liu, 2008, 2009; Stein and Shurson, 2009); however, most of these data are based on the by-products from corn and wheat with less published data on the composition of sorghum DDGS, the predominant type in Australia. However, the amino acid and approximate analyses of sorghum DDGS were reported by Urriola *et al.* (2009). They showed higher CP, NDF and ADF in sorghum DDGS (sDDGS) than in maize DDGS (mDDGS). There concentrations of Lys and Arg were also less in sDDGS than in mDDGS.

Moreover, there is less information available on the variability between batches from the same ethanol plant. Thus, this chapter aims to comprehensively investigate the nutrient characteristics and variability of different DDGS batches prior to conducting the main feeding experiments.

3.2 MATERIALS AND METHODS

3.2.1 Sorghum DDGS samples

Six sorghum DDGS samples from different batches were obtained from an ethanol production plant operated by Manildra Pty Ltd in Nowra, New South Wales, Australia. The samples were produced at different times from different production batches over a period of 4.5 months in 2009.

3.2.2 Laboratory analyses

In order to conduct proximate analysis, representative DDGS samples were finely ground and kept in sealed glass bottles at 4 ºC until required for analysis. All the analyses were conducted in duplicate and results were expressed based on dry matter content. The methods described by the Association of Official Analytical Chemists (AOAC, 2002) were employed for DM,

gross energy (GE), ash, and crude protein (CP). To assure the reliability of analytical procedures, relevant standards or controls for each method were included and simultaneously analysed with the samples.

To determine DM content, approximately 2-3 g of each sample was accurately weighed into pre-weighed crucibles in duplicate. Samples were then placed in a forced-air convection oven (Qnaltex Universal Series 2000, Watson Victor Ltd., Perth, Australia) at a constant temperature of 105 ºC for 2 days. Samples were weighed repeatedly until constant weight was reached. The difference between pre-weighed samples and the dry weight gave the DM content. The nitrogen content of DDGS samples was measured using a LECO FP-2000 automatic nitrogen analyser according to the Dumas' combustion technique (Leco Corporation, St. Joseph, MI, USA). The combustion of nitrogen-containing organic matter in pure oxygen at high temperature leads to the release of nitrogen which is measured by thermal conductivity detection. The crude protein in the current study was then calculated by multiplying the nitrogen content by the factor of 6.25. The ether extract (EE) of the DDGS samples was measured gravimetrically by the Soxhlet extraction method. Approximately 2 g of samples was weighed into paper thimbles and extracted for 24 h with chloroform in a Soxhlet extraction unit. The EE was then determined by the weight difference expressed as a proportion of DM of DDGS samples. The GE contents of samples were determined using an adiabatic bomb calorimeter (IKA® WERKE, C7000, GMBH and CO., Staufen, Germany) and benzoic acid as standard.

3.2.3 Total, resistant and digestible starch

The Megazyme total starch kit (AMG/AA 05/2006) was employed to determine total starch and resistant starch contents according to the method developed and described by McCleary *et al.* (1994), which is recognized by the AOAC (Method 996.11). The ground samples (0.5 mm size) were accurately weighed (100±2 mg) into pre-weighed 25 mL screw-capped reaction tubes and 0.2 mL of 80 % ethanol was added to each tube to wet the samples. Enzymatic hydrolysis was commenced by adding 3 mL of thermo-stable α-amylase (3000 U/mL; 45 U/mg at pH 6.0, Megazyme) in MOPS buffer (50 mM, pH 7.0). Thereafter, 0.1 mL amyloglucosidase (3300 U/mL on soluble starch, pH 4.5) was added and then incubated at 50 ºC for 1 h. An aliquot of 0.1 mL was subsequently incubated with 2.25 mL of GOPOD reagent at 50 ºC for 20 min. Finally, the absorbance was read at 510 nm against a reagent

blank. The same procedure was used for the measurement of resistant starch, but samples were treated with DMSO at 100 ºC before enzymatic hydrolysis. The difference between total and resistant starch is equivalent to digestible starch of the samples.

3.2.4 Phytate phosphorus

The method described by Haug and Lantzsch (1983) was used to determine phytate-P content of DDGS samples. Firstly, ferric solution was prepared by dissolving 0.2 g ammonium iron sulphate 12 H_2O in 100 mL 2N HCl made up to 1000 mL with distilled water. A second solution was made by dissolving 10 g of 2, 2´- bipyridine (Merck KGaA, 5427) followed by adding 10 mL thioglycolic acid (Merck Art, 700) in distilled water, made up to 1000 mL. Approximately 0.1 g of finely ground DDGS sample was weighed into 16 mL reaction tubes and 10 mL of 0.2 M HCl were added. Thereafter, 2 mL of ferric solution were added and the tubes were placed in boiling water for 30 min and subsequently cooled to room temperature. Two mL of bipyridine solution were then added to the tubes and mixed thoroughly. Within one minute of adding the last solution, the phytate contents of the DDGS samples were determined colorimetrically, by reading the absorbance at 519 nm against water as the blank. The standard curve obtained from the standard solution (Haug and Lantzsch, 1983) was used to determine the amount of phytate in the samples.

3.2.5 Non-starch polysaccharides

Measurements of free sugars, insoluble and soluble non-starch polysaccharides (NSP) were performed by gas-liquid chromatography (GLC) as the alditol acetate derivatives of monosaccharides. All DDGS samples were finely ground to pass a 0.5 mm sieve. About 100-200 mg of samples were weighed and placed in screw-capped glass tubes followed by addition of 10 mL of hexane. The tubes containing samples were thoroughly mixed, sonicated for 15 min, centrifuged (2000xg, 15 min), and the supernatant was decanted to extract the fat. Thereafter, 5 mL 80 % ethanol was added to the residue and the samples were incubated at 80 °C for 10 min to remove the free sugars by collecting the supernatant after centrifugation. A series of hydrolysis, reduction and acetylation were applied to the samples according to the methods described by Theander and Westerlund (1993). Using a vacuum rotary evaporator, the supernatants were dried at 40 °C for 6 h, followed by adding 3 mL of 1 M H_2SO_4 and hydrolysed at 100 °C for 2 h. The samples were left at room temperature to

cool and subsequently an aliquot of 0.4 mL was transferred into a clean 25 mL reaction tube before mixing with 0.1 mL of 28% NH_3. Following that, the samples were thoroughly mixed and 50 μL of internal standard (allose 4 mg/mL) were precisely added. The slurry was again dried in a vacuum rotary evaporator for 4 h at 40 °C. Thereafter, the residues were dissolved in 0.2 mL distilled water and the reduction of monosaccharides was performed by treatment with sodium borohydrate ($NaBH_4$) (0.3 mL, 50 mg sodium borohydrate per mL 3 M NH_4OH) at 40 °C for 1 h. Glacial acetic acid was used to decompose the excess amount of $NaBH_4$. In the acetylation process, 5 and 0.5 mL acetic anhydride and 1-methylimidazole were added to the residual alditol acetate derivates, respectively, followed by decomposition of any excess acetic anhydride with 5 mL of distilled water. Finally, the alditols were extracted with 2 mL of dichloromethane and the volatile alditol derivates of monosaccharides were analysed using a Varian 3400 GC equipped with a Varian series 8200 auto-sampler, a capillary column (BPX70, 25 m, 0.32 mm, SEG International, Australia), and a flame ionisation detector (FID) set at 280 °C. During analysis, the temperature of the column was held at 195 °C for 1 min and then raised by 5 °C/min until 225 °C was reached and held for 4 min.

The analysis of soluble and insoluble NSP portions was continued on the insoluble residue. The samples were extracted with 80 % ethanol before drying to a slurry using nitrogen, followed by incubation at 100 °C for 30 min to gelatinise the starch. After cooling, the mixture was digested by a thermo-stable α-amylase (E.C. 3.2.1.1) at 95 °C for 30 min, and subsequently with amyloglucosidase (E.C. 3.2.1.3) incubated at 55 °C for 16 h. The samples were then centrifuged for 30 min at 2000 x g. To determine insoluble NSP the insoluble residue was dried under nitrogen and hydrolysed with 12 M H_2SO_4 for 1 h at 30 °C after hydrolysis in 1 M H_2SO_4 for 2 h at 100°C. After cooling the hydrolysate, an aliquot of 0.8 mL was accurately pipetted into a clean 25 mL reaction tube followed by adding 0.2 mL of 28% NH_3. The sample was then vortexed to mix and precisely 50 μL of two internal standards (inositol, 4 mg/mL and allose 4 mg/mL) were added before drying in a vacuum rotary evaporator for 8 h at 40 °C. Similar to the procedure previously described for free sugars, reduction and acetylation were carried out. Soluble NSPs were precipitated in 80 % ethanol by taking 4 mL of the supernatant after removal of starch by mixing with 16 mL of absolute ethanol. The precipitate was dried under a stream of nitrogen at 40 °C before 1 mL of 2 M triflouroacetic acid was added. Hydrolysis of the mixture was undertaken at 125 °C for 1 h. The mixture was cooled to room temperature and 50 μL of two internal standards (inositol 4 mg/mL and allose 4 mg/mL) were precisely added. Under a stream of nitrogen, the

triflouroacetic acid was removed by co-distillation with distilled water. The dry residue was then mixed in 0.2 mL of distilled water followed by reduction and acetylation of monosaccharides as described in the preceding section. The levels of polysaccharides were calculated from the levels of the component sugars using polymerisation factors of 0.88 for pentoses (ribose, xylose and arabinose), 0.9 for hexoses (mannose, galactose and glucose), 0.89 for deoxysugars (fucose and ribose) and 0.91 for rhamnose (Theander and Westerlund, 1993).

3.2.6 Amino acid composition

The concentration of amino acids were determined using pre-column derivatisation 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 (2001). Amino acids were detected by UV absorbance. Briefly, approximately 100 mg of samples was hydrolysed in 20% HCL for 24 h at 110 ºC. Following hydrolysis, amino butyric acid (AABA) was added to each sample as an internal standard. Thereafter, an AccQ Tag Ultra Derivatization Kit (Waters Corp. USA; 70 µL borate buffer plus 20 µL AccQ Tg solution, incubated 10 min at 50 ºC) was used to derivatise 10 µL of solution. The HPLC analysis was based on the method of Cohen (2001), but adapted for use with ACQUITY Ultra Performance LC (UPLC; Waters Corp. State USA) system. The column employed was an ACQUITY UPLC BEH C18 1.7 µL column (water) with detection at 260 nm and a flow rate of 0.7 mL per min. All samples were run in duplicate and results were reported as an average. All of the above procedures were carried out by the Australian Proteome Analysis Facility (APAF), Macquarie University, NSW, Australia.

3.2.7 Mineral composition

Minerals were analysed using the inductively coupled plasma (ICP) method (Vista MPX-radial) following the protocol of Anderson and Henderson (1986). The sealed chamber digest (SCD) method was also used for P, S, K, Na, Mg and trace elements. This digest is the most appropriate for ICP analyses in which final oxidation occurs in the high temperature plasma of the ICP. Ground samples, approximately 0.5 g, were weighed into 50 mL borosilicate reagent bottles and exact weights and vial numbers were entered on a record sheet. After that,

2 mL of a 7:3 (v/v) mixture of $HClO_4$ (70%) and H_2O_2 (30%) was added to each tube and capped tightly. These were left overnight at room temperature to digest before 1 mL of H_2O_2 was added, and the bottles tightly sealed and placed into a warming oven at 80 ºC for 30 min. After cooling slightly, an additional 1 mL of H_2O_2 was added and they were left for one hour longer for further digestion. The final volume was adjusted to 25 mL of total volume using distilled water and mixed thoroughly. The samples were briefly stored at 2 ºC to reduce adsorption onto the plastic and inhibit the growth of microorganisms, prior to reading the absorbance at 785 nm against a blank.

3.3 RESULTS

3.3.1 Proximate analyses

The results for proximate analyses of DM, GE, CP, fat and ash contents of the 6 different DDGS batches are shown in Table 3.1. Mean values of DM of different DDGS batches varied between 890.5 and 931 g/kg, while CP content revealed a higher CV compared to DM, with mean values of 287.1 to 310.4 g/kg in batches 5 and 3, respectively. The range of GE content of samples was narrow, being highest for batch 4 and lowest for batch 2. Fat contents were found to be widely variable with 20.0 g/kg difference between batches 5 and 3 as the two extremes for lipids. Batch 1 had the highest ash content, while the lowest was observed in batch 3 with a CV of 4.74.

Table 3.1 Nutrient composition of distillers dried grain with solubles from 6 different batches

sDDGS batches	DM (g/kg)	CP (g/kg)	GE (MJ/kg)	Lipids (g/kg)	Ash (g/kg)
1	907.1	289.3	19.14	98.7	51.6
2	890.5	291.9	18.69	94.6	49.0
3	918.0	310.4	19.52	89.5	44.6
4	931.0	303.4	19.59	90.7	48.5
5	914.0	287.1	19.47	110.1	48.2
6	908.5	290.9	19.15	103.0	49.7
Mean	*911.5*	*295.5*	*19.30*	*97.8*	*48.6*
CV (%)	*1.47*	*3.13*	*1.76*	*8.04*	*4.74*

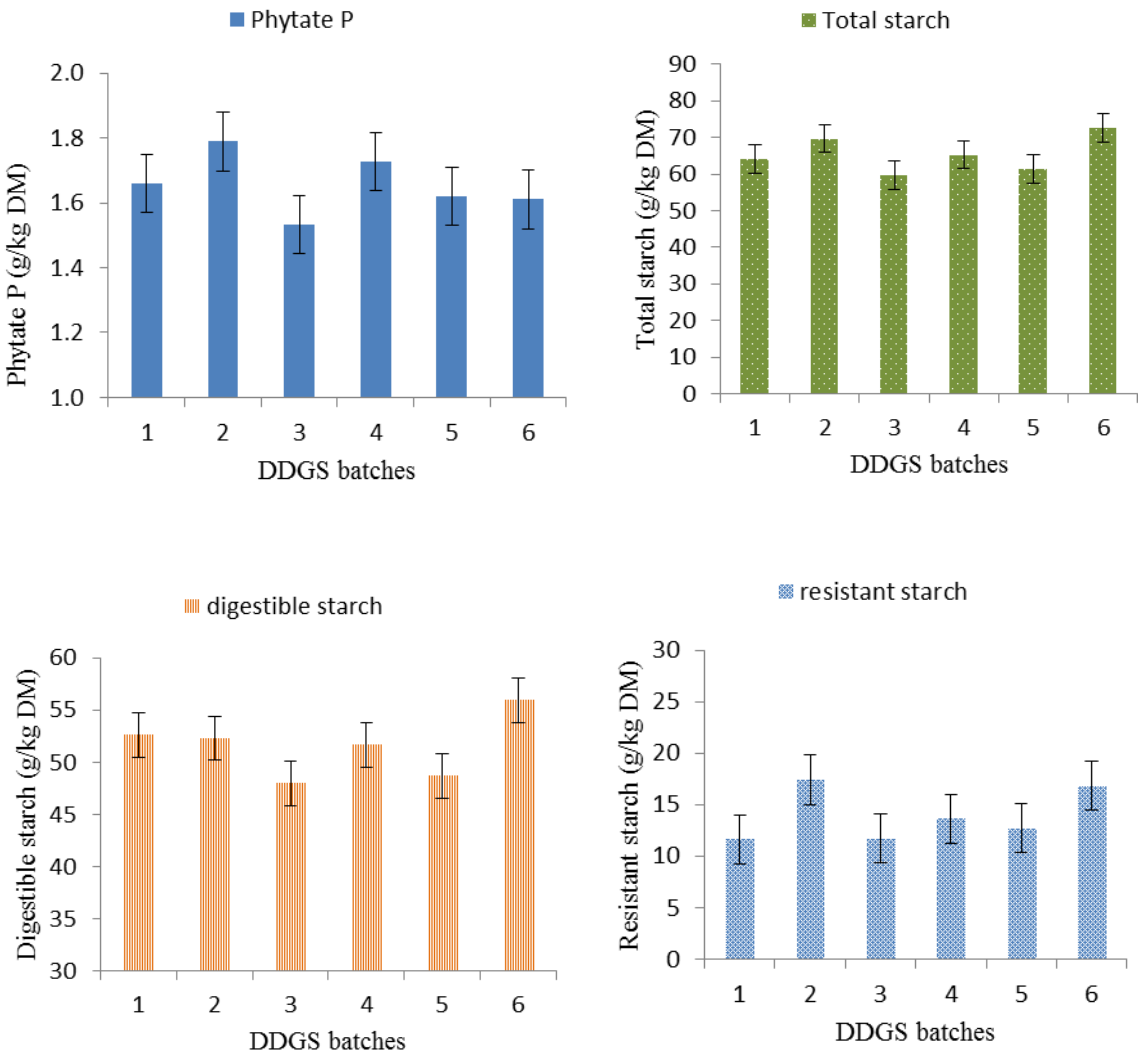

Figure 3.1 Total, digestible, resistant starch and phytate contents of DDGS samples from different batches. The bars indicate standard deviation for the 6 DDGS samples.

3.3.2 Level of total, digestible and resistant, starch and phytate P

As shown in Figure 3.1, total starch content varied from 59.7 g/kg in batch 3 to 72.7 g/kg in batch 6, with a CV of 7.31 %. Most of the starch content was found to be digestible, ranging from 48 to 55.9 g/kg in batch 3 and 6, respectively (CV=5.2 %). Resistant starch contents were found to be more variable that digestible and total starch, showing a CV of 18 % in a range of 11.6 to 17.4 g/kg DM. The analyses of phytate phosphorus also revealed an average of 1.66 g/kg DM, varying from 1.53 to 1.79 g/kg in batch 3 and 2 as the two extreme samples, respectively (CV=5.11 %).

Table 3.2 Composition of soluble and insoluble NSP and free sugar contents of distillers' dried grains with solubles from 6 different batches (g/kg)

	Soluble sugars								
sDDGS (batches)	Rham	Fuc	Rib	Arab	Xyl	Mann	Galac	Glu	Total
1	0.07	0.00	0.36	7.28	12.84	3.09	4.38	3.59	28.04
2	0.00	0.00	0.39	7.26	13.45	3.30	3.91	3.95	28.62
3	0.06	0.00	0.40	7.78	11.41	2.47	3.34	2.10	24.39
4	0.05	0.00	0.41	7.67	12.45	1.84	2.67	1.68	23.66
5	0.06	0.00	0.43	9.02	14.27	2.55	4.23	2.42	29.21
6	0.09	0.04	0.42	7.38	12.89	4.55	4.83	5.84	32.01
Mean	*0.05*	*0.01*	*0.40*	*7.73*	*12.88*	*2.97*	*3.89*	*3.26*	*27.65*
CV (%)	*0.50*	*2.24*	*6.18*	*8.61*	*7.45*	*31.34*	*20.00*	*47.05*	*11.30*
	Insoluble NSP								
sDDGS (batches)	Rham	Fuc	Rib	Arab	Xyl	Mann	Galac	Glu	Total
1	0	0.25	0.28	36.97	56.55	8.96	5.12	86.19	172.90
2	0	0.51	0.27	36.52	57.21	12.10	5.00	96.27	184.98
3	0	0.23	0.31	41.25	57.31	13.77	4.81	81.79	177.44
4	0	0.37	0.27	44.78	63.37	11.91	5.24	94.49	196.05
5	0	0.33	0.24	37.08	52.84	10.28	4.62	82.53	167.07
6	0	0.19	0.25	36.14	55.76	11.24	4.92	95.28	181.47
Mean	*0*	*0.31*	*0.27*	*38.79*	*57.17*	*11.38*	*4.95*	*89.42*	*179.99*
CV (%)	-	*37.31*	*9.07*	*8.96*	*6.04*	*14.50*	*4.47*	*7.47*	*5.60*
	Free sugars								
sDDGS (batches)	Rham	Fuc	Rib	Arab	Xyl	Mann	Galac	Glu	Total
1	0	0	0.22	6.70	11.52	7.96	2.95	31.28	60.62
2	0	0	0.21	6.46	10.34	6.04	2.63	23.48	49.16
3	0	0	0.16	5.72	9.58	5.99	2.26	28.77	52.47
4	0	0	0.19	7.14	11.31	6.72	2.78	27.72	55.86
5	0	0	0.24	9.11	12.52	6.19	2.63	25.32	56.61
6	0	0	0.23	6.92	10.81	6.49	2.85	25.77	53.08
Mean	*0.00*	*0*	*0.21*	*7.01*	*11.01*	*6.57*	*2.68*	*27.06*	*54.63*
CV (%)	-	-	*14.05*	*16.27*	*9.23*	*11.24*	*9.02*	*10.28*	*7.24*

Rham: rhamnose; Fuc: fucose; Rib: ribose; Arab: arabinose; Xyl: xylose; Mann: mannose; Galac: galactose; Glu: glucose.

3.3.3 Non starch carbohydrate and free sugar composition

The composition of soluble and insoluble NSP as well as free sugar contents of DDGS samples are presented in Table 3.2. The total carbohydrate contents averaged 262.2 g/kg of the DDGS samples, which were dominated by the insoluble fraction, followed by free sugars, while only 27.6 g/kg soluble NSP was detected. Xylose and arabinose were found to be the

main sugars for soluble and free sugar composition. For the soluble fraction, xylose, arabinose, galactose, glucose, mannose, ribose and mannose made up the NSP in decreasing order. The analysis of insoluble NSP showed the highest amount of glucose followed by xylose and arabinose. For free sugars, after xylose and arabinose, the concentration of glucose, mannose, galactose and ribose made up the free sugar composition of DDGS samples in decreasing order. Compared to the remaining sugars, the CV was found to be higher for mannose and fucose for the insoluble and soluble fractions of NSP.

3.3.4 Mineral contents

The mineral composition of DDGS samples is summarised in Table 3.3. In different DDGS batches, the content of Mo, Na, Fe and S were found to be most variable, in decreasing order among all minerals analyses. Calcium and phosphorus content of DDGS samples remained relatively consistent for different samples, with a CV of 2.95 and 5.01 %, respectively. The coefficient of variation for the rest of the minerals was found between 5.94 and 9.96 %.

Table 3.3 Mineral composition of distillers' dried grain with solubles from 6 different batches

sDDGS samples	Ca g/kg	P g/kg	S g/kg	K g/kg	Mg g/kg	Mn g/kg	Mo µg/kg	Na g/kg	Cu µg/kg	Fe mg/kg	Zn mg/kg
1	1.4	9.3	3.9	11.6	4.3	0.11	0.11	3.37	13.2	0.22	0.06
2	1.4	9.2	3.7	11.3	4.2	0.12	0.34	3.69	13.8	0.20	0.06
3	1.4	8.5	4.5	10.5	3.7	0.10	0.76	3.77	12.7	0.14	0.05
4	1.4	9.5	4.4	11.6	4.2	0.13	0.86	3.70	14.6	0.15	0.05
5	1.3	9.9	4.0	12.7	4.5	0.12	0.33	5.16	12.5	0.14	0.05
6	1.4	9.1	3.7	11.1	4.1	0.11	0.88	3.68	12.9	0.19	0.06
Mean	*1.4*	*9.2*	*4.0*	*11.5*	*4.1*	*0.11*	*0.55*	*3.90*	*12.1*	*0.17*	*0.05*
CV (%)	*2.95*	*5.01*	*8.54*	*6.35*	*6.38*	*9.12*	*59.84*	*16.31*	*5.94*	*19.85*	*9.96*

3.3.5 Amino acid composition

Table 3.4 shows the amino acid concentrations of DDGS samples from different batches. Among the amino acids, lysine, methionine and tyrosine were the most variable, in a decreasing order. The remaining amino acids were relatively consistent between different batches, with CVs from 2.77 to 6.4 %. On average, batch 1 had the highest amino acid contents, while batch 2 had the lowest.

Table 3.4 Amino acid composition of distillers' dried grain with solubles from different batches produced by an ethanol plant (g/kg DM)

sDDGS	His	Ser	Arg	Gly	Aspartic acid	Glutamic acid	Thr	Ala	Pro	Lys	Tyr	Met	Val	Ile	Leu	Phe
1	6.7	14.3	13.2	12.7	21.3	58.5	11.4	21.9	22.3	5.4	8.7	4.5	16	12.1	31.3	14.3
2	6.0	12.5	11.9	12.0	18.9	52.6	10.2	19.0	19.7	4.1	7.2	4.1	14.4	10.6	26.7	12.6
3	6.6	14.1	13.0	12.6	21.1	57.8	11.3	21.6	22.1	5.3	8.6	4.4	15.9	11.9	30.9	14.1
4	6.3	13.3	12.6	12.5	19.4	53.3	10.6	19.7	20.3	4.6	7.8	4.1	14.8	11.0	27.9	13.2
5	6.3	12.9	11.8	11.9	18.8	52.0	10.3	20.1	20.3	4.4	7.5	3.5	14.7	11.3	28.7	13.1
6	6.3	12.6	12.1	12.1	18.6	51.6	10.1	19.1	20.2	4.0	7.5	3.6	14.6	11.2	27.7	12.8
Mean	*6.4*	*13.3*	*12.4*	*12.3*	*19.6*	*54.3*	*10.7*	*20.2*	*20.8*	*4.6*	*7.8*	*4.0*	*15.0*	*11.3*	*28.9*	*13.3*
CV (%)	*3.93*	*5.76*	*4.74*	*2.77*	*6.13*	*5.61*	*5.34*	*6.15*	*5.27*	*12.85*	*7.92*	*10.12*	*4.63*	*4.96*	*6.40*	*5.21*

3.4 DISCUSSION

This study probably represents the most comprehensive analyses of sDDGS produced in Australia. Australia's ethanol industry is based largely on sugarcane. Manildra, the source of samples tested in this study, produces a range of products other than ethanol. The end products of this process, including DDGS, may be different from those originating from a core ethanol plant. However, the results of proximate composition of sDDGS in the recent analysis were in range of those reported by Urriola *et al.* (2009) and Jenkins *et al.* (2007). For example, the CP content of three sDDGS sample were reported by Jenkins et al. (2007) being 29.3, 25.6 and 30%. Cromwell *et al.* (1993) also reported an average of 29.7, 10.7 and 5.3 % for protein, fat and ash contents of mDDGS from nine different samples, respectively. The CV reported for DM by the same authors was also consistent with a value of 1.47 % obtained in sDDGS presented here. It appears that lower variation exists in DM of DDGS samples compared to ash, lipid and protein composition (Spiehs *et al.*, 2002).

Indeed, the results revealed that Lys is the most variable amino acid in Australian DDGS originating from sorghum, and this supports the data in the literature (Cromwell *et al.*, 1993; Stein *et al.*, 2006; Urriola *et al.*, 2009). Moreover, Met was also found to be relatively variable and the content of Lys and Met were lower than the average reported in aforementioned works. This is strongly supported by the data reported by Urriola *et al.* (2009) where less concentration of Lys and Arg was found in sDDGS than in mDDGS. However, the content of amino acids, Lys and Met in particular, was found to be slightly lower for the present analyses compared to the data reported by Urriola *et al.* (2009). In general, Lys is highly susceptible to heat damage, therefore the Lys content and the digestibility may vary depending upon various drying practices and production processes. This could be attributed to the Maillard reaction between ε-amino group of Lys and reducing end of carbohydrates that substantially reduces the true amino acid availability, particularly Lys (Fastinger *et al.*, 2006). Besides, the samples obtained for the analyses were brownish in colour, which may also be an indicator of estimating Lys content as shown by Batal and Dale (2006) and Fastinger *et al.* (2006). The starch content of DDGS is believed to be approximately 6 % as most of the starch is converted to ethanol upon saccharification, enzymatic hydrolysis and fermentation (Belyea *et al.*, 2004; Liu, 2008). The current analyses also revealed similar results. However, the high digestible starch can be an indication of inefficiency of converting all the starch to ethanol; thus it may still be of value for animal feeding. Total carbohydrate of sDDGS was found to be slightly lower than that of mDDGS,

which Choct and Peterson (2009) found to be approximately 40 %. Different raw material, processing and presence of high residual starch in mDDGS may explain this discrepancy. However, although a range of sugars were found in sDDGS it was, indeed, dominated by arabinoxylans and this concurs with some other reports (Wu, 1994; Choct and Petersen, 2009). This probably points to a potential for using exogenous enzymes when it is incorporated in non-ruminant diets. The limited variability observed in the contents of NSP and free sugars of sDDGS indicates that, presumably, the process of ethanol production has little effect on the carbohydrate composition except starch. This is expected because the carbohydrate composition remains relatively unchanged until the saccharification stage when the conversion of starch and dextrose to simple sugars takes place (Liu, 2011). Therefore, in the case of having consistent conversion of starch to ethanol, the composition of NSP will only increase, but composition remains relatively unaffected. Upon these analyses, it was attempted to test the effect of different exogenous enzymes in a series of *in vitro* assays, but the results were inconsistent, probably due to inappropriateness of enzymes and conditions for an *in vitro* assay or even the amount of ingredient itself; therefore, results of those tests have not been included.

Despite relatively the low variation between DDGS batches in the current study, Na, Fe and S were still found to be more variable than other elements. In particular, Na appeared to be unusually high and variable between the 6 samples. There are several factors that may explain this observation. Exogenous supplementation of some mineral compounds during the process of ethanol production may influence mineral content of the final by-product. Considering Na, in particular, sodium hydroxide may be used by ethanol plants to sanitise the equipment (Liu, 2011) or, along with sulphuric acid, to adjust the pH of mashes for optimum enzyme activity in the stage of liquefaction or to provide the requirements for yeast during the fermentation (Belyea *et al.*, 2006). The phosphorus content of sDDGS is in the range of 0.5 to 1% reported in other studies (Spiehs *et al.*, 2002; Batal and Dale, 2003). The low phytate P detected in sDDGS samples indicates that most of the P in DDGS is phosphate P. Noureddini *et al.* (2009) did not report the presence of phytate in DDGS with HPLC analysis. These findings point to a possibility of degradation of phytate, presumably due to activity of yeast phytase in the process of ethanol production, supporting the higher bioavailability of P in DDGS compared to main grains (Pedersen *et al.*, 2007).

3.5 CONCLUSION

Depending upon the basal grain used, such as sorghum, wheat, maize or barley, the composition of DDGS may differ. However, in terms of magnitude of variability between different batches, the current analyses did not show a high coefficient of variation for most of the nutrients, and this may indicate less need for concern for the variability of DDGS when it is sourced from the same ethanol plant. However, the comprehensive analyses of DDGS prior to inclusion in poultry diet still appear to be unavoidable, particularly for protein, amino acids and minerals.

CHAPTER 4

IMPACT OF VARYING LEVELS OF SORGHUM DISTILLERS' DRIED GRAINS WITH SOLUBLES AND XYLANASE SUPPLEMENTATION ON PERFORMANCE, INTESTINAL FUNCTION AND ILEAL RESIDUAL NON-STARCH POLYSACCHARIDES IN BROILER CHICKENS

Abstract

An experiment was conducted to investigate the effect of sorghum distillers' dried grains with solubles (sDDGS) and xylanase supplementation in starter diets (0 to 21d) and subsequently grower diets (22 to 35d) for broiler chickens. A total of 432 Cobb 500 day-old male broiler chicks were used in a 4 x 2 factorial design (0, 100, 200 or 300 g sDDGS/kg with or without a xylanase enzyme). Each treatment was replicated 6 times with 9 birds per replicate cage. Compared to the control diet, feed intake was significantly increased (P<0.001) with an inclusion of dietary sDDGS in the diet during the starter, grower and the entire periods of this study. Body weight gain (BWG) was unaffected by the inclusion of sDDGS or xylanase except for the last 2 weeks of trial when birds that received 20 and 30% sDDGS had higher (P<0.001) BWG. Feed conversion ratio (FCR) increased (P<0.05) as the sDDGS in diets rose to 10 % during the starter phase of feeding and the whole period of trial but remained same for 20 and 30 % as for 10 % of inclusion. Over the starter period, xylanase supplementation markedly improved (P<0.05) FCR particularly for the highest inclusion of sDDGS. While starch digestibility was not altered by dietary sDDGS or enzyme supplementation, protein digestibility deteriorated (P<0.001) as sDDGS rose to 30% in the diet. Addition of xylanase to the diets significantly reduced (P<0.001) the concentration of xylose in the ileum of the birds. While dietary treatments had no impact on pancreatic enzyme activities, the activities of sucrase and maltase in the jejunal mucosa were reduced as birds were offered 20 and 30% sDDGS. Incorporation of sDDGS also increased (P<0.01) total short-chain fatty acids concentration in the caecum of broiler chickens. Overall, the results showed that diets containing large amounts of sDDGS will benefit from xylanase supplementation, particularly in terms of FCR.

4.1 INTRODUCTION

There has been an increase in the use of distillers' dried grains with solubles (DDGS) in livestock diets as a result of dramatic developments in ethanol production in the past few years, which has led to the availability of large amounts of this by-product all around the world. In general, the starch component of the grain is converted to ethanol and carbon dioxide during fermentation, resulting in a threefold increase in content of all other nutrients. Although DDGS were primarily fed to ruminants because of their high fibre content and nutrient variability, the high contents of protein, fat and mineral compared to the native grain make this by-product an attractive ingredient for non-ruminants. In an early study, Waldroup *et al.* (1981) reported no reduction in body weight or feed utilization when broilers were fed diets containing up to 25 % DDGS. More recently, Thacker and Widyarante (2007) found that 15 % of DDGS can be incorporated into diets with no detrimental effect on broiler performance. In another report, Wang *et al.* (2008) tested the feasibility of using high level of DDGS in broiler diets and found that up to 30 % DDGS can be incorporated in the diets if the price was justified. However, application of DDGS in large amounts in poultry diets has faced some limitations, including the presence of non-starch polysaccharides (NSP), variability, bulk density and flow-ability. Therefore, it has been shown that high levels of DDGS can adversely affect bird performance. In this regard, other works (Wang *et al.*, 2007a; Wang *et al.*, 2007b) indicate the reduced feed utilization and poor performance when birds were fed diets containing 30 % DDGS.

Distillers' dried grains with solubles consist of a greater proportion of NSP compared to their respective native grains, a component that is difficult for non-ruminant animals to digest. The presence of NSP in maize DDGS and their composition has been evaluated by Choct and Peterson (2009) who reported an average of 40 % total NSP, most of which was insoluble and composed mainly of glucose, xylose and arabinose. This indicates that the NSP in DDGS are cellulose and arabinoxylans, as is the case in maize.

In regard to the application of DDGS in poultry diets, most researchers have focused on the material from maize and wheat and less work has been done on DDGS from sorghum. Thus far, little information has been made available regarding the influence of any single enzyme particularly xylanase targeting NSP, in diets containing sorghum DDGS for broilers. However, Metayer *et al.* (2009) found an improvement in the growth performance of birds fed 15% DDGS along with a cocktail of carbohydrases. Liu *et al.* (2011) also demonstrated

beneficial impact of exogenous xylanase supplementation of broiler diets containing DDGS on nutrient digestibility. However, the actual effects of DDGS inclusion on digestive function have not been extensively evaluated, and the reason for the poor performance caused by high levels of DDGS inclusion remains unclear. Therefore, the aims of this study were to evaluate the effects of different levels of sorghum DDGS and xylanase supplementation on bird performance and digestive function, with a particular emphasis on NSP degradation and to better understand the potential limitations and challenges associated with high DDGS incorporation in broiler diets.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design and treatments

Eight treatments were allocated to each of the starter and grower diets in a 4 × 2 factorial design comprising 0, 100, 200 or 300 g sDDGS/kg diet (designated control, LDDG, MDDG and HDDG, respectively) to test the effect of enzyme inclusion and determine the optimum level of sDDGS, formulated to meet or exceed the National Research Council (NRC) nutrient requirements for broiler chickens. The energy and protein content of diets were calculated to be the same for all basal diets. Two levels of xylanase (Ronozyme WX, 0 or 0.25 g/kg) were tested. Ronozyme WX, kindly donated by DSM Nutritional Products Australia Pty Limited, is derived from *Thermomyces lanuginosus spp*. The enzyme is an endoxylanase, hydrolyzing arabinoxylans and xylans in the feed with a minimum 1000 fungal xylanase unit (FXU) per gram. The DDGS in this experiment was obtained from an ethanol plant (Manildra, NSW, Australia) and was derived from sorghum as the predominant grain used in ethanol production in Australia. The sample had brownish colour and a coarse appearance. The material was analysed for nutrient contents prior to feed formulation. To assess the effect of enzyme supplementation on NSP degradation and performance, the diets contained maize and soy protein concentrate as main feed ingredients.

Titanium dioxide (TiO_2) was added as an indigestible marker in the starter diets (0.5 g/kg diet) that were fed for the first 21 days of the trial, to measure nutrient digestibility. The birds were then transferred to a finisher diet and raised to 35 days of age. The composition of the experimental diets is shown in Table 4.1. The starter diets were fed as mash.

4.2.2 Bird management and housing

Four hundred and thirty-two day-old male birds (Cobb 500, initial weight 47 ± 0.6 g) were obtained from a local hatchery (Baiada Pty Ltd, Tamworth, NSW, Australia), and randomly assigned to 48 cages comprising 8 treatments and 6 replicates, with 9 birds per replicate. The birds were reared in two environmentally controlled rooms. The temperature was maintained at 34ºC for the first 5 days and then gradually decreased to 21ºC by the end of the third week. Birds had access to feed and water *ad libitum*. A photoperiod of 18 hours per day was applied throughout the experiment. Feed intake and body weight were recorded weekly but the data are presented for days 0-21, days 21-35 and days 0-35. Feed conversion ratio (FCR) was also calculated and corrected for mortality when applicable.

4.2.3 Sampling procedures

At 21 days, 3 birds per pen were randomly selected, weighed and humanly euthanased and dissected. The full weight of the proventriculus and gizzard, small intestine, liver, spleen, bursa and yolk were recorded. Digesta content was removed from ileum by gently squeezing out the content between Meckel's diverticulum and 4 cm above the ileocaecal junction (defined as ileum) into plastic containers. A sub-sample of the digesta was kept on ice prior to centrifugation and measurement of viscosity. The ileal digesta from each pen were pooled and mixed to obtain homogenous samples, then were immediately frozen, freeze-dried and stored at -4 ºC until required for chemical analysis.

The contents of caeca were also collected and kept in air-tight containers at -20 ºC for volatile fatty acid analysis. The entire pancreas of one bird from each pen was weighed and snap-frozen in liquid nitrogen for digestive enzyme assays. A subsample of jejunum was also taken and snap-frozen in liquid nitrogen for later analysis of protein and digestive enzyme activities.

Table 4.1 Composition of the starter and grower basal diets containing graded level of sDDGS

Ingredients	Starter diets (sDDGS g/kg diet)				Grower diets (sDDGS g/kg diet)			
	0	100	200	300	0	100	200	300
Maize	677.4	604.3	520	439.3	738.5	668.2	586.4	492
Soy Co Meal (67% CP)	267.6	221.8	188	151.6	221	175	140	115.5
Sorghum DDGS	-	100.0	200.0	300.0	-	100.0	200.0	300
Dicalcium phosphate	18.5	18.5	18.5	18.5	15.5	15.5	15.5	15.5
Vegetable oil	6.8	23.2	40.6	56.9	2.3	18.3	33.7	51.2
limestone	13.9	13.9	13.9	13.6	13.9	13.9	13.9	13.9
salt	4.5	4.5	4.7	3.7	3.5	3.5	3.5	3.5
Vitamin-mineral premix[1]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
DL- Methionine	2.3	2.3	2.3	2.3	1.1	1.1	1.1	1.1
L- Lysine	0.4	2.9	3.4	5.5	1.1	1.7	3.4	4.8
Choline chloride	1.1	1.1	1.1	1.1	0.6	0.3	-	-
TiO_2	5.0	5.0	5.0	5.0	-	-	-	-
Total	1000	1000	1000	1000	1000	1000	1000	1000
Nutrient composition (g/kg diet)								
Crude protein	228.0	228.0	228.0	228.0	206.6	206.6	206.6	206.6
Metabolisable energy (MJ/kg DM)	13.35	13.35	13.35	13.35	13.38	13.38	13.38	13.38
Lys	13.7	13.8	13	13.2	11.5	11.2	11.2	11.3
Met	6.0	5.8	5.8	5.7	4.5	4.3	4.2	4.2
Met + Cys	8.5	8.4	8.5	8.5	6.9	6.8	6.8	6.9
Calcium	10.1	10.2	10.2	10.2	9.5	9.5	9.5	9.6
Available Phosphorus	4.6	4.9	4.9	4.9	4.4	4.3	4.5	4.4

[1]Supplied per kg of diet (mg): vitamin A (as all-trans-retinol) 3.6 mg, cholecalciferol 0.09 mg, vitamin E (as D-tocopherol) 44.7 mg, vitamin K3 2 mg, thiamine 2 mg, riboflavin 6 mg, pyridoxine hydrochloride 5 mg, vitamin B12 0.2 mg, biotin 0.1 mg, niacin 50 mg, D-calcium pantothenate 12 mg, folic acid 2 mg, Mn 80 mg, Fe 60 mg, Cu 8 mg, I 1 mg, Co 0.3mg and Mo 1mg.

4.2.4 Chemical analysis and measurements

4.2.4.1 Titanium oxide measurement

The method described by Short *et al.* (1996) was adopted with some modifications to measure TiO_2 contents of diets and ileal digesta. Basically, approximately 0.1 g of freeze-dried ileal digesta or feed sample was weighed and placed in porcelain crucible, then ashed for 13h at 580 ºC. Thereafter, all samples were dissolved in 5 mL 7.4M sulphuric acid upon

cooling. The samples were then gently warmed up using a digital temperature-controlled heating plate (HP30 Digital, IKA® LABORTECHNIK, Germany) for approximately 30 min at 200 ºC followed by another 30 min at 250 ºC until completely dissolved. After cooling, the solution was poured quantitatively into a 50 mL volumetric flack through a Whatman 504 filter paper. This was performed by several rinsing of crucible with approximately 15 mL of distilled water. Subsequently, 10 mL hydrogen peroxide (30 % v) were added to each flask and diluted to 50 mL using distilled water. Reaction with hydrogen peroxide resulted in a typical orange colour, the intensity of which is dependent on the concentration of titanium in the sample. Six titanium standard solutions (0, 0.005, 0.01, 0.015, 0.02 and 0.03 mg/mL) were also similarly prepared in volumetric flasks and read along with all other samples at 410 nm using a Hitachi 150-20 UV spectrophotometer (Hitachi Science Systems Ltd., Ibaraki, Japan). The titanium contents of samples were eventually converted to mg/mL from standard curve then expressed as mg/g of the sample.

4.2.4.2 Nutrient and NSP content

The protein content of the diets and digesta was measured according to the method of Association of Official Analytical chemist international (AOAC, 2002) using a LECO FP-2000 automatic analyser (Kjeldahl N × 6.25). Starch was determined as glucose, following digestion, as described in Section 3.2.3. The total NSP composition of the digesta and diets samples was determined by gas chromatography according to the method described by Englyst and Hudson (1987) and Theander and Westerlund (1993) as described in Section 3.2.5.

4.2.4.3 Nutrient digestibility

Digestibility coefficient of nutrients was calculated using indigestible marker as follow:

Subsequent digestibility coefficients for different nutrients were calculated using the following formula:

$$\text{Apparent ileal digestibility coefficient} = \frac{(NT/Ti)_d - (NT/Ti)_i}{(NT/Ti)_d}$$

where:

$(NT/Ti)_d$ was the ratio of nutrient and titanium in diet

$(NT/Ti)_i$ was the ratio of nutrient and titanium in ileal digesta

4.2.4.4 Viscosity

About 0.5 mL of thawed supernatant was used to measure viscosity with a Brookfield DVIII viscometer at 25 ºC with a CP 40 cone. The shear rate was from 5 to 500/s at which the samples did not exhibit shear thinning. The remaining supernatant and pellets were used for nutrient analysis after being recombined and freeze-dried.

4.2.5 Brush-border Enzyme analyses in jejunum

The method developed by Shirazi-Beechey *et al.* (1991) was adopted to isolate brush-border membrane vesicles from jejunal tissue with minor modification described by Iji (1998).

Jejunum sample preparation

Around 2 g jejunum tissue was first weighed and cut into smaller pieces into 20mL of buffer (100 mM mannitol, 2mM N-[2-hydroxyethyl] piperazine-N´[2ethanesulfonic acid] (HEPES/Tris, pH 7.1)) and defrosted. The samples were then vibromixed at high speed for approximately 1 min and filtered through a Buchner funnel, to isolate the mucosa from muscular debris. The mixture was blended at about 13000/min for 30 second using a homogeniser (Utra Turrax T25 basic, IKA, Labortedrik, NC, U.S.A). Two aliquots of the homogenate were transferred to Eppendorf tubes for protein and enzyme analyses.

Protein analysis

The method described by Bradford (1976) was adopted to assess the recovery rate of the membrane by estimating the protein concentration of the homogenate. The method is based on the reaction of the red form of Coomassie Brilliant Blue G-250 (CBB), which turns blue on binding to protein. Briefly, homogenate was first diluted 15 times with distilled water. Forty µl of the dilute homogenate was pipetted into tubes. Two 2 mL of CBB were added and vibromixed. The absorbance was read at 595 nm after 5 minutes but within 1 h. Bovine serum albumin was used a standard solution at concentration of 2 mg/mL. The Lowry

software (Elsevier BIOSOFT, Cambridge, UK) was employed to calculate actual concentrations of protein from absorbance values.

Maltase and Sucrase

The activities of disaccharidases, α-glucosidase (maltase, EC. 3.2.1.20) and β-fructofuranosidase (sucrase, EC. 3.2.1.26) were tested employing the method outlined by Dahlqvist (1964), with modifications (Iji, 1998). Initially, fresh substrate was prepared from 100 mM maltose or sucrose respectively in succinate buffer (4mM sodium succinate, 90 mM sodium chloride, pH 6.0). Accurately, 25 µL of homogenates were incubated in 475 µL of substrate buffer for 30 minutes at 39ºC. The glucose released from incubation was then quantified by GOD-perid kit obtained from Boerhinger-Mannheim Australia (Castle Hill, NSW, Australia). The amount of glucose was finally measured spectrophotometrically at 610 nm after development of colour for 30 min at room temperature.

Alkaline phosphatase

Alkaline phosphatase was measured by the method described by Forstner *et al.* (1968) and Holdsworth (1970). Briefly, an incubation mixture was set up to consist 20 µL of sample, 800 µL 50 mM Tris buffer, pH 10.1, 100 µL 50 mM $MgCl_2$ and 100 µL of 10 mM phosphatase substrate. The mixture was maintained for 20 minutes at room temperature. The reaction was subsequently terminated by rapidly pipetting in 100 µL 40% trichloroacetic acid. In order to accomplish further colour development, 100 µL of incubated mixture was transferred into fresh tubes and 2 mL of 0.4 M NaOH were added. The standard solution consisted of 0, 50 and 100 µL of p-nitrophenol at 1 µmol/mL concentration. Eventually, the mixture was vibromixed and read at 410 nm.

4.2.6 Protein content and enzyme activities of pancreas

Fractionation of pancreas was done according to the method described by Nitsan *et al.* (1974). The tissue was weighed and then cut into 20 mL ice-cold distilled water, followed by homogenisation at moderate speed (13000/min) for one minute. The mixture was centrifuged at 30000 x g for 20 minutes. Aliquots of the supernatant were frozen until used for protein and enzyme assays. The protein content of the pancreas was assayed by the same procedure described for jejunal samples.

Chymotrypsin amidase

The method of Serviere-Zaragoza *et al*. (1997) was adopted to measure chymotrypsin amidase activity in pancreas. The substrate solution consisted 0.1 mM of succinyl-$(Ala)_2$-Pro-*p*-nitroanilide, 50 mM Tris-HCL buffer with 20mM $CaCl_2$, pH 7.5. Fifty µL of diluted sample were added to 1 mL fresh substrate solution prior to incubation at 39 ºC for one hour. The reaction was then terminated by rapidly pipetting 500 µL of 30 % acetic acid. The mixture was vibromixed and absorbance was spectrophotometrically measured at 410 nm against water as blank.

Lipase

The assay was conducted according to the method described by Winkler and Stuckmann (1979). Concisely, the substrate was freshly made by dissolving 30 mg of p-nitrophenylpalmitate in 10 mL of isopropanol then thoroughly mixed in 90 mL of 0.05M Sorensen phosphate buffer, pH 8.0 containing 207 mg sodium deoxycholate. The standard solution was 1µmol/mL of *p*-nitrophenol, same as the standard used for alkaline phosphatase assay. Freshly made substrate was first warmed to 39º C, then 50 µL of sample was incubated in duplicate with 200 µL of substrate for 15 minutes at 39º C in a water bath. The reaction was terminated by adding 4 mL 0.4 M NaOH, subsequently mixed and read at 410 nm against distilled water as blank.

4.2.7 Short chain fatty acid measurements

The concentrations of short chain fatty acids (lactic and volatile fatty acids) were assayed according to the method described by Jensen *et al.* (1995). Briefly, frozen caecal samples were thawed and mixed thoroughly by vigorous shaking. About 1.5 g of caecal samples were accurately weighed and diluted with 1mL 0.01M ethylbutyric acid as an internal standard. Following centrifugation at 5 ºC and 15000 x g for 15 min, approximately 1 mL of supernatant was taken out and mixed with 2 mL ether and 0.5 mL of concentrated hydrochloric acid. To make a blank and internal standard solution, supernatant was replaced by one mL water and one mL standard acid mixture, respectively. All samples including blank and internal standard were again centrifuged at 5 ºC, 8000 x g for 15 min then 400 µL of supernatant were mixed with 40 µL of *N*-tert-butyldimethylsilyl-*N*-

methyltrifluoroacetamide in a gas chromatograph vial. The vials were placed on a heating block for 20 min prior to maintaining at room temperature for 48h. Eventually, short chain fatty acids were quantified on a Varian CP3400 CX gas chromatograph (Varian analytical instrument, Palo Alto, CA, USA).

4.2.8 Statistical analysis

Data were analyzed using both ANOVA of General Linear Models and regression procedure of SAS (SAS/STAT Version 9.1, SAS Institute Inc., Cary, NC, USA). Data were regressed for the levels of sDDGS and xylanase to assess main factor effects and interactions. Xylanase was treated as a fixed factor and cage was experimental unit for all variables. The mean values were separated by least squares means option of SAS when a significant F-value was detected at $P \leq 0.05$. The tendency was considered as $0.06 \leq P \leq 0.10$.

4.2.9 Animal ethics

The Animal Ethics Committee of the University of New England approved the procedures of the current experiment with an approval number of AEC 09/173.

4.3 RESULTS

4.3.1 Bird performance and visceral organ weight

There was a significant increase ($P<0.001$) in feed intake with increasing levels of dietary sDDGS during the first three weeks and the entire period of this study. Feed intake of the birds was not affected by enzyme supplementation up to 20 % of DDGS in the diets.

Table 4.2 Effects of xylanase supplementation and different levels of sDDGS on the performance of broiler chickens

Treatments		Feed intake (g/bird)			Body weight gain(g/bird)			Feed conversion ratio		
sDDGS (g/kg)	Xylanase	1-21d	21-35d	1-35d	1-21d	21-35d	1-35d	1-21d	21-35d	1-35d
0	-	884.8^{b}	1689.8^{bd}	2574.6^{c}	679.5	1163.7^{b}	1843.3	1.31^{cd}	1.46	1.40^{c}
	+	894.8^{b}	1710.4^{e}	2605.3^{c}	698.5	1152.3^{b}	1850.8	1.30^{d}	1.50	1.41^{c}
100	-	1020.5^{a}	1864.3^{ed}	2884.7^{ab}	696.8	1157.0^{b}	1853.9	1.48^{ab}	1.61	1.56^{ab}
	+	1013.9^{a}	1822.4^{ed}	2836.3^{b}	698.2	1159.8^{b}	1858.0	1.45^{abc}	1.59	1.53^{ab}
200	-	1016.7^{a}	2005.8^{a}	3022.5^{ab}	682.8	1321.2^{a}	2004.1	1.50^{ab}	1.52	1.51^{ab}
	+	992.7^{a}	1954.7^{ab}	2947.4^{ab}	699.3	1284.8^{ab}	1984.2	1.42^{abcd}	1.53	1.49^{bc}
300	-	1017.2^{a}	2047.7^{ab}	3064.9^{a}	660.0	1266.0^{ab}	1926.0	1.54^{a}	1.62	1.59^{a}
	+	971.6^{a}	1892.9^{b}	2864.5^{ab}	704.3	1220.5^{ab}	1925.0	1.38^{bcd}	1.56	1.49^{bc}
	SEM	*9.27*	*21.39*	*25.05*	*8.64*	*17.73*	*16.86*	*0.018*	*0.02*	*0.01*
Source of variation		----------*Probability*----------								
sDDGS		<0.001	<0.001	<0.001	NS	<0.001	NS	<0.02	NS	<0.01
Xylanase		NS	NS	NS	NS	NS	NS	<0.02	NS	NS
sDDGS × xylanase		NS	NS	NS	NS	NS	NS	NS	NS	NS

[a] Mean values on the same column not sharing a superscript are significantly different (P<0.05).
NS: Not significant, SEM: Standard error of mean

There was no significant effect of dietary sDDGS or of xylanase on body weight gain (BWG) in this experiment except for the finisher phase during which birds offered MDDG and HDDG had a higher (P<0.01) BWG (Table 4.2). Feed conversion ratio (FCR) was significantly increased (P<0.001) by raising the level of sDDGS in diets during the first 3 weeks of feeding. At that time, the effect of xylanase supplementation on FCR was not significant up to 20% sDDGS inclusion while FCR was significantly improved (P<0.05) in the birds fed HDDG in the starter feeding period. The FCR, however, was not affected by dietary DDGS or addition of xylanase over the last 2 weeks of study. Moreover, when assessed over the entire period of trial, FCR was adversely affected (P<0.01) by increasing level of DDGS, being poorest at 30% DDGS inclusion with no enzyme added. Similar to the result obtained for 0-21d, the effect of enzyme was only significant at the greatest level of sDDGS inclusion (30%).

As shown in Table 4.3, there was no significant effect of sDDGS at up to 20 % inclusion on the weight of the proventriculus and gizzard but the two organs were heavier (P<0.05) in the birds fed HDDG than those in the control group. The relative weight of the small intestine increased (P<0.01) in line with dietary sDDGS inclusion. However, liver, spleen and pancreas relative weights were not affected either by sDDGS level or by enzyme supplementation.

Table 4.3 Effects of increasing levels of sDDGS and xylanase on visceral organ weight (g/100g body weight) of 21-day-old birds

sDDGS (g/kg)	Xylanase	Proventriculus and gizzard	Small intestine	Liver	Spleen	Bursa	pancreas
0	-	4.45^{b}	5.69^{d}	3.09	0.08	0.15^{d}	0.35
	+	4.59^{b}	6.22^{bcd}	3.29	0.10	0.19^{bcd}	0.40
100	-	4.33^{b}	5.96^{dc}	3.13	0.10	0.23^{abc}	0.36
	+	4.72^{ab}	6.10^{dc}	3.52	0.11	0.18^{cd}	0.38
200	-	4.75^{ab}	6.92^{ab}	3.39	0.09	0.19^{abcd}	0.36
	+	4.62^{b}	6.21^{bcd}	3.46	0.08	0.24^{ab}	0.38
300	-	5.18^{a}	7.52^{a}	3.62	0.08	0.22^{abc}	0.38
	+	4.84^{ab}	6.51^{bc}	3.42	0.09	0.25^{a}	0.33
	SEM	*0.063*	*0.100*	*0.081*	*0.003*	*0.008*	*0.008*
Source of variation		----------*Probability*----------					
sDDGS		<0.05	<0.001	NS	NS	<0.05	NS
Xylanase		NS	NS	NS	NS	NS	NS
sDDGS × xylanase		NS	<0.05	NS	NS	NS	NS

[a] Mean values on the same column not sharing a superscript are significantly different (P<0.05).
NS: Not significant, SEM: Standard error of mean

The birds fed HDDG also had a higher (P<0.05) relative weight of bursa than the other groups of birds.

4.3.2 Nutrient digestibility, viscosity and NSP content of the ileal digesta

Digestibility of protein was adversely affected (P<0.001) in the birds fed diets MDDG and HDDG by approximately 8 to 11 percentage units, and xylanase did not ameliorate it (Table 4.5). There was no effect of sDDGS inclusion or of xylanase on the digestibility of starch. Ileal viscosity tended (P=0.06) to increase with increasing levels of dietary sDDGS, but xylanase supplementation tended (P=0.09) to counter this effect.

The total NSP profile showed that at 20 and 30% sDDGS levels, there were significant increases in xylose and glucose concentrations in the ileum, whereas arabinose and ribose concentrations were not affected. At the 30% inclusion rate, ileal rhamnose and fucose levels tended to increase.

Table 4.4 Effects of increasing levels of sDDGS and xylanase on tissue protein content, activities of pancreatic enzymes, nutrient digestibility and ileal viscosity of 21-day-old birds

sDDGS (g/kg)	Xylanase	Protein [1]	Chymotrypsin[2]	Lipase[3]	Starch digestibility	Protein digestibility	Viscosity (mpa.s[4])
0	-	22.7	0.23	0.04	0.93	0.785[a]	2.42
	+	20.0	0.21	0.04	0.92	0.796[a]	2.18
100	-	24.8	0.23	0.03	0.95	0.751[ab]	2.67
	+	21.1	0.29	0.03	0.91	0.753[ab]	2.53
200	-	27.5	0.17	0.03	0.91	0.705[bcd]	2.70
	+	21.8	0.23	0.03	0.93	0.724[bc]	2.57
300	-	24.6	0.25	0.04	0.92	0.669[d]	3.07
	+	24.6	0.31	0.04	0.90	0.694[cd]	2.46
	SEM	*1.03*	*0.02*	*0.002*	*0.004*	*0.0061*	*0.086*
Source of variation		----------	----------	*Probability*	----------	----------	----------
sDDGS		NS	NS	NS	NS	<0.001	0.06
Xylanase		NS	NS	NS	NS	NS	0.09
sDDGS ×xylanase		NS	NS	NS	NS	NS	NS

[1] Concentration in wet tissue (mg/g)

[2] Specific activity (μmol *p*-nitroaniline / mg protein per min)

[3] Units per min according to porcine lipase standard

[4] Centipoise, cp = 1/100 dyne second per square centimetre

[a] Mean values on the same column not sharing a superscript are significantly different (P<0.05).

NS: Not significant, SEM: Standard error of mean

Table 4.5 Effects of graded levels of DDGS and xylanase on non-starch polysaccharides composition of the ileum (g/kg) in 21-day-old birds

Treatment	Xylanase	Sugars								
sDDGS (g/kg)		Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	Total NSP
0	-	2.5[a]	6.4[a]	2.4	83.6	72.4[c]	9.9[c]	64[a]	51[c]	259
	+	2.3[ab]	6.0[ab]	2.2	75.4	67.4[c]	10.5[c]	61[a]	76[b]	267
100	-	2.1[bc]	5.2[bc]	2.4	76.7	76.8[bc]	13.3[b]	53[b]	87[ab]	281
	+	1.9[cd]	4.8[cd]	2.3	70.5	69.2[c]	12.9[b]	50[bc]	91[ab]	270
200	-	1.6[ef]	4.2[d]	2.4	74.9	85.1[ab]	15.9[a]	44[c]	104[a]	295
	+	1.6[de]	4.3[cd]	2.4	74.1	75.0[bc]	15.7[a]	45[c]	103[a]	285
300	-	1.3[fg]	3.2[e]	2.3	71.7	89.9[a]	16.3[a]	35[d]	107[a]	290
	+	1.2[g]	3.2[e]	2.4	71.6	77.7[bc]	15.8[a]	32[d]	100[a]	271
	SEM	*0.034*	*0.113*	*0.047*	*1.441*	*1.293*	*0.184*	*0.954*	*2.806*	*4.06*
Source of variation		----------*Probability*----------								
sDDGS		<0.0001	<0.0001	NS	0.0769	<0.001	<0.0001	<0.0001	<0.0001	0.0772
Xylanase		NS	NS	NS	NS	<0.001	NS	NS	NS	NS
sDDGS × xylanase		NS	NS	NS	NS	NS	NS	NS	NS	NS

[a] Mean values on the same column not sharing a superscript are significantly different (P<0.05).
NS: Not significant, SEM: Standard error of mean

Irrespective of sDDGS inclusion, supplementation of the diets with xylanase markedly reduced (P<0.001) xylose concentration in the ileal digesta. The response to xylanase was, however, only significant (P<0.001) at the highest inclusion of sDDGS.

4.3.3 Digestive enzyme activities

The protein content of the jejunal mucosa was increased (P<0.01) by xylanase supplementation, but this was only significant in chicks in HDDG group. Activities of maltase (P<0.001) and sucrase (P<0.01) in the jejunum declined as dietary DDGS rose to 20 % and beyond. Alkaline phosphatase was, however, unaffected by the levels of sDDGS in the diets. In addition, there was no significant influence of enzyme supplementation on sucrase, maltase and alkaline phosphatase activities in the jejunal mucosa. Neither dietary sDDGS nor enzyme supplementation affected protein content and activities of pancreatic enzymes.

Table 4.6 Effects of increasing levels of sDDGS and xylanase on tissue protein content and activities of digestive enzymes in jejunum of 21-day-old birds

sDDGS (g/kg)	Xylanase	Protein[1]	Maltase[2]	Sucrase[2]	Alkaline phosphatase[3]
0	-	34.5^{bc}	0.94^{a}	0.072^{a}	0.49
	+	34.6^{bc}	0.84^{ab}	0.066^{ab}	0.52
100	-	34.8^{bc}	0.65^{bc}	0.048^{bc}	0.51
	+	43.6^{ab}	0.65^{bc}	0.048^{bc}	0.40
200	-	39.7^{abc}	0.38^{d}	0.042^{c}	0.42
	+	43.5^{ab}	0.38^{d}	0.052^{abc}	0.44
300	-	32.4^{c}	0.47^{cd}	0.050^{bc}	0.55
	+	44.9^{a}	0.33^{d}	0.046^{bc}	0.38
	SEM	*1.14*	*0.03*	*0.0026*	*0.022*
Source of variation		----------*Probability*----------			
sDDGS		NS	<0.001	<0.01	NS
Xylanase		0.01	NS	NS	NS
sDDGS × xylanase		NS	NS	NS	NS

[1]Concentarion in mucosa (mg/g)

[2] Specific activity (µmol glucose/mg protein per min).

[3]Specific activity (µmol *p*-nitrophenol/mg protein per min).

[a] Mean values on the same column not sharing a superscript are significantly different (P<0.05).

NS: Not significant, SEM: Standard error of mean

4.3.4 Fermentation in caeca

The concentration of SCFA in the caeca of birds at d 21 is shown in Table 4.7. Results showed that the total caecal SCFA concentration was increased by dietary inclusion of sDDGS but the effect was only significant (P<0.01) when the level of sDDGS rose to 20 and 30 % of the diet. Irrespective of the sDDGS level in the diet, there was no effect of xylanase inclusion on total organic acid concentration in the caeca. A similar effect was observed for the butyric (P<0.01) and acetic acids (P<0.001) in the caecal digesta where inclusion of DDGS increased the concentration.

In contrast, the concentration of propionic acid decreased (P<0.01) as sDDGS increased to 30 % compared to birds fed the control diet. However, enzyme supplementation had no effect on the concentrations of propionic and acetic acids in caecal content. The butyric acid concentration in the caeca was higher (P<0.05) when xylanase was added to the diets while the concentrations of acetic acid tended (P=0.10) to increase.

Table 4.7 Concentration of SCFA in the caeca of broiler chickens fed on diets containing increasing levels of sDDGS with or without xylanase supplementation

sDDGS (g/kg)	Xylanase	Caecal SCFA concentration (µmol/g digesta)			
		Acetic acid	Propionic acid	Butyric acid	Total SCFA
0	-	52.6^{c}	8.4^{abc}	6.1^{cd}	80.1^{d}
	+	67.4^{bc}	10.0^{ab}	7.9^{bcd}	94.6^{bcd}
100	-	67.3^{bc}	10.0^{ab}	5.4^{d}	87.3^{cd}
	+	77.4^{ab}	10.6^{a}	9.2^{abcd}	101.8^{abc}
200	-	87.4^{a}	8.1^{bc}	11.1^{ab}	114.9^{a}
	+	86.4^{a}	7.2^{c}	12.2^{a}	112^{ab}
300	-	75.7^{ab}	8.1^{bc}	9.3^{abc}	101.8^{abc}
	+	78.9^{ab}	7.5^{c}	11.4^{ab}	104.4^{abc}
	SEM	*1.85*	*0.27*	*0.46*	*2.34*
Source of variation		----------*Probability*----------			
sDDGS		<0.001	<0.05	<0.01	<0.01
Xylanase		NS	NS	<0.05	NS
sDDGS × xylanase		NS	NS	NS	NS

[a] Mean values on the same column not sharing a superscript are significantly different (P<0.05).
NS: Not significant, SEM: Standard error of mean

4.4 DISCUSSION

4.4.1 Bird performance

In corroboration with the results obtained by Olukosi *et al.* (2010) and Wang *et al.* (2008), the present study showed an increase in feed intake when DDGS were included at high levels. Similarly, Youssef *et al.* (2008) showed higher feed intake in birds fed a 15 % DDGS diet compared to a control group during 12-35 days of feeding. This result, however, was in contrast to that of Thacker and Widyaratne (2007) who found no effect of wDDGS on feed intake and performance of broilers at up to 20 % inclusion.

Distillers' dried grains with solubles contain a very high level of NSP, which are not digested by the endogenous enzymes of birds, so that birds eat more to overcome the dilution effect of the NSP in order to satisfy their energy and nutrient needs. Although diets were formulated to be isoenergetic, the difference in feed intake suggests that the value of AME for the DDGS and therefore diets have been different than calculated values. Therefore, such difference may also be responsible for the effect on growth parameters. Another possible explanation may lie in the development of the small intestine as well as the gizzard shown in the birds fed diets containing large amounts of sDDGS in the present study. A more developed gut with a better functioning gizzard may have facilitated an increased intake of feed. Indeed, the birds were markedly heavier during the last 2 weeks of the current study. Similar findings have been reported by other authors (Olukosi *et al.*, 2010; Oryschak *et al.*, 2010b; Shim *et al.*, 2011). The NSP present in DDGS are mainly cellulosic and arabinoxylans in nature (Choct and Petersen, 2009), which are less prone to degradation by exogenous enzymes. However, in the current study, xylanase supplementation improved FCR in birds fed the highest level of DDGS inclusion. Metayar *et al.* (2009) reported a 4 and 5 % reduction in FCR when broilers were fed diets containing 10 and 15 %, sDDGS, respectively, with enzyme supplementation. As observed in this study, xylanase supplementation markedly reduced luminal xylose concentration, indicating that the enzyme had an impact on the arabinoxylan structure of the NSP. Perhaps this may have led to partial destruction of the cell walls, which encapsulated nutrients such as protein. Furthermore, a significant level of simple sugars (*i.e.*, 2-12 sugar units) was reported to be present in DDGS (Choct and Petersen, 2009), which may have been degraded by the enzyme to yield monomeric carbohydrates for absorption in the intestine.

Overall, the results of the experiments on DDGS in literature are contradictory. For instance, Olukosi *et al.* (2010) observed improvement in all performance parameters when 10% DDGS was included in the diet of broiler chickens. They postulated that several factors, such as higher N intake, fermentation in the hind gut and possibly the high fat content of the diets containing DDGS may contribute to better performance. It is clear from the literature that DDGS are a highly variable ingredient and their composition depends largely on the processing conditions under which they were produced. Thus, there is no surprise that the nutritional value of DDGS in poultry will also vary accordingly. The current study, however, has investigated sorghum DDGS, which to the author's knowledge, is the first such paper and hence, no direct comparison can be made as to the variability or otherwise of sDDGS in broiler chickens.

4.4.2 NSP, viscosity and digestive enzyme activities

As previously mentioned, DDGS contain high levels of NSP, in particular arabinoxylans, which are known to contribute to poor performance via possible increase in digesta viscosity, encapsulation of nutrients, and alteration in the gut microflora. All these can lead to poor digestion and impaired absorption of nutrients in chickens. For example, the tendency observed for the viscosity of the ileal digesta is in agreement with the results reported by Loar *et al.* (2010). In addition to viscosity, the poorer feed efficiency observed by increasing level of DDGS with no enzyme supplementation may be attributed to the presence of NSP, lower protein digestibility and ostensibly lower activity of digestive enzymes. It is known that the last step of digestion of most dietary fractions, including carbohydrates and protein, is limited by membrane-bound enzymes in the small intestine (Iji 1999).

In the present study, the maltase and sucrase activities were markedly reduced at 20 and 30 % sDDGS inclusion, which may be attributable to the presence of NSP physically complexing with intestinal enzymes and causing a reduction in nutrient digestion (Ikeda and Kusano, 1983). Furthermore, a possible restriction to the amount of substrates in the mid and lower parts of the intestine for pancreatic enzymes may reduce the activities of the intestinal enzymes, especially disaccharidase, through proteolysis (Tivey and Shulman, 1991). It is also possible that a high level of fermentation end-products may impair the activities of carbohydrases (Iji, 1999). The foregoing may account for the impaired activities of sucrase

and maltase observed in broilers fed high levels of sDDGS (20 and 30 %) in the current study.

4.4.3 Nutrient unitisation

There are quite a few reports on N retention in poultry fed maize DDGS. Leytem *et al.* (2008) found a linear decrease in apparent retention of N with increasing DDGS inclusion in broiler diets. They also reported a 19 % decrease in N digestibility at a 20 % inclusion rate compared to a negative control group. The results of the present study agree with their findings, where protein digestibility was reduced. This is expected as sorghum cell walls exist in a tightly packed matrix, encapsulating nutrients including protein and starch (Selle *et al.*, 2010). The protein in sorghum is characterized by a high level of kafirin, which are highly resistant to proteolysis in poultry (Selle *et al.*, 2010). The partial improvement of protein digestibility observed in the current study as a result of enzyme addition seems to suggest that the enzyme partially destroyed the cell wall architecture, making the encapsulated nutrients readily exposed to digestion.

The lack of difference between treatments in term of starch digestibility may be attributed to the low soluble NSP content of DDGS (Choct and Peterson 2009).

4.4.4 Fermentation in caeca

It has been well documented that the caeca are the primary site of fermentation in the avian gut with SCFA as the major end-products. These acids are produced mainly due to degradation of NSP by exogenous enzymes as well as enzymes produced by the caecal microflora (Jamroz *et al.*, 2002; Jozefiak *et al.*, 2004). Thus far, no data are available on the effect of sDDGS on fermentation end-products in broilers. However, it has been shown that different cereals, including rye, triticale and wheat, may significantly influence the fermentation process in broilers whereas enzymes appear to be not as important as cereal type (Jozefiak *et al.*, 2007). In the present study, the inclusion of a xylanase in the diet only had an impact on butyric acid concentration while all the other SCFA were unaffected by increasing levels of sDDGS. Nevertheless, Choct *et al.* (1996; Choct *et al.*, 1999) showed an increase in total VFA in the caeca by the addition of a xylanase to a wheat-based diet.

Similarly, a 41 % increase in SCFA was found by Jamroz *et al.* (1996) in birds fed on a triticale-based diet supplemented with a xylanase. The present results did not establish a significant effect of xylanase on total organic acid concentrations, which is in agreement with results reported by Lazaro *et al.* (2003) and Jozefiak *et al.* (2007). The modes of action of enzymes, xylanase in particular, have been extensively explained to be a multifaceted mechanism where viscosity reduction effected by partial degradation of soluble NSP, nutrient release by partial destruction of insoluble cell wall architecture, and modulation of microbial activities via release of low molecular weight carbohydrates can all play a part in alleviating the adverse effects of NSP on nutrient digestion and absorption in non-ruminant animals (Choct *et al.*, 1996; Choct *et al.*, 1999; Silva and Smithard, 2002).

4.5 CONCLUSION

It is concluded from this study that high levels of sDDGS inclusion up to 30 % in broilers are feasible with the supplementation of appropriate enzymes. Enzyme supplementation may particularly be useful during the starter phase of feeding. However, the ability of a xylanase to totally overcome the negative impact of high DDGS in broiler diets is still limited. The source and activity level of the xylanase may also have been responsible for the results obtained and different outcomes will be possible using a different xylanase. Further studies are required to examine various cocktails of carbohydrases tailored for the substrates in terms of activities and affinities in order to degrade a substantial amount of the NSP present in DDGS as an energy source.

CHAPTER 5

TESTING THE SYNERGISTIC EFFECTS OF PROTEASE AND XYLANASE ON SORGHUM DISTILLERS' DRIED GRAINS WITH SOLUBLES FOR BROILER CHICKENS

Abstract

Individual or combined effects of xylanase and protease on the nutritive value of diets containing sorghum distillers dried grains with solubles (sDDGS) in broiler chickens were investigated. A total of 480 day-old male broiler chickens were assessed in a 3×2×2 factorial design (0, 150 or 300 g sDDGS/kg diet, with or without xylanase, and with or without protease). Each of the 12 treatments was replicated 5 times, accommodating 8 birds per replicate. Feed intake and body weight gain (BWG) of the birds were increased by inclusion of sDDGS to the diets independent of enzyme supplementation. Feed conversion ratio (FCR) deteriorated with the incorporation of sDDGS into the diets at both levels. Protease improved feed consumption and BWG of the birds across the 21 d study. Regardless of sDDGS and protease, xylanase significantly improved FCR at any period of the trial. Digestibility of protein and most amino acids was adversely affected by the inclusion of 150 and 300 g/kg sDDGS. While protease individually improved amino acid digestibility in birds offered diets containing the highest amount of sDDGS (300 g/kg), an admixture of xylanase and protease did not result in further improvement in amino acid digestibility. Addition of xylanase reduced the concentration of insoluble non-starch polysaccharides (NSP) in the ileum. Noticeably, the response of birds to xylanase supplementation on the concentrations of arabinose, xylose and total insoluble NSP was compromised when xylanase and protease were added to the diet simultaneously. To conclude, the application of xylanase and protease in combination was beneficial for the performance of the birds fed sDDGS, in particular FCR.

5.1 INTRODUCTION

The results from the preceding experiment (Chapter 4), revealed a lower protein digestibility when diets contained sDDGS, which also contributed to the observed poor feed conversion in the birds receiving diets containing 30% DDGS. Similar observations were reported by Olukosi *et al.* (2010) when 100 g/kg mDDGS was incorporated to the diets of broiler chickens. It has also been widely documented that DDGS has a lower amino acid (AA) digestibility in poultry compared to the parent grain. The reason for this variation in AA digestibility is most likely due to the effect of the various processing and drying techniques involved in the production of DDGS, and inherent chemical composition of the material. Bandegan *et al.* (2009) found that all AA digestibility estimates for standardized and apparent coefficients of wheat DDGS were lower in broiler chickens compared to figures for wheat as a main grain with lysine the least digestible amino acid. In another study, Kim *et al.* (2010) showed higher AA digestibility for corn germ than for both conventional and high-protein DDGS samples in broiler chickens. This poor AA availability is further exacerbated when sorghum is used as the main grain owing to its low protein digestibility. A substantial portion of sorghum protein is composed of kafirin, which is known to negatively impact digestibility of AA due to resistance to proteolysis (Selle *et al.*, 2010). Kafirin resistance to pepsin hydrolysis is further compounded by the lack of improvement in protein digestibility when sorghum is treated with moist heat or cooked (Oria *et al.*, 1995). This considered, application of exogenous protease appears to be a logical approach to enhancing protein, and hence amino acid digestibility of DDGS.

In general, protease has been primarily used in combination with other enzymes to improve performance of broiler chickens (Marsman *et al.*, 1995; Shapiro and Nir, 1995; Cowieson and Adeola, 2005; Cowieson *et al.*, 2006; Cowieson and Ravindran, 2008; Tiwari *et al.*, 2010) although there exist few reports on the individual application of protease in broiler diets containing DDGS. In the study conducted by Olukosi *et al.* (2010) a mixture of carbohydrases and protease was not effective in improving the performance of birds fed DDGS-containing diets although it improved the performance of the control birds, which were fed a low-density diet. Irrespective of the inclusion of DDGS, there are some contradictory reports with regard to the influence of protease on broiler performance. Recently, Kalmendal and Tauson (2012) observed an improvement in feed conversion ratio in broilers fed diets supplemented with xylanase and protease individually or in combination

with no sub-additive effect of the two. Using a mono component protease, live performance and CP digestibility in broilers were also restored (Angel *et al.*, 2011). Simbaya *et al.* (1996) ascribed a synergistic effect of protease with carbohydrase and phytase on growth of broilers over 4-11 days of age. Similarly, Ghazi *et al.* (2003) reported an improvement in nutritive value of soybean meal when protease was added to broiler diets. However, when protease and α-glucosidase were used simultaneously, the responses in terms of TME and nitrogen digestibility of the birds were compromised compared to individual application of either enzyme.

The NSP content remains the primary concern for using DDGS in broiler diets, regardless of the source grain. In addition, for sorghum DDGS, there is clear evidence of poor protein digestion. Therefore, the present study was conducted to test the effect of protease and xylanase supplementation and the synergistic effect of both enzymes in diets containing different levels of DDGS for broiler chickens.

5.2 MATERIALS AND METHODS

5.2.1 Experimental design and diets

A 3×2×2 factorial design was employed in the present study to test the effect of varying levels of sDDGS, xylanase, protease and their interactions. All diets were formulated to meet the minimum requirements for Cobb 500 broilers. Three levels of dietary sDDGS were formulated at 0, 150 and 300 g/kg, partly replacing corn, soybean meal and soy protein concentrate in the basal diet. The composition of the experimental diets is shown in Table 5.1.

Protein and energy contents of the experimental diets were calculated and maintained at the same level of 22.48 % and 13 MJ/kg, respectively. Two levels of xylanase supplementation, with or without xylanase, were created by adding Ronozyme WX (DSM Nutritional Products Australia Pty Ltd) at 0 and 0.25 g per kg of diets. Protease (Ronozyme ProAct) was also supplemented at 0 and 0.2 g per kg of experimental diets. To measure digestibility of nutrients, all experimental diets were supplemented with 0.5 % (w/w) of titanium dioxide (TiO_2) (Sigma-Aldrich) as an indigestible marker. All diets were prepared, mixed and pelleted at the University of New England.

Table 5.1 Ingredients and calculated nutrient compositions of experimental basal diets (g/kg)

Ingredients (g/kg)	Experimental basal diets (sDDGS g/kg)		
	Control (0)	150	300
Maize	580.5	511.9	436.6
Soy bean meal dehulled, 47.5 % CP	262.0	178.9	105.0
Soy co meal, 65% CP	80.0	80.0	75.0
Sorghum DDGS	0.0	150.0	300.0
Canola oil	28.7	29.9	33.0
Sodium chloride	4.5	3.0	1.8
Choline Chloride	0.4	0.4	0.4
Dicalcium phosphate	21.5	19.0	18.0
Limestone	12.3	13.8	14.6
Vitamin and mineral premix [1]	2.5	2.5	2.5
DL-Methionine	2.3	2.4	2.5
L-Lysine HCL	0.3	2.8	5.2
Threonine	0.0	0.4	0.8
Titanium oxide	5.0	5.0	5.0
Total	1000	1000	1000
Calculated nutrient composition (g/kg)			
ME (MJ/kg)	12.98	12.98	12.98
CP	225.7	225.7	225.8
Calcium	10.4	10.4	10.5
Available phosphorus	5.2	5.2	5.3
Lysine	12.9	12.9	12.8
Methionine	5.8	5.8	5.8
Methionine and cystine	9.4	9.4	9.3
Threonine	8.8	8.9	8.9
Tryptophan	2.9	2.6	2.2
Sodium	2.0	2.1	2.1
Chloride	3.0	2.9	2.9

[1]Contained vitamins and minerals in milligrams per kilogram of diet as follow: vitamin A (as all-trans retinol), 12,000 IU; cholecalciferol, 3,500 IU; vitamin E (as d-α-tocopherol), 44.7 IU; vitamin B_{12}, 0.2 mg; biotin, 0.1 mg; niacin, 50 mg; vitamin K_3, 2 mg; pantothenic acid, 12 mg, folic acid, 2 mg; thiamine, 2 mg; riboflavin, 6 mg; pyridoxine hydrochloride, 5 mg; D-calcium panthothenate, 12 mg; Mn, 80 mg; Fe, 60 mg; Cu, 8 mg; I, I mg; Co, 0.3 mg; and Mo, 1 mg.

5.2.2 Housing and general management

A total of 480 male day-old Cobb 500 broiler chickens, vaccinated for Marek's disease and infectious bronchitis, were collected from a commercial hatchery (Baiada hatchery, Tamworth, NSW, Australia). Sixty multi-tiered brooder cages (42 × 75 × 25 cm) with wire floor were used to house the birds in climate-controlled rooms. In this experiment, the cages

were randomly assigned to each of twelve treatments, each replicated five times, with 8 birds per replicate.

Temperature was set at 33-34º C on the first day of the experiment and then gradually decreased by 1º C every second day until a stable temperature of 24º C was reached by 21 days. A lighting program of 18h light and 6h darkness was maintained throughout the trial except the first day when birds had 24 h of light. Birds had access to feed and water *ad libitum* from a waterer and a feeding trough attached to each cage.

5.2.3 Performance parameters and protein efficiency ratio

Feed consumption and body weight were recorded on a cage basis at weekly intervals. Feed conversion ratio (FCR), corrected for mortality, was then calculated. The body weight gain of the birds was expressed as a fraction of actual crude protein intake to calculate protein efficiency ratio (PER).

5.2.4 Sample collection and processing

One bird on d 7 and three birds on d 21 per replicate were randomly selected, weighed and subsequently euthanased by cervical dislocation. The gastrointestinal tract excised; the small intestine was divided into three segments; the duodenum (from the gizzard outlet to the end of the pancreatic loop), the jejunum (from the end of pancreatic loop to the Meckel's diverticulum), and the ileum (from the Meckel's diverticulum to 3 cm above the ileo-caecal junction). The length and weight of each segment of the small intestine were measured. The proventriculus, gizzard, liver, pancreas, spleen and bursa were also weighed. The ileal contents of sampled birds were collected by gentle squeezing of the ileum into ice-cold plastic containers, and pooled by replicate cage.

5.2.5 Nutrient digestibility and viscosity of ileal digesta

Protein, dry matter, starch, amino acids, insoluble NSP and free sugar contents of ileal digesta were determined as described in Sections 3.2.2, 3.2.3, 3.2.6 and 3.2.5, respectively. Ileal viscosity was measured as described in Section 4.3.2. Titanium content and subsequent

digestibility coefficients for different nutrients were also determined as described in Section 4.2.4.1.

5.2.6 Animal ethics

All the experimental procedures were approved by the Animal Ethics Committee of the University of New England with approval number AEC 09/173.

5.2.7 Statistical analysis

The twelve treatments of 3 diets (control, 15 and 30 % DDGS), 2 levels of xylanase (with or without) and protease (with or without) in a 3×2×2 factorial arrangement were subjected to statistical analysis using 3-way ANOVA of GLM procedure of SAS (SAS/STAT Version 9.1, SAS Institute, 2003) to assess the main effects and 2 or 3-way interactions. Data were checked for normal distribution prior to conducting statistical analyses. If a significant effect was detected, differences among treatments were separated by the Fisher's Least Significant Differences test. All statements of significance are considered on a P-value less than 0.05 unless otherwise specified. Due to high number of treatments, only values for the treatment effects were presented in the tables.

5.3 RESULTS

5.3.1 Bird performance

The performance of the birds is summarised in Table 5.2. Dietary inclusion of sDDGS increased (P<0.001) FI and impaired (P<0.001) FCR of the birds during the first week as well as a tendency (P=0.08) to improve BWG. At the same time, there were no significant differences in FI or BWG between birds received 15 % and 30 % sDDGS. Birds given diets containing sDDGS also had higher FI (P<0.001) and BWG (P<0.001) and poorer FCR (P<0.001) when calculated across the 21 d study. Despite the lack of enzyme effect in the first week, supplementation of xylanase alone reduced (P<0.01) feed consumption and improved (P<0.01) FCR when assessed for the entire period of study (d 1 to 21). At the same time, independent of xylanase and sDDGS, birds fed diets supplemented with

Table 5.2 Effects of xylanase, protease individually or in combination and dietary levels of sDDGS on growth performance of birds at the starter phase of feeding

Treatments			Feed intake (g/bird)		Body weight gain (g/bird)		Feed Conversion Ratio	
sDDGS (g/kg)	Xylanase	Protease	d1-7	d1-21	d1-7	d1-21	d1-7	d1-21
0	-	-	125.5	1020.8	103.7	732.8	1.21	1.39
	+	-	123.9	960.1	99.6	713.9	1.25	1.35
	-	+	129.4	1103.9	102.3	764.6	1.26	1.45
	+	+	131.0	1068.4	105.1	769.4	1.25	1.39
150	-	-	141.7	1249.0	107.6	844.7	1.33	1.48
	+	-	137.0	1220.8	107.2	844.8	1.28	1.45
	-	+	147.6	1289.5	112.4	899.5	1.32	1.44
	+	+	147.1	1235.9	110.0	877.2	1.29	1.41
300	-	-	141.6	1293.4	103.4	820.7	1.42	1.58
	+	-	148.3	1285.4	107.4	844.3	1.39	1.52
	-	+	146.7	1297.7	101.9	865.2	1.45	1.50
	+	+	143.5	1228.8	100.5	838.1	1.43	1.47
SEM			*1.285*	*6.537*	*1.146*	*7.409*	*0.007*	*0.011*
Main effects and interactions			----------*Probability*----------					
sDDGS			<0.001	<0.001	0.08	<0.001	<0.001	<0.001
Xylanase			NS	<0.01	NS	NS	NS	<0.01
Protease			NS	<0.05	NS	<0.01	NS	NS
sDDGS × xylanase			NS	NS	NS	NS	NS	NS
sDDGS × protease			NS	<0.01	NS	NS	NS	<0.05
Xylanase × protease			NS	NS	NS	NS	NS	NS
sDDGS × xylanase × protease			NS	NS	NS	NS	NS	NS

Each value correspondent to each treatment group represents the mean of 5 replicates.
NS: Not significant, SEM: Standard error of mean
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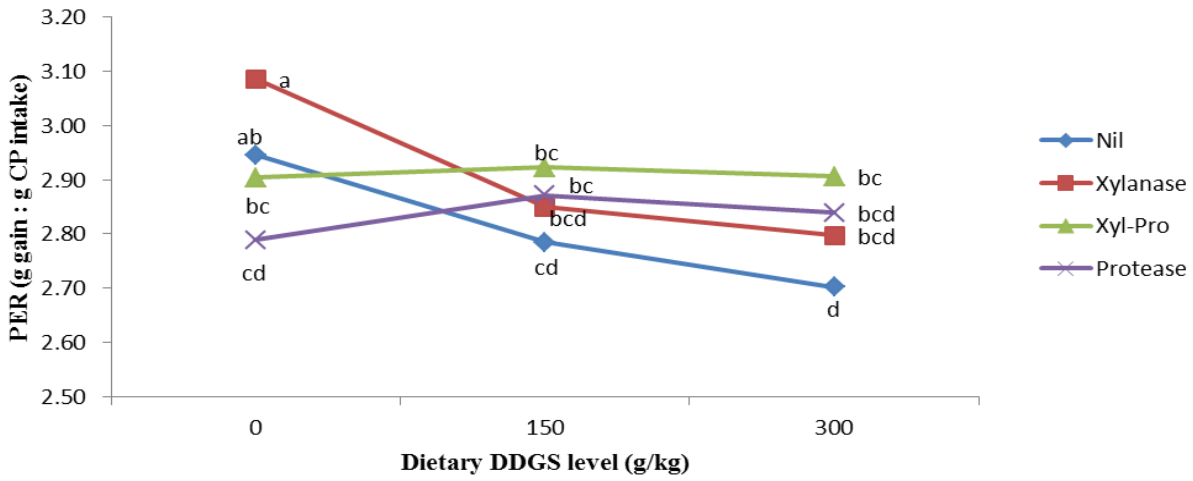

Figure 5.1 Effect of dietary DDGS, xylanase and protease on protein efficiency ratio (PER) of birds from d 1 to 21

protease consumed more feed (P<0.05) and had higher (P<0.01) BWG compared to those birds fed protease-free diets. No effect of protease on FCR of the birds was observed.

In terms of feed consumption, a significant interaction (P<0.01) attributed to sDDGS inclusion and protease was observed over the entire period of study where protease increased feed intake with 15 % sDDGS inclusion but remained unchanged with 30 % sDDGS. There was also an interaction of DDGS x protease for FCR of the birds from d 1 to 21. Application of xylanase and protease together did not reveal significant synergy for the growth performance of the birds.

The effects of sDDGS, xylanase, protease and the combination of the two on PER are presented in Figure 5.1. Independently, inclusion of sDDGS reduced (P<0.05) PER only in the birds given 30 % DDGS. Xylanase supplementation improved (P<0.01) PER regardless of sDDGS and protease. In addition, a significant interaction (P<0.001) between sDDGS and protease was observed for d 1-21. Supplementation of xylanase and protease together increased (P<0.05) PER only in birds fed diet with 30 % DDGS. However, the application of either enzyme individually did not exhibit such an effect.

Table 5.3 Effects of xylanase, protease individually or in combination and dietary levels of sDDGS on relative weight of visceral organs of broilers at 7 and 21 days of age[1]

Treatments			Proventriculus		Gizzard		Liver		Pancreas		Spleen		Bursa	
sDDGS (g/kg)	Xylanase	Protease	7 d	21 d	7 d	21 d	7 d	21 d	7 d	21 d	7 d	21 d	7 d	21 d
			g/100g body weight											
0	-	-	0.67	0.59	3.05	1.52	3.98	3.39	0.43	0.43	0.11	0.09	0.13	0.15
	+	-	0.86	0.51	3.37	1.61	4.10	3.42	0.45	0.30	0.11	0.09	0.18	0.16
	-	+	0.90	0.53	3.11	1.95	4.33	3.65	0.48	0.39	0.11	0.10	0.12	0.19
	+	+	0.83	0.49	3.33	1.53	4.78	3.36	0.47	0.34	0.12	0.11	0.11	0.15
150	-	-	0.92	0.49	3.81	1.75	4.59	3.40	0.51	0.39	0.10	0.11	0.15	0.23
	+	-	0.90	0.55	3.66	1.94	4.58	3.29	0.44	0.35	0.10	0.08	0.15	0.23
	-	+	0.98	0.52	3.65	1.61	4.18	3.15	0.45	0.33	0.10	0.08	0.14	0.25
	+	+	1.03	0.52	3.72	1.53	4.03	3.59	0.47	0.30	0.08	0.08	0.14	0.20
300	-	-	1.00	0.53	3.80	1.89	4.65	3.43	0.44	0.32	0.09	0.07	0.16	0.25
	+	-	0.97	0.58	3.93	1.76	4.72	3.50	0.45	0.32	0.08	0.08	0.15	0.22
	-	+	0.95	0.55	3.79	1.89	4.78	3.65	0.47	0.34	0.08	0.08	0.19	0.20
	+	+	1.00	0.49	4.12	1.67	4.74	3.54	0.48	0.31	0.09	0.08	0.15	0.21
SEM			*0.016*	*0.010*	*0.054*	*0.032*	*0.055*	*0.049*	*0.008*	*0.008*	*0.003*	*0.002*	*0.005*	*0.006*
Main effects and interactions			*Probability*											
sDDGS			<0.001	NS	<0.001	<0.01	<0.01	NS	NS	0.10	<0.05	NS	NS	<0.001
Xylanase (Xyl)			NS	NS	NS	NS	NS	NS	NS	<0.01	NS	NS	NS	NS
Protease (Pro)			0.08	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
sDDGS × xylanase			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
sDDGS × protease			NS	NS	NS	<0.05	<0.01	NS	NS	NS	NS	NS	NS	NS
Xylanase × protease			NS	NS	NS	<0.05	NS	NS	NS	NS	NS	NS	NS	NS
sDDGS × xylanase × protease			0.07	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value correspondent to each treatment group represents the mean of 5 replicates.
NS: Not significant, SEM: Standard error of mean

Table 5.4 Effects of xylanase, protease individually or in combination and dietary levels of sDDGS on relative weight of small intestinal regions at 7 and 21 days of age[1]

Treatments			Relative weight (g/100g BW)							
sDDGS (g/kg)	Xylanase	Protease	Duodenum		Jejunum		Ileum		Small intestine	
			Day 7	Day 21	Day 7	Day 21	Day 7	Day 21	Day 7	Day 21
0	-	-	1.86	1.04	2.29	1.35	2.06	1.00	6.21	3.40
	+	-	1.95	0.98	2.42	1.34	2.18	0.94	6.55	3.26
	-	+	1.94	1.04	2.32	1.56	1.99	0.97	6.25	3.57
	+	+	2.09	0.88	3.05	1.47	2.23	0.96	7.36	3.31
150	-	-	2.37	1.17	2.92	1.70	2.50	1.14	7.79	4.01
	+	-	2.28	1.06	2.99	1.43	2.32	1.07	7.58	3.57
	-	+	2.43	1.00	2.99	1.61	2.61	1.16	8.03	3.76
	+	+	2.66	0.98	2.85	1.52	2.60	1.07	8.11	3.57
300	-	-	2.20	1.13	3.33	1.76	2.44	1.15	7.97	4.03
	+	-	2.41	1.12	3.19	1.63	2.89	1.11	8.50	3.87
	-	+	2.22	1.15	3.10	1.67	2.31	1.22	7.63	4.03
	+	+	2.36	1.08	3.18	1.69	2.62	1.22	8.16	3.99
SEM			*0.067*	*0.025*	*0.081*	*0.025*	*0.085*	*0.014*	*0.204*	*0.051*
Main effects and interactions			*Probability*							
sDDGS			<0.01	NS	<0.01	<0.001	0.08	<0.001	<0.01	<0.001
Xylanase (Xyl)			NS	NS	NS	0.07	NS	NS	NS	<0.05
Protease (Pro)			NS	NS	NS	NS	NS	NS	NS	NS
sDDGS × xylanase			NS	NS	NS	NS	NS	NS	NS	NS
sDDGS × protease			NS	NS	NS	NS	NS	NS	NS	NS
Xylanase × protease			NS	NS	NS	NS	NS	NS	NS	NS
sDDGS × xylanase × protease			NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value correspondent to each treatment group represents the mean of 5 replicates.
NS: Not significant, SEM: Standard error of mean

Table 5.5 Effects of xylanase, protease individually or in combination and dietary levels of sDDGS on relative length of small intestinal regions at 7 and 21 days of age[1]

Treatments			Relative length (g/100g body weight)							
sDDGS (g/kg)	Xylanase	Protease	Duodenum		Jejunum		Ileum		Small intestine	
			Day 7	Day 21	Day 7	Day 21	Day 7	Day 21	Day 7	Day 21
0	-	-	9.8	2.95	23.2	7.12	23.9	7.0	35.9	38.1
	+	-	11.9	3.21	27.1	6.76	28.5	6.9	42.2	42.2
	-	+	11.3	3.09	25.7	7.03	24.6	7.3	40.1	38.9
	+	+	11.7	2.88	26.8	6.54	26.1	6.8	41.4	39.4
150	-	-	11.9	2.98	24.3	6.62	24.3	6.8	39.2	37.8
	+	-	11.7	2.80	26.8	6.17	26.2	6.8	41.4	39.2
	-	+	11.8	2.76	25.4	6.25	27.1	6.7	40.1	40.1
	+	+	13.2	2.77	26.9	6.40	26.1	6.8	43.0	39.3
300	-	-	12.5	2.80	27.4	6.58	24.5	6.9	42.7	38.0
	+	-	12.5	2.51	27.2	6.28	27.6	6.4	42.2	40.4
	-	+	11.1	3.10	24.5	7.23	24.1	7.2	38.8	38.6
	+	+	12.5	2.67	27.9	6.23	28.1	6.6	43.1	40.9
SEM			*0.30*	*0.043*	*0.52*	*0.080*	*0.48*	*0.09*	*0.791*	*0.507*
Main effects and interactions			*Probability*							
sDDGS			NS	<0.05	NS	<0.05	NS	NS	NS	NS
Xylanase (Xyl)			NS	NS	NS	<0.05	<0.05	NS	0.09	NS
Protease (Pro)			NS	NS	NS	NS	NS	NS	NS	NS
sDDGs × xylanase			NS	NS	NS	NS	NS	NS	NS	NS
sDDGs × protease			NS	NS	NS	NS	NS	NS	NS	NS
Xylanase × protease			NS	NS	NS	NS	NS	NS	NS	NS
sDDGS × xylanase × protease			NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value correspondent to each treatment group represents the mean of 5 replicates.
NS: Not significant, SEM: Standard error of mean

5.3.2 Organ weights and intestinal length

Table 5.3 presents the weights of proventriculus, gizzard, liver, spleen and bursa at d 7 and 21. On the d 7, the relative weights of proventriculus, gizzard and liver were increased by dietary inclusion of sDDGS. However, a reduction (P<0.05) was observed for spleen weight when diets containing 30 % sDDGS were fed to the birds. There were no significant effects of xylanase and protease supplementation on organ weights at d 7 and d 21, except there was a decrease (P<0.001) in the relative weight of pancreas as a result of xylanase addition to the diets at d 21. On d 21, only the relative weights of the bursa and gizzard were increased (P<0.001) as a result of incorporation of sDDGS in the diets. There were significant interactions between sDDGS, protease for the relative weights gizzard and liver at d 21 and 7, respectively.

The relative weights of the duodenum, jejunum, ileum and small intestine are summarised in Table 5.4. The duodenum weight was increased (P<0.05) when sDDGS was added to the diets at both levels of 15 and 30 % at d 7 but not at d 21. A heavier jejunum (P<0.01, d7 and d21) was also noticed in the birds fed sDDGS compared to control group of birds. The inclusion of sDDGS (15 and 30 %) also resulted to higher weight of ileum at d 21 (P<0.001) and when calculated for the entire small intestine at d 7 and 21 (P<0.01). Independently, xylanase alone also decreased (P<0.05) the total weight of the small intestine with no interaction between any of the three factors. Interestingly, xylanase tended (P=0.07) to reduce the jejunum weight at d 21. Supplementation of protease to diets did not alter the weights of small intestine or any of the intestinal regions.

Table 5.5 shows the results on the relative lengths of the small intestinal sections. Adding 30 % sDDGS to the diet increased (P<0.05) the duodenum and jejunum lengths at d 21. At the same time, xylanase addition to the diets resulted in a shorter (P<0.05) jejunum regardless of sDDGS and protease effects. There was no effect of protease on the relative length of the small intestine. For these parameters, no interaction between sDDGS and the enzymes or the three factors was observed.

5.3.3 Ileal viscosity and nutrient digestibility

The results of ileal nutrient digestibility are presented in Table 5.6. Dry matter digestibility deteriorated (P<0.001) as the sDDGS level rose to 30 % in the diets. There were no significant effects of either xylanase or protease when they were added individually but

surprisingly the combination of the two enzymes resulted in poorer (P<0.01) dry matter digestibility for the control diet where there was an interaction (P<0.01) between xylanase and protease. The birds fed sDDGS (15 and 30 %) showed poorer (P<0.001) protein digestibility than those received sDDGS-free diets. Neither of the enzymes nor their combination resulted in any improvement in protein digestibility. There was no effect of any treatment on ileal strach digestibility.

As shown in Table 5.7, incorporation of 30 % sDDGS in diets led to a higher ileal viscosity (P<0.01). Both xylanase and protease consistently decreased (P<0.05) ileal viscosity with the response being more pronounced with the combination of the two enymes in birds fed 30 % sDDGS. There was an interaction (P<0.05) between sDDGS and protease for ileal vicosity where protease reduced the viscosity only in the bird fed 30 % sDDGS.

Table 5.6 Effects of xylanase and protease individually or in combination and dietary level of sDDGS on nutrient digestibility coefficients and ileal viscosity at d 21[1]

Treatments			DM	CP	Starch	Viscosity
sDDGS (g/kg)	Xylanase	Protease				
0	-	-	0.67^{ab}	0.76^{ab}	0.94	2.46^{cbd}
	+	-	0.69^{a}	0.78^{a}	0.94	2.28^{cd}
	-	+	0.68^{ab}	0.77^{a}	0.93	2.29^{cd}
	+	+	0.64^{cd}	0.75^{ab}	0.92	2.17^{d}
150	-	-	0.64^{cd}	0.71^{c}	0.94	2.26^{cd}
	+	-	0.66^{bc}	0.72^{c}	0.93	2.10^{d}
	-	+	0.65^{bcd}	0.73^{bc}	0.93	2.23^{d}
	+	+	0.65^{bcd}	0.73^{bc}	0.93	2.25^{d}
300	-	-	0.62^{ef}	0.67^{e}	0.93	3.13^{a}
	+	-	0.62^{ef}	0.68^{de}	0.93	2.68^{b}
	-	+	0.63^{de}	0.70^{cd}	0.94	2.63^{bc}
	+	+	0.60^{f}	0.68^{d}	0.93	2.40^{cbd}
SEM			*0.003*	*0.003*	*0.002*	*0.037*
Main effects and interactions			*----------Probability----------*			
sDDGS			<0.001	<0.001	NS	<0.001
Xylanase			NS	NS	NS	<0.05
Protease			NS	NS	NS	<0.05
sDDGS × xylanase			NS	NS	NS	NS
sDDGS × protease			NS	NS	NS	<0.05
Xylanase × protease			<0.01	<0.05	NS	NS
sDDGS × xylanase × protease			NS	NS	NS	NS

[1]Each value correspondent to each treatment group represents the mean of 5 replicates.
NS: Not significant, SEM: Standard error of mean
[a] Means within a continuous column not sharing same superscript differ significantly (P<0.05).

The ileal amino acid digestibility coefficients at d21 are summarised in Tables 5.7 and 5.8. Inclusion of sDDGS adversely affected the digestibility of all measured amino acids, except lysine, in this study. Xylanse alone did not affect amino acid digestibility and no interaction between xylanase and sDDGS was observed. Irrespective of sDDGS and xylanase, methionine digestibility was improved by supplementation of protease. There was an interaction (P<0.05) between protease and dietary level of sDDGS for glutamic acid, proline and methionine, where the protease had a more pronounced effect when sDDGS was added to the diets. In addition, sDDGS and protease tended (P<0.09) to interact on the digestibility of aspartic acid, alanine, valine and leucine. When the diets contained 30 % sDDGS, protease improved (P<0.05) ileal digestibility of all amino acids, except lysine (lysine excluded) compared to the same diet without the protease. However, the combination of the two enzymes did not have such an effect. There was also significant interaction between xylanase and protease for all amino acids, independent of sDDGS level. The enzymes had no effect on the amino acid digestibility of the control diet.

5.3.4 Insoluble NSP and free sugars in the ileum

The concentration of insoluble NSP in the ileal contents of birds at d 21 is presented in Table 5.9. The incorporation of sDDGS in the diets increased (P<0.001) the total ileal insoluble NSP level, being the highest in birds fed 30 % sDDGS. Xylanase reduced (P<0.05) the concentration of insoluble NSP whereas protease did not. There was an interaction between xylanase and protease for the total amount of insoluble NSP (P<0.01), arabinose (P<0.05), xylose (P<0.05) and galactose (P<0.01). A detailed analysis of insoluble NSP in the ileal digesta revealed that in diets supplemented with xylanase, the concentrations of arabinose (P<0.05) and xylose (P<0.001) were significantly reduced, whereas other sugars remained unaffected by it. Irrespective of enzyme supplementation, inclusion of sDDGS increased the concentration of all constituent sugars of the insoluble NSP fraction, except ribose, which showed a reduction (P<0.05) as sDDGS rose to 30 % in the diets compared to the control diet. Moreover, the reduction of arabinose and xylose cause by xylanase addition was compromised when xylanase and protease were added to the diet simultaneously. This was confirmed by significant (P<0.05) interaction between xylanase and protease for concentration of arabinose and xylose, regardless of sDDGS

Table 5.7 Effects of xylanase, protease individually or in combination and dietary levels of sDDGS on ileal amino acid digestibility at d 21[1]

Treatments			His	Ser	Arg	Gly	Asp	Glu	Thr	Ala
sDDGS (g/kg)	Xylanase	Protease								
0	-	-	0.78^{ab}	0.73^{abc}	0.84^{ab}	0.72^{abc}	0.74^{ab}	0.81^{a}	0.68^{abc}	0.75^{a}
	+	-	0.79^{a}	0.76^{a}	0.85^{a}	0.74^{a}	0.75^{a}	0.82^{a}	0.71^{a}	0.78^{a}
	-	+	0.78^{ab}	0.74^{ab}	0.84^{ab}	0.73^{ab}	0.73^{ab}	0.80^{ab}	0.70^{ab}	0.76^{a}
	+	+	0.76^{abc}	0.72^{abc}	0.82^{abc}	0.70^{bcd}	0.71^{abc}	0.79^{abc}	0.67^{abcd}	0.74^{ab}
150	-	-	0.74^{cd}	0.69^{cde}	0.81^{bcd}	0.68^{d}	0.68^{cde}	0.76^{cde}	0.64^{de}	0.68^{cd}
	+	-	0.75^{bcd}	0.71^{bc}	0.82^{abc}	0.70^{bcd}	0.70^{bc}	0.77^{bcd}	0.66^{bcd}	0.70^{c}
	-	+	0.75^{bcd}	0.72^{abc}	0.82^{abc}	0.70^{bcd}	0.70^{bc}	0.77^{bcd}	0.66^{bcd}	0.71^{bc}
	+	+	0.74^{cd}	0.70^{cd}	0.81^{bcd}	0.69^{cd}	0.69^{c}	0.76^{cde}	0.65^{cd}	0.69^{cd}
300	-	-	0.68^{f}	0.64^{f}	0.77^{e}	0.62^{f}	0.63^{f}	0.70^{f}	0.59^{f}	0.62^{e}
	+	-	0.70^{ef}	0.66^{def}	0.79^{de}	0.64^{ef}	0.65^{def}	0.72^{f}	0.60^{ef}	0.63^{e}
	-	+	0.73^{de}	0.70^{cd}	0.81^{cd}	0.67^{de}	0.68^{cd}	0.75^{de}	0.64^{de}	0.68^{cd}
	+	+	0.70^{ef}	0.65^{ef}	0.79^{de}	0.63^{ef}	0.64^{ef}	0.73^{ef}	0.60^{ef}	0.65^{de}
SEM			*0.003*	*0.004*	*0.003*	*0.003*	*0.004*	*0.003*	*0.004*	*0.004*
Main effects and interactions			*----------Probability----------*							
sDDGS			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Xylanase			NS	NS	NS	NS	NS	NS	NS	NS
Protease			NS	NS	NS	NS	NS	NS	NS	NS
sDDGS × xylanase			NS	NS	NS	NS	NS	NS	NS	NS
sDDGS × protease			0.10	NS	0.10	NS	0.09	<0.01	NS	<0.05
Xylanase × protease			<0.05	<0.01	0.06	<0.01	<0.05	<0.05	0.01	<0.05
sDDGS × xylanase × protease			NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value correspondent to each treatment group represents the mean of 5 replicates.
NS: Not significant, SEM: Standard error of mean
[a] Means within a continuous column not sharing same superscript differ significantly (P<0.05).

Table 5.8 Effects of xylanase, protease individually or in combination and dietary level of sDDGS on ileal amino acid digestibility at d 21[1]

Treatments			Pro	Lys	Tyr	Met	Val	Ile	Leu	Phe
sDDGS (g/kg)	Xylanase	Protease								
0	-	-	0.75^{ab}	0.79	0.71^{abc}	0.84^{bc}	0.74^{abc}	0.76^{ab}	0.75^{abc}	0.76^{abc}
	+	-	0.77^{a}	0.80	0.75^{a}	0.87^{a}	0.76^{a}	0.77^{a}	0.77^{a}	0.78^{a}
	-	+	0.76^{ab}	0.80	0.74^{ab}	0.86^{ab}	0.75^{ab}	0.77^{a}	0.76^{ab}	0.77^{ab}
	+	+	0.73^{abc}	0.78	0.72^{abc}	0.84^{abc}	0.73^{abcd}	0.74^{abc}	0.74^{abcd}	0.75^{abc}
150	-	-	0.70^{cd}	0.79	0.65^{ef}	0.78^{de}	0.70^{de}	0.72^{cde}	0.70^{def}	0.73^{cd}
	+	-	0.72^{bc}	0.81	0.66^{def}	0.79^{d}	0.72^{bcd}	0.74^{abc}	0.72^{bcde}	0.75^{abc}
	-	+	0.72^{bc}	0.81	0.68^{cde}	0.79^{d}	0.72^{abcd}	0.74^{abc}	0.73^{bcd}	0.76^{abc}
	+	+	0.70^{cd}	0.80	0.66^{def}	0.78^{de}	0.71^{cd}	0.73^{bc}	0.71^{cdef}	0.74^{bcd}
300	-	-	0.64^{e}	0.79	0.63^{f}	0.72^{f}	0.64^{f}	0.67^{f}	0.64^{g}	0.68^{e}
	+	-	0.66^{e}	0.80	0.66^{def}	0.75^{ef}	0.66^{f}	0.68^{ef}	0.66^{fg}	0.70^{de}
	-	+	0.70^{cd}	0.82	0.70^{bcd}	0.81^{cd}	0.70^{de}	0.72^{cde}	0.71^{cde}	0.74^{bcd}
	+	+	0.67^{de}	0.79	0.66^{def}	0.79^{d}	0.67^{ef}	0.68^{def}	0.68^{efg}	0.71^{de}
SEM			*0.004*	*0.003*	*0.005*	*0.003*	*0.004*	*0.004*	*0.004*	*0.004*
Main effects and interactions			*Probability*							
sDDGS			<0.001	NS	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Xylanase			NS	NS	NS	NS	NS	NS	NS	NS
Protease			NS	NS	NS	<0.01	NS	NS	NS	NS
sDDGS × xylanase			NS	NS	NS	NS	NS	NS	NS	NS
sDDGS × protease			<0.05	NS	NS	<0.001	0.09	NS	0.07	NS
Xylanase × protease			<0.05	NS	<0.05	<0.01	<0.05	<0.05	<0.05	<0.05
sDDGS × xylanase × protease			NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value correspondent to each treatment group represents the mean of 5 replicates.
NS: Not significant, SEM: Standard error of mean
[a] Means within a continuous column not sharing same superscript differ significantly (P<0.05)

Table 5.9 Effects of xylanase, protease individually or in combination and dietary levels of sDDGS on concentrations (g/kg TiO_2) of insoluble NSP in the ileum at d 21[1]

Treatments			Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	Total
sDDGS (g/kg)	Xylanase	Protease	(g/kg TiO_2)								
0	-	-	119.5	273.0^{ab}	56.7^{d}	3373.7^{cde}	2854.2^{f}	131.9^{e}	4173.0^{a}	557.9^{c}	10242.7^{bcd}
	+	-	112.7	227.6^{bc}	62.5^{cd}	2879.4^{fg}	2340.8^{g}	151.7^{e}	3664.4^{b}	556.7^{c}	8873.2^{ef}
	-	+	134.3	270.4^{ab}	92.2^{abcd}	2775.7^{g}	2385.4^{g}	168.1^{de}	3331.9^{b}	536.9^{c}	8603.8^{f}
	+	+	152.5	291.6^{a}	123.7^{ab}	3032.2^{efg}	2576.3^{fg}	223.2^{cd}	3720.1^{b}	696.2^{c}	9600.4^{def}
150	-	-	107.4	199.5^{cd}	70.6^{bcd}	3661.8^{abc}	3910.7^{cd}	243.6^{c}	2930.2^{c}	1012.0^{b}	10748.7^{abc}
	+	-	110.7	189.9^{cde}	60.9^{cd}	3224.3^{def}	3431.3^{e}	240.7^{c}	2694.6^{c}	1007.4^{b}	9710.1^{cde}
	-	+	108.3	188.6^{cde}	85.0^{abcd}	3464.2^{bcde}	3662.4^{de}	266.2^{c}	2672.5^{c}	1041.6^{b}	10177.0^{bcd}
	+	+	104.2	186.9^{cde}	64.4^{cd}	3477.4^{bcd}	3496.4^{de}	239.6^{c}	2877.1^{c}	988.6^{b}	10130.4^{bcd}
300	-	-	116.2	177.9^{cde}	116.4^{abc}	3845.5^{ab}	4704.6^{a}	406.5^{a}	2029.1^{d}	1525.6^{a}	11435.9^{a}
	+	-	123.5	196.4^{cd}	141.5^{a}	3576.0^{abcd}	4145.8^{bc}	423.1^{a}	1961.3^{d}	1396.8^{a}	10591.0^{abcd}
	-	+	88.5	132.4^{e}	68.6^{cd}	3994.2^{a}	4772.3^{a}	327.3^{b}	2035.5^{d}	1468.0^{a}	11400.8^{a}
	+	+	111.4	170.8^{de}	99.7^{abcd}	3824.7^{ab}	4414.8^{ab}	383.4^{ab}	2075.1^{d}	1481.1^{a}	11117.3^{a}
SEM			*4.62*	*13.72*	*8.12*	*112.15*	*251.35*	*28.37*	*216.61*	*109.37*	*263.65*
Main effects and interactions			*Probability*								
sDDGS			NS	<0.001	<0.05	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Xylanase			NS	NS	NS	<0.05	<0.01	0.10	NS	NS	<0.05
Protease			NS	NS	NS	NS	NS	NS	NS	NS	NS
sDDGS × xylanase			NS	NS	NS	NS	NS	NS	NS	NS	NS
sDDGS × protease			NS	0.06	<0.01	NS	NS	<0.01	0.06	NS	NS
Xylanase × protease			NS	NS	NS	<0.05	<0.05	NS	<0.01	NS	<0.01
sDDGS × xylanase × protease			NS	NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value correspondent to each treatment group represents the mean of 5 replicates.
NS: Not significant, SEM: Standard error of mean
[a] Means within a continuous column not sharing same superscript differ significantly (P<0.05).

Table 5.10 Effects of xylanase, protease individually or in combination and dietary levels of sDDGS on composition of free sugars (g/kg marker) in the ileum at d 21[1]

Treatments			Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	Total
sDDGS (g/kg)	Xylanase	Protease					(g/kg TiO_2)				
0	-	-	30.4^{ab}	7.1	25.1	83.5^{f}	19.1^{f}	146.6	1390.6	1676.1	3375.8^{d}
	+	-	32.5^{ab}	1.7	35.5	96.9^{f}	32.2^{f}	144.4	1380.2	1681.7	3404.2^{d}
	-	+	26.3^{abc}	7.6	22.2	82.4^{f}	17.7^{f}	144.7	1403.0	1849.3	3550.9^{bcd}
	+	+	35.1^{abc}	8.9	38.8	114.8^{f}	39.7^{f}	172.3	1549.3	2216.3	4174.2^{ab}
150	-	-	29.2^{bc}	12.1	42.0	220.7^{a}	259.4^{e}	185.8	1179.2	1642.1	3565.8^{abcd}
	+	-	26.5^{cd}	12.2	37.2	252.0^{cd}	432.6^{bc}	141.2	1029.2	1828.5	3758.3^{abcd}
	-	+	22.7^{bc}	3.6	37.8	205.6^{e}	231.8^{e}	165.4	997.7	1585.8	3248.6^{d}
	+	+	26.8^{bc}	7.8	42.9	272.6^{bc}	458.3^{b}	170.4	1216.3	1892.1	4084.6^{abc}
300	-	-	17.8^{d}	9.1	41.3	298.3^{b}	393.2^{cd}	178.2	775.4	1723.2	3434.7^{cd}
	+	-	18.6^{d}	9.2	40.9	423.2^{a}	849.4^{a}	166.3	717.1	1603.0	3826.2^{abcd}
	-	+	15.4^{d}	9.4	45.5	280.8^{bc}	367.5^{d}	174.8	732.6	1646.2	3271.0^{d}
	+	+	18.6^{d}	11.5	40.5	421.4^{a}	848.1^{a}	190.7	762.6	1936.6	4230.1^{a}
SEM			*1.83*	*0.92*	*2.02*	*34.64*	*84.94*	*4.92*	*86.70*	*52.49*	*100.95*
Main effects and interactions			----------*Probability*----------								
sDDGS			<0.001	NS	<0.05	<0.001	<0.001	<0.05	<0.001	NS	NS
Xylanase			0.08	NS	NS	<0.001	<0.001	NS	NS	0.08	<0.01
Protease			NS	NS	NS	NS	NS	NS	NS	0.10	NS
sDDGS × xylanase			NS	NS	NS	<0.001	<0.001	NS	NS	NS	NS
sDDGS × protease			NS	0.06	NS	NS	NS	NS	NS	NS	NS
Xylanase × protease			0.09	NS	NS	NS	NS	NS	NS	NS	<0.05
sDDGS × xylanase × protease			NS	NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value correspondent to each treatment group represents the mean of 5 replicates.
NS: Not significant, SEM: Standard error of mean
[a] Means within a continuous column not sharing same superscript differ significantly (P<0.05)

inclusion in this study. Protease interacted with sDDGS (P<0.01) for the concentrations of ribose and mannose.

As shown in Table 5.10, in contrast to insoluble NSP content, inclusion of sDDGS did not alter the total free sugar concentration in the ileum of the birds. Considering individual sugars, the concentrations of xylose and arabinose, in the form of free sugars, were significantly higher (P<0.001) in the diets containing sDDGS compared to control diets. Xylanase supplementation, however, markedly increased (P<0.001) the concentrations of xylose and arabinose in the same diet groups, with a significant (P<0.001) xylanase x sDDGS interaction. While protease had no effect on the concentration of free sugars, a significant interaction (P<0.05) was found between protease and xylanase for increase in the total concentrations of free sugars in the ileal contents of the birds.

5.4 DISCUSSION

5.4.1 Growth performance

Similar to the observations made in Chapter 4, increasing levels of sDDGS elevated feed intake, improved BWG and impaired FCR. These findings concur with recent reports on DDGS from different grains (Olukosi *et al.*, 2010; Oryschak *et al.*, 2010a; Shim *et al.*, 2011). Similar to the present study, Lumpkins *et al.* (2004) reported a depression in feed utilization by feeding birds up to 18 % DDGS attributed to a possible marginal lysine deficiency. However, the results are in disagreement with Loar *et al.* (2010) who found a linear decrease in BWG and FI with increasing DDGS levels in broiler diets. Thacker and Widyarante (2007) demonstrated no differences in growth performance parameters of broiler chickens fed diets containing up to 20 % DDGS. It is, however, well documented that variation exists between different DDGS samples depending upon the processing methods used, and the source grains (Belyea *et al.*, 2004; Batal and Dale, 2006; Fastinger *et al.*, 2006; Martinez-Amezcua *et al.*, 2007). This may, at least partially, explain the different outcomes when DDGS is tested in different experiments. In the current study, energy and protein levels of diets were maintained at the same levels but lower nutrient digestibility, protein in particular, as well as development of the gut may have contributed to a higher FI of the birds received DDGS. Furthermore, Oryschak *et al*. (2010a) postulated that a possible underestimation of the AME value of DDGS may be responsible for differences observed in feed consumption. Besides,

expected greater hindgut fermentation products as the DDGS level rises in diets may play a role in providing energy, resulting in the improvement associated with birds fed diets containing DDGS (Olukosi *et al.*, 2010). Nevertheless, it is hard to canvass a firm explanation as to how birds performed better in terms of BWG and FI compared to the control group of birds.

The positive effect of xylanase on FI and BWG observed in the present study was in line with results of the preceding experiment (Chapter 4) as well as those reported by Liu *et al.* (2011). The reduction in FI observed as xylanase was added to the diet is possibly linked to the breakdown of insoluble NSP with some free sugars yield. The relationship between xylanase and DDGS at 3 weeks of age highlights the different effects of xylanase on the levels of DDGS. It is of importance to note that there is a dearth of research regarding the application of protease in broilers fed DDGS. Besides, this experiment, to my knowledge, is the first to investigate the interaction between xylanase and protease in diets containing sorghum DDGS. Nevertheless, the benefit of the same protease used in the current study has been recently reported in broilers (Angel *et al.*, 2011; Kalmendal and Tauson, 2012). The lack of significant synergy between the two enzymes is in close agreement with the result of experiment reported by Kalmendal and Tauson (2012). However, a synergistic effect of protease and carbohydrases was evaluated in wheat-canola based diets by Simbaya *et al.* (1996) who demonstrated a dose-response improvement in feed efficiency by a combination of phytase, protease and carbohydrases, compared to the individual supplementation of either enzyme.

The significant improvement in BWG following an increase in FI as a result of protease supplementation suggests that protein quality in the experimental diets, particularly for DDGS-containing diets, plays a pivotal role in dictating the magnitude of response to exogenous protease. Compared to enzyme-free diets, approximately 55 g increase in BWG was observed in birds given the diets containing 15 % sDDGS with protease. Birds fed the control diet gained only 32 g extra in response to protease supplementation. This was confirmed by the strong interaction between protease and DDGS for FCR, showing the highest response to the protease when 30 % DDGS was added to the diets. These results were supported by PER data which showed a decrease in protein expense against bird's gain by simultaneous supplementation of both enzymes in the birds fed DDGS. However, the lower PER of the birds fed DDGS was in strong agreement with results reported by Youssef *et al.* (2009). Despite the lack of interaction between the two enzymes in performance response, comparing the values of individual enzymes with a combination of the two revealed a

marginal rather than full additive improvement for FCR in birds fed DDGS. This may illustrate the potential use of an admixture of xylanase and protease in DDGS-rich diets.

5.4.2 Organ weight and intestinal growth

The significant increase in proventriculus and gizzard weights of birds fed diets containing DDGS up to 7d of age may indicate the ability of this material to induce gut development. This is in accordance with the observation made in the previous experiment (Chapter 4) and those reported by Loar *et al.* (2012) . However, in the experiment conducted by Loar *et al.* (2012) no effects on the relative weight of small or large intestine were observed.

However, the specific effect of high levels of DDGS on gut development has not been reported. Despite the lack of data on the effect of DDGS, it has been well documented that feeding fibrous materials to birds can stimulate the development of the upper gastrointestinal tract (Gonzalez-Alvarado *et al.*, 2007; Gonzalez-Alvarado *et al.*, 2008; Jimenez-Moreno *et al.*, 2009). Therefore, the same explanation can be applied here for DDGS where the high insoluble fibre content can play a stimulating role in gizzard development and possibly other segments of the intestinal tract. This effect is likely due to the greater resistance of the fibrous and coarse material in sDDGS to grinding and possibly a higher concomitant mechanical stimulation compared to the control group of birds.

The higher relative weights of bursa and liver (d7) in the birds given sDDGS-containing diets may likely be due to better gut development. It is well known that the gut is the largest immune organ in the body, thus anything affecting the gut will also affect the immune system.

Indeed, in the current experiment, feeding DDGS increased the size of the total GIT. It seems that the effect of fibre on the size of the gut is dependent on the region considered. In the present study, the magnitude of differences was more apparent in the jejunum and ileum than in the duodenum. The large amounts of insoluble fibre in diets containing DDGS may absorb water into their matrix, consequently swell as they pass through the GIT, and resulting in bulkier digesta and eventually a larger GIT (Gonzalez-Alvarado *et al.*, 2007). There is much research on the effect of microbial enzyme supplements on intestinal growth in birds but not any on birds fed DDGS. However the reduction in the length of the jejunum, ileum and small intestine by xylanase supplementation was in line with previous reports on broilers

(Brenes *et al.*, 1993; Lazaro *et al.*, 2004). The reduction in the intestinal length may have implications on energy maintenance and therefore justify the improved FCR observed in the birds. However, a more detail study on energy utilisation is needed to prove such influence.

5.4.3 Nutrient digestibility and viscosity of ileal digesta

It is obvious that there exists a link between dietary DDGS and ileal digestibility of CP and DM in this experiment. The lower CP digestibility with DDGS inclusion observed is in agreement with the previous experiment (Chapter 4) and also data reported by Olukosi *et al.* (2010). However, the effect on DM digestibility in the present study was in contrast with those reported by Olukosi *et al.* (2010), due likely to differences in inclusion level as well as the source of DDGS. The DM digestibility became necessarily lower in DDGS-containing diets due to the fact that energy density was kept constant with fat when the level of fibre increased by incorporating DDGS in diets. In regard to the effect of enzyme, a 3 % improvement (albeit non-significant) in the CP digestibility coefficient of the birds receiving 30 % DDGS as a result of protease addition may explain, at least partly, the improved growth performance of the relevant birds. Using the same mono-component protease, Angel *et al.* (2011) found 6% improvement in CP digestibility of a low protein density diet (Maize-soybean meal diet). Similar to the result of the current experiment, Kalmendal and Tauson (2012) reported no additional improvement of CP digestibility by simultaneous application of same type of xylanase and protease.

A similar finding was observed for DM digestibility. In this regard, the lack of effect of xylanase on CP digestibility was in accordance with the previous experiment conducted with the same material (Chapter 4) and the data reported in a study conducted with grower pigs (Yanez *et al.*, 2011). In contrast, Liu *et al.* (2011) demonstrated that xylanase improved DM digestibility of diets containing maize DDGS in broiler chickens. Overall, a low N retention reported by others (Leytem *et al.*, 2008; Applegate *et al.*, 2009) and a poor digestibility value for protein in the current trial with high inclusions of DDGS remain limiting factors contributing to poor feed efficiency of the birds.

The lack of effect of protease and xylanase or their combination on DM and CP digestibility of the control diet may relate to the fact that the diet had little substrate for the enzymes to act on. Irrespective of DDGS levels, the current trial revealed an improvement in FCR in the

birds offered protease or xylanase but not in the nutrient digestibility, which is in agreement with the results of other experiments examining protease supplementation in different diets (Mahagna *et al.*, 1995; Thacker, 2005; Yu *et al.*, 2007). For example, Thacker (2005) noticed improved FCR when birds were offered a wheat-based diet supplemented with protease whereas there was no significant improvement on DM and energy digestibility as well as nitrogen retention. Nevertheless, a further elucidation for the mode of action of exogenous protease in the diets of broilers is still required. Isaksen *et al.* (2010) proposed an augmentation in endogenous peptidase production thereby reducing the requirements of amino acids and energy as a result of protease addition to the diets. It is also postulated that exogenous enzymes may present an opportunity to minimize endogenous enzyme production as well as reduction in secretory investment, hence possibly leading to an improvement in performance. Furthermore, it can be conceded that exogenous protease may exhibit its beneficial effects indirectly through maintenance requirement, secretion and recovery; however, it is hard to ascertain until further examinations are conducted. Such studies should include assessment of endogenous secretion of nutrients and possibly net energy requirements of the birds offered exogenous enzymes. Nevertheless, a partial improvement of CP and DM digestibility in the current study may provide evidence for the enhancement of feed efficiency of the birds fed sDDGS-containing diets, particularly those supplemented with protease.

This study is the first to report the effect of exogenous xylanase and protease on apparent ileal digestibility of amino acids in diets containing sorghum DDGS. In contrast to DM and CP digestibility values, the response to protease was more pronounced for AID of amino acids, showing 2-9 % unit improvement for Met digestibility at different levels of DDGS inclusion. Despite the lack of effect of protease on AID of most amino acids, the interaction between DDGS and protease for His, Glu, Pro and Met as well as a strong tendency for Leu, Val, Arg, Asp and Ala indicate that exogenous enzymes, protease in particular, are probably more efficient on less digestible diets. This was previously documented by other studies in broiler chickens (Cowieson and Ravindran, 2008; Cowieson and Bedford, 2009; Cowieson, 2010).

In the current study, it is obvious that protease was more effective on AID when used alone rather than in combination with xylanase. This may highlight a possible negative interaction

between xylanase and protease, which prevented synergy between the two enzymes in terms of AID of amino acids.

5.4.4 Insoluble NSP and free sugar composition of ileal content

Although a wide range of sugars were found in the ileal content of the birds fed diet containing sDDGS, arabinoxylan appeared to dominate the insoluble NSP fraction. This was expected because corn contains arabinoxylan as the main NSP fraction. As discussed before, the proportion of NSP in the ileal content may also contribute to the viscous environment, which was apparent at the highest level of sDDGS in the current experiment. Moreover, the high concentration of insoluble NSP may explain the lower DM and CP digestibility observed in the birds offered sDDGS.

The significant interaction observed between the level of sDDGS and xylanase would explain the magnitude of response to xylanase in terms of arabinose and xylose (insoluble) disappearance in the ileum of the birds, which was most apparent at the highest level of sDDGS. This provides evidence for the positive effect of xylanase addition on FCR of the birds in this experiment. Furthermore, the adverse interaction of the two enzymes on the level of simple sugars like xylose and arabinose suggests a possible interference on xylanase by protease. In this regard, there have been some reports on the negative impact of protease on the effectiveness of other exogenous enzymes (Naveed *et al.*, 1998; Ghazi *et al.*, 2003; Olukosi *et al.*, 2007). For example, Naveed *et al.* (1998) reported that improved performance resulted by carbohydrases on lupins was compromised when broiler diets were supplemented with a mixture of carbohydrases and protease. In addition, they proposed a possible inactivation of carbohydrases through digestion by the exogenous protease. This explanation, however, is also supported by the results of the current experiment in terms of free sugar composition of the ileal contents of the birds. For instance, a higher concentration of free sugars was found by using protease and xylanase together. A likely explanation may lie in possible degradation of xylanase by exogenous protease that may have resulted to accumulation of free sugars in the small intestine. Therefore, the potential of free sugars degradation by xylanase may not have been fully realised when protease was added. In addition, the soluble NSP may have contributed to the concentration of residual free sugars

which were not measured in the present work as a very low concentration of soluble NSP was expected from sDDGS.

Little is known about the effect of protease on NSP degradation and free sugar disappearance as they pass through the intestinal tract of the birds. However, protease has been predominantly used in combination with other enzymes and the effect on nutrient digestibility, including NSP, has been documented. For instance, Kocher *et al.* (2002) demonstrated an enhancement in NSP faecal digestibility when soybean meal-based diets were supplemented with a mixture of protease and carbohydrases, but neither protein digestibility nor the growth performance of broiler chickens was affected. Similarly, in an experiment conducted on young pigs, Yin *et al.* (2001) demonstrated that adding β-glucanase, xylanase and protease enhanced digestibility of NSP and NDF, leading to improved growth. Nevertheless, the mechanism of the interaction between these two enzymes remains unclear until the mode of action of protease is fully understood.

5.5 CONCLUSION

In the present study, application of xylanase and protease were beneficial for the growth performance of broiler chickens fed sDDGS, particularly for the FCR, which revealed a degree of synergy. However, more research is required to identify the optimum inclusion of protease and xylanase to degrade a substantial amount of NSP and undigested protein in the sDDGS. Although, xylanase degraded the insoluble NSP fraction to release substantial amounts of free sugars, whereas the combination of xylanase with protease appeared to diminish this effect of xylanase.

CHAPTER 6

ROLE OF PROTEASE AND XYLANASE IN DIETS CONTAINING DISTILLERS' DRIED GRAINS WITH SOLUBLES FOR BROILER CHICKENS UNDER NECROTIC ENTERITIS CHALLENGE

Abstract

A 2×2×2 factorial experiment was conducted to investigate the effect of a high level of sorghum distillers' dried grains with solubles (sDDGS) (200 g/kg), with or without a combination of protease and xylanase in broiler chickens, under a necrotic enteritis disease challenge. A total of 576 male broiler chicks were randomly assigned to 8 experimental treatments, each replicated 6 times, with 12 birds per replicate for 35 d. Oral inoculation of the challenged group with Eimeria *spp. occurred on d 9, followed by 3 consecutive inoculations of Clostridium perfringens* (C.p) *from d 14 to 16. Microbial profile and the concentration of short-chain fatty acids (SCFA) in ileum and caeca of the birds were evaluated on 13d and 17d before and after* C.p. *inoculations. On d 17, 21 and 35, blood samples from one bird of each replicate were collected to detect total serum immunoglobulin (IgA, IgG and IgM) by ELISA assays. Disease challenge adversely influenced (P<0.01) body weight gain (BWG) and feed conversion ratio (FCR) of the birds in the third week and across the 35-day study. Incorporation of sDDGS markedly depressed BWG (P<0.001) and FCR (P<0.001) only in week 3, revealing significant interaction (P<0.01) between challenge and sDDGS inclusion. Despite the lack of a significant effect, challenged birds on sDDGS diets supplemented with enzymes tended to maintain BWG in week 3. On d 17, infected birds had a higher number of ileal coliform, lactic acid bacteria, lactobacilli and total anaerobic bacteria, while birds that received diets containing DDGS had lower total anaerobic bacteria. Inoculation of birds with* C.p. *resulted in higher counts of* C.p. *(P<0.01) in both ileal and caecal contents while* Eimeria *did not alter* C.p. *counts. Despite the lack of enzyme effect, inclusion of 20 % DDGS, markedly increased the proliferation of* C.p. *in caeca at d17 with a significant interaction between challenge and sDDGS. On day 17, more severe NE-related lesions (P<0.05) were found in birds fed sDDGS-containing diets than in birds fed*

the control diet. The concentration of IgA at 21d (P<0.001) and 35d (P<0.05) was elevated in the challenged birds, but remained unchanged at d13. Furthermore, infected birds had higher total IgG at d21 and 35 and IgM at any of three time evaluations. Irrespective of challenge and enzyme, incorporation of DDGS to the diets improved (P<0.01) the IgA and IgG titre at d13 but decreased (P<0.05) IgA, IgG at d 21 and IgM at d 35. No effect of enzyme was observed on the immune response of birds except an increase in serum IgG at d 21. In conclusion, incorporating a high level of sDDGS in the diet of broiler chickens may increase susceptibility to NE. Supplementation with a blend of xylanase and protease did not show a significant mitigation effect in infected birds fed DDGS or control diet.

6.1 INTRODUCTION

Necrotic enteritis (NE) is caused by *Clostridium perfringens*. This bacterium is ubiquitous in nature and can be found in soil, dust, faeces, litter, feed and the intestines of most healthy animals and humans (McReynolds *et al.*, 2009; Palliyeguru *et al.*, 2010). The spores of *C. perfringens* are ingested continuously via poultry feed and it is generally believed that presence of predisposing factors is required to cause the disease (van Immerseel *et al.*, 2009). There are a number of toxins involved with the a-toxin (Truscott and Al-Sheikhly, 1977) and netB (Keyburn *et al.*, 2008) being the key agents. Signs of NE include an increase in feed conversion ratio (FCR), depression in feed ingestion, reduction in weight gain, malabsorption of nutrients, diarrhoea, severe necrosis of the intestinal tract and increased mortality in the case of the acute form (Hofacre *et al.*, 2003; van Immerseel *et al.*, 2004; Lensing *et al.*, 2010). This disease, however, is more common in the subclinical form than in clinical outbreaks in broiler flocks (Hofshagen and Kaldhusdal, 1992; Palliyeguru *et al.*, 2010). Necrotic enteritis has been partially controlled by the use of coccidiostat, and in-feed antibiotics (IFAs). In the past few years, the prevalence of this disease has emerged as a considerable problem following the restrictions on the use of IFAs in animal diets (McDevitt *et al.*, 2006; Grilli *et al.*, 2009). However, various predisposing factors such as environment, health and dietary components may promote colonisation of *C. perfringens* leading to the onset of NE (McDevitt *et al.*, 2006). Therefore, understanding the effective control measures and predisposing factors contributing to the development of NE is fundamentally important.

Dietary components are known to have a conclusive role in the incidence of NE via the alteration of the bacterial community and intestinal balance in favour of the proliferation of

the causative bacterium (Apajalahti *et al.*, 2001; Palliyeguru *et al.*, 2010). It has been shown that a high level of wheat (Branton *et al.*, 1987; Riddell and Kong, 1992), barley (Kaldhusdal and Hofshagen, 1992; Riddell and Kong, 1992) and fishmeal (Truscott and Al-Sheikhly, 1977) may precipitate outbreaks of NE. For instance, Annett *et al.* (2002) found that wheat- and barley-based diets increased clostridial proliferation compared with corn-based diets. It is, however, believed that the presence of arabinoxylans and β-glucans in wheat, rye, barley and oat not only interfere with digestion, but also influence intestinal bacteria (Riddell and Kong, 1992).

Recently, the incorporation of distillers' dried grains with solubles (DDGS) in poultry diets has drawn attention, but information concerning the impact of this by-product on the health and immune response of poultry, particularly at a high level of inclusion, is sparse. Perez (2010) found that recovery of young pigs under challenge of pathogenic *Escherichia coli* was accelerated by either DDGS or cellulose. However, such a preventive effect of DDGS was not observed in broilers infected with *E. acervulina* (Perez *et al.*, 2011). Moreover, in a study on young pigs, including DDGS in the diet reduced the severity of intestinal lesions associated with *Lawsonia interacellularis* infection (Whitney *et al.*, 2006c). Weber *et al.* (2008) also demonstrated an upregulation of the expression of both proinflammatory and anti-inflammatory cytokines in intestinal tissue of weanling pigs fed diet containing 7.5 % DDGS. Nonetheless, there is a dearth of research on the effect of DDGS on broiler health, particularly associated with NE, and further evaluation is warranted.

Although a really effective alternative to antibiotics or coccidiostats that confronts *Clostridium* has not yet been found (Lensing *et al.*, 2010), attempts are being made to provide products that may at least alleviate the detrimental effects of the causative agents. In this quest, enzymes such as xylanase may have a positive role on animal performance by breaking down the NSP, reducing viscosity and improving nutrient digestion and absorption, hence reducing substrate availability for microbial growth in the ileum (Bedford and Classen, 1992; Choct *et al.*, 1999; Jia *et al.*, 2009b). Choct *et al.* (1999) reported a reduction in fermentation products in the small intestine of broilers when diets were supplemented with xylanase. This resulted in the suppression of *C. perfringens* in the small intestine, but may not always be associated with protection against necrotic enteritis (Riddell and Kong, 1992; Jackson *et al.*, 2003; van Immerseel *et al.*, 2004). Such effects may substantially vary, depending on feed components, particularly cereal types, used in the experimental diets of different challenge models.

On the other hand, it has been documented that dietary protein sources (Palliyeguru *et al.*, 2010) and also amino acid balance (Wilkie *et al.*, 2005) may affect the proliferation of *C. perfringens* within the caeca and ileum of poultry. Additionally, low protein digestibility may provide a significant amount of this nutrient, thereby accelerating the proliferation of bacteria, in particular *C. perfringens*, which is highly proteolytic (Jia *et al.*, 2009a). In this regard, exogenous protease may have an application, which has been shown to have a mitigating influence in detrimental consequences of the coccidia infection (Peek *et al.*, 2009). Nevertheless, the possible complementary effect of a combination of xylanase and protease under NE challenge has yet to be evaluated.

Hence, the aim of the present study was to investigate the impact of a high dietary sorghum DDGS inclusion on the development of NE and the effectiveness of a blend of xylanase and protease in broiler diets. Moreover, the interrelationship of DDGS and exogenous enzymes on immunological response of the birds as well as modification of the microflora and fermentation products were evaluated.

6.2 MATERIALS AND METHODS

6.2.1 Experimental design, diets and bird husbandry

A 2×2×2 factorial arrangement of treatments was employed in a randomised complete design to investigate the effects of three factors, namely, diets (0 and 200 g/kg sDDGS), a blend of xylanase and protease supplementation (with or without) and necrotic enteritis challenge (challenged or unchallenged), and their interactions.

Five hundred and seventy-six Cobb 500 male broilers (initial weight, 48 ± 0.66 g), vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease were collected from a local hatchery (Baiada Hatchery, Tamworth, NSW, Australia). Birds were randomly assigned to 48 floor pens (600 mm × 600 mm × 300 mm dimension and with a floor area of 0.36 m^2/pen) placed on litter of wood shavings in climate-controlled rooms. Each treatment was replicated 6 times, starting with 12 chicks per pen. To avoid cross contamination between challenged and unchallenged groups of birds, challenged cages were separated completely from unchallenged groups of birds by placing them in identical climate-controlled rooms with

exactly the same management applied to the two groups of birds. In addition, the rooms were thoroughly cleaned and disinfected prior to the commencement of the study.

Four treatments were allocated to each of the starter and grower basal diets comprising mainly of wheat, sorghum, barley and soybean meal as shown in Table 6.1. Two phases of feeding were adopted, starter diets from 1 to 21d and grower diets from 22 to 35d. All diets were formulated to meet the minimum requirements for Cobb 500 (2008) broiler chickens. Two levels of sDDGS were created by substitution of 0 and 200 g/kg sorghum DDGS in lieu of mainly sorghum and protein sources. Xylanase (Ronozyme WX, DSM, Nutritional Products, Ltd.) and protease (Ronozyme ProAct, DSM, Nutritional Products, Ltd.) were supplemented at the recommended levels of 0.3 and 0.2 g/kg of the experimental diets, respectively. All birds had access to feed and water *ad libitum* throughout the study. Feed was provided from a separate feeding trough per pen, and water was supplied via nipple drinkers. The room temperature was maintained at 34 $^{\circ}$C during the first 5 days and then gradually decreased to 23 $^{\circ}$C by the third week. A lighting program of 16 h light and 6 h darkness was applied to the birds except for the first day, which had 24 h light. Birds were weighed weekly; feed consumption was also measured for each experimental pen, and FCR was adjusted for mortality on a pen basis. All the birds were monitored for general health at least twice a day. Mortality was recorded as it occurred and the carcass was necropsied for the detection of necrotic enteritis.

6.2.2 Experimental challenge model (necrotic enteritis)

The model described by Wu *et al.* (2010) was used with some modifications to induce subclinical necrotic enteritis in broiler chickens. The dietary fishmeal component of this model was omitted to reduce interference with DDGS as the main test factor in the current experiment.

All the birds received their experimental diets until d 9 when challenged groups of birds were orally gavaged a suspension of 2500 oocytes each of *E. acervulina*, *E. maxima* and *E. brunetti* in 1 ml PBS. The *E. acervulina* isolate was obtained from Bioproperties Pty Ltd. (Glenorie NSW, Australia), whereas the other species were sourced from Bioproperties Pty Ltd. (Melbourne, VIC, Australia). The purification of all three species had already been made by serial passages through 3-week-old *Eimeria*-free chickens followed by storage in 2%

(w/v) potassium dichromate at 4 ºC prior to inoculation. The rest of the birds (unchallenged group) received 1 ml of sterile PBS in lieu of *Eimeria spp*.

A primary poultry isolate of C. *perfringens* type A was also obtained from the CSIRO laboratory (Geelong, VIC, Australia) and stored in thioglycollate broth (USP alternative, Oxoid, CM391) containing 30% (v/v) glycerol, at -20ºC. Fresh preparation of challenge inoculum was performed by growing the bacterium overnight at 39 ºC in 1000 mL of thioglycollate broth with 10 and 5 g starch and casitone per litre, respectively.

Subculturing of the stock culture of *C. perfringens* had been previously performed in cooked meat media (Oxoid, CM81). Three consecutive oral gavages of *C. perfringens* were undertaken on d 14, 15 and 16 for all challenged groups with 3.5×10^8 CFU per ml of thioglocollate broth. Unchallenged group of birds received 1 mL of sterile thioglocollate broth before the inoculation of the challenged group of birds to avoid cross-contamination which may have otherwise occurred.

Plate 6.1 Experimental facilities used in this study

Table 6.1 Ingredient and nutrient composition of experimental diets

Ingredients (g/kg)	Starter diets (d 0 - 21)		Grower diets (d 22 - 35)	
	Control	sDDGS	Control	sDDGS
Wheat	313.7	306.5	262.0	261.8
Barley	118.2	119.3	70.2	84.2
Sorghum	190.0	93.8	330.0	221.0
Soybean meal -48%	235.0	150.0	203.2	110.0
Sorghum DDGS	0.0	200.0	0.0	200.0
Canola oil	43.2	53.0	56.1	60.0
Isolated soy protein (SCM) - 65% CP	55.7	33.8	38.0	20.8
Dicalcium phosphate	20.9	15.1	19.6	16.6
Limestone	11.9	15.0	10.6	12.5
Salt	4.5	2.3	3.8	1.8
Vitamin and mineral premix [1]	2.0	2.0	2.0	2.0
DL-methionine	2.5	2.7	2.5	2.9
L- lysine	1.7	5.5	1.5	5.2
Choline Cl-70%	0.4	0.0	0.0	0.0
Threonine	0.3	1.0	0.5	1.2
Nutrient composition				
ME (MJ/kg)	12.34	12.31	12.98	12.98
Crude protein	231.9	231.9	208.3	208.2
Crude fibre	29.3	41.0	26.9	38.9
Ca	10.0	10.0	9.2	9.2
Available P	5.0	5.0	4.6	4.6
Met	5.6	6.0	5.3	5.5
Lys	12.8	12.8	10.6	10.6
Met + Cys	9.1	9.1	8.4	8.4
Thr	8.2	8.3	7.7	7.7
Na	2.1	2.1	1.7	1.9

[1] Contained vitamins and minerals (mg/kg): Vitamin A (as all-trans retinol), 12,000 IU; cholecalciferol, 3,500 IU; vitamin E (as d-α-tocopherol), 44.7 IU; vitamin B12, 0.2 mg; biotin, 0.1 mg; niacin, 50 mg; vitamin K3, 2 mg; pantothenic acid, 12 mg; folic acid, 2 mg; thiamine, 2 mg; riboflavin, 6 mg; pyridoxine hydrochloride, 5 mg; D- calcium panthothenate, 12 mg; Mn, 80 mg; Fe, 60 mg; Cu, 8 mg; I, 1 mg; Co, 0.3 mg; and Mo, 1 mg.

6.2.3 Sample collection and processing

Three birds on d 13 and 2 birds on d 17 per pen were randomly selected, weighed and euthanased by cervical dislocation. Birds from the unchallenged group were processed first to minimise the likelihood of cross-contamination. Thereafter, birds were dissected to remove the small intestine. The contents of the ileum and caeca were collected by gently squeezing the digesta into plastic containers. Samples from each replicate were pooled together and subsamples of each caeca and ileum were transferred to 10 mL McCartney bottles containing

anaerobic solution in order to enumerate the bacteria. Around 0.5 g of fresh digesta from the caeca and ileum were mixed with 4.5 ml of distilled water and pH values were eventually measured using a combined glass/reference electrode (Ecoscan, Eutech, Singapore). The rest of the samples were frozen immediately to -20 ºC until further analyses were conducted. The bursa of fabricius and spleen of the sample birds were also weighed on both days of sampling and expressed as g/100g of BW.

Lesion scores of NE

The small intestine from each sampled bird was removed aseptically, incised longitudinally and subjected to a gross pathologic diagnosis of NE based on the presence of intestinal lesions typical of naturally occurring and experimentally induced NE according to the descriptions of Prescott *et al.* (1978), Broussard *et al.* (1986) and Branton *et al.* (1996) (Plate 6.2). The scoring was conducted blindly so that the person carrying out the score was not aware of the treatment for the particular bird. Scores were given from 0 to 4 depending on lesion severity.

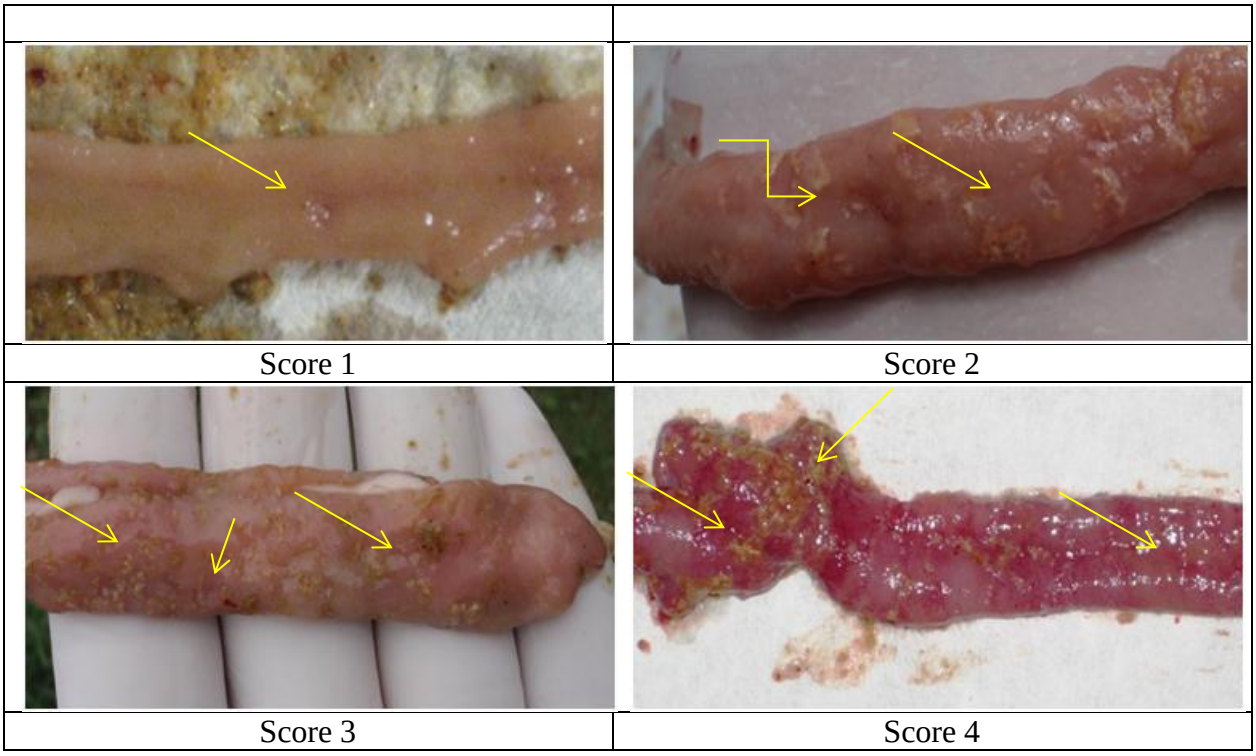

Plate 6.2 Intestinal NE lesion scores

6.2.4 Enumeration of intestinal bacteria

About 1 g of fresh digesta from the ileal and caecal contents of the birds was transferred into 15-mL pre-weighed McCartney bottles containing 10 mL of anaerobic broth. The suspension

was then transferred into a plastic bag and subsequently homogenised for 2 min under CO_2 atmosphere using a MiniMix® bag mixer (Interscience, St. Norm, France). A serial 10-fold dilution was then conducted on the suspensions by adding 1 mL of the mixed sample to 9 mL of anaerobic broth, diluting from 10^{-1} to 10^{-5} (for ileal samples) and 10^{-1} to 10^{-6} (for the caecal samples), as described by Miller and Wolin (1974). From the last three dilutions, a sample of 0.1 mL each was taken and plated on the appropriate medium for enumeration of microbial populations. Total anaerobic bacteria were determined using anaerobic roll tubes containing Wilkins-Chalgren anaerobe agar (CM 0619) incubated at 37 °C for 7 d. Coliforms and lactose-negative enterobacteria, which develop as red and colourless colonies, respectively, were cultured on MacConkey agar (CM 0007) and incubated aerobically at 39 °C for 24 h, prior to enumeration. Lactobacilli were cultured and counted on Rogosa agar (CM 0627) in anaerobic conditions using anaerobic AnaeroGen™ sachets (AN0025A, Oxoid Ltd, Hampshire, UK) at 39 °C for 48 h. Lactic acid bacteria were counted on De Man Rogosa and Sharp (MRS) agar (CM 0361) incubated anaerobically at 39 °C for 48 h. The population of *C. perfringens* was determined on Tryptose-Sulfite-Cycloserine and Shahidi-Ferguson Perfringens agar base (CM 0587 TSC and SFP) mixed with egg yolk emulsion (Oxoid, SR0047) and Perfringens (TSC) selective supplement (Oxoid, SR0088E) where inoculum was spread between two layers of prepared agar. After incubation period for all species, colonies were carefully counted, converted into a logarithmic equivalent and expressed as $\log_{10}$ CFU/g of wet digesta.

6.2.5 Determination of SCFA

Short-chain fatty acids (lactic and volatile fatty acids) were measured according to the method described by Jensen *et al.* (1995) as described in Section 4.2.4.

6.2.6 Total serum antibody measurements by enzyme-linked immunosorbent assay (ELISA)

A sandwich ELISA assay was used to determine the total antibody titre concentrations of IgY, IgM and IgA at 3 time points; before the *C. perfringens* challenge, 7d after the first *C. perfringens* inoculation and finally at the end of the experiment at 35d. On the designated

days, blood samples were taken from the jugular vein into 7 mL serum tubes. Blood samples were then allowed to clot at room temperature for 2 h and subsequently centrifuged at 2500 x g for 5 min to separate serum from the cells. All serum samples were immediately frozen at -20 °C until antibody assays were performed.

Antibody assays were begun by adding 100 µL of diluted coating antibody (0.05 M carbonate-bicarbonate, pH 9.6) to each well of microtitre plates (96 wells, Nunc-Immuno Plate, Nunc A/S Denmark) and incubated at room temperature for 1 h. The samples were then washed three times with wash solution (50 mM Tris, 0.14 M NaCl containing 0.05 % Tween 20, pH 8.0) followed by addition of 200 µL of blocking solution (50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin) to each well. The microtitre plates were again subjected to incubation by leaving the plates at room temperature for 30 min in the dark. Plates were subsequently washed five times using the same wash solution.

Serum samples were first diluted 1:50000, 1:10000 and 1:2000 for IgY, IgM and IgA assays, respectively. Subsequently 100 µL of dilute sera or the standard were added to each well, and microplates were in incubated for 1 h in the dark and at room temperature (RT). Washing was subsequently repeated five times with washing buffer. Thereafter, depending on the type of Ig measured, 100 µL of diluted conjugated chicken detection antibody (Bethyl Laboratories Inc., Montgomery, TX, USA) was added to each well and incubated at RT for 1 h in the dark followed by washing with wash solution (5 times). In the next step, 100 µl of TMB (3,3´,5,5´-tetramethylbezidine) microwell per oxidase substrate system (0.4 g/litre TMB in an organic base and H_2O_2 at a concentration of 0.02 % (v/v) in a citric acid buffer, Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) were added to each well. The plates were incubated in the dark at RT for 15 min. The reaction was terminated by pipetting 100 µL of 0.18 M H_2SO_4 into each well. Absorbance was measured on an ELISA microplate reader (Titertek Multiskan® PLUS MKII, Labsystems, Finland). All the samples and the standard chicken reference serum samples (Bethyl Laboratories Inc., Montgomery, TX, USA) were assayed in duplicate.

6.2.7 Animal ethics

The Animal Ethics Committee of the University of New England approved all the experimental procedures of this experiment (AEC 11/066).

6.2.8 Statistical analysis

The eight treatments of 2 diets (with or without DDGS), 2 levels of enzymes (with or without) and challenge (with or without) in a 2×2×2 factorial arrangement were subjected to statistical analysis using 3-way ANOVA of GLM procedure of SAS (SAS/STAT Version 9.1, SAS Institute, 2003) to assess the main effects and 2- or 3-way interactions. Data were checked for normal distribution prior to statistical analyses. If a significant effect was detected, differences between treatments were separated by a Least Significant Difference test (Fisher's test). Differences between mean values were considered significant at $P \leq 0.05$, unless otherwise specified.

6.3 RESULTS

6.3.1 Bird performance, mortality and lesion scores

As presented in Table 6.2, feed consumption was not affected by disease challenge or enzyme supplementation, while sDDGS markedly ($P<0.001$) increased FI in all periods of this trial except for week 3. A significant interaction was noticed between sDDGS and enzyme when FI was assessed for the finisher phase ($P<0.05$) of feeding as well as across the 35-d study ($P<0.01$) when enzyme increased the feed intake in birds given sDDGS. Disease challenge and enzyme supplementation also tended ($P=0.07$) to interact in the third week, arising from relatively higher FI in the challenged birds that were offered diets supplemented with enzymes.

The disease challenge adversely influenced ($P<0.01$) BWG in the third week, and when assessed from 1 to 35d. Incorporation of sDDGS depressed ($P<0.01$) BWG when birds were challenged with necrotic enteritis that became apparent in week 3 as birds received *C. perfringens* inoculations. This was accompanied by significant interaction between sDDGS and the challenge, revealing the lowest BWG in the birds that received the DDGS-containing diet with no enzyme supplementation. Nevertheless, BWG was higher in other periods of the study in the birds that received sDDGS irrespective of challenge and enzyme addition. Enzymes had no effect on BWG but tended ($P=0.07$) to increase values between 14 and 21d. Challenged birds that received diets containing sDDGS and enzymes exhibited similar BWG to the unchallenged group of birds which received the same diet for the whole period of

study. Over the same period, the interaction between sDDGS and microbial enzymes tended towards significance (P=0.09), regardless of whether or not the birds had been challenged.

In the first two weeks of the experiment, FCR of the birds was unaffected by dietary treatments or disease challenge. However, inoculation of the birds with *Eimeria* spp. and *C. perfringens* resulted in poorer (P<0.001) FCR compared to the unchallenged group of birds, and this was independent of sDDGS and enzyme supplementation in week 3. At the same period of the trial, feeding diets containing sDDGS markedly increased (P<0.001) FCR of the birds. There was an interaction (P<0.01) between sDDGS and disease challenge, with the poorest FCR in the birds offered sDDGS and challenged. No significant impact of the blend of xylanase and protease was observed in terms of FCR in this experiment. Overall, FCR of the birds (1-35d) was also unaffected by sDDGS or enzymes, although these two factors tended (P=0.06) to interact between 1 and 35d.

The NE scores revealed a significant impact of disease challenge resulting in higher NE scores in all 3 regions of the small intestine of broilers when considered as a separate factor from the diet and enzyme effects. Inclusion of sDDGS to the diet markedly increased (P<0.001) NE scores in the jejunum and ileum, and, when the entire small intestine was assessed. There was no effect of enzyme supplementation on lesion score. Mortality was significantly higher in the birds receiving challenge treatment in week 3 and across the 35 d study. However, the effect of dietary treatment was not significant on mortality.

Table 6.2 Effects of sDDGS inclusion, enzyme supplementation and necrotic enteritis challenge on broiler performance

Treatments		Feed intake (g/b)				Body weight gain (g/b)				Feed Conversion ratio (g feed/g gain)			
sDDGS (g/kg)	Enzyme	0-14	14-21	21-35	0-35	0-14	14-21	21-35	0-35	0-14	14-21	21-35	0-35
0 (unchallenged)	-	590	679	2046^{bc}	3315^{cd}	434^{bc}	416^{ab}	1031	1881^{bcd}	1.36	1.63^{bcd}	2.02	1.77^{cd}
	+	583	670	1970^{c}	3223^{d}	425^{c}	436^{a}	1005	1866^{bcd}	1.37	1.54^{d}	1.96	1.73^{d}
200 (unchallenged)	-	628	711	2092^{bc}	3431^{bc}	459^{a}	437^{a}	1068	1963^{ab}	1.37	1.63^{bcd}	1.97	1.75^{cd}
	+	621	688	2204^{ab}	3513^{ab}	499^{a}	428^{a}	1138	2036^{a}	1.33	1.61^{cd}	1.94	1.73^{d}
0 (challenged)	-	578	664	2058^{bc}	3300^{cd}	415^{c}	368^{cd}	1014	1797^{cd}	1.39	1.83^{b}	2.04	1.84^{abc}
	+	580	674	1920^{c}	3174^{d}	419^{c}	384^{bc}	959	1762^{d}	1.38	1.76^{bc}	2.03	1.81^{bcd}
200 (challenged)	-	636	665	2185^{ab}	3487^{ab}	454^{ab}	297^{e}	1048	1800^{cd}	1.40	2.26^{a}	2.09	1.94^{a}
	+	628	714	2302^{a}	3644^{a}	458^{a}	339^{d}	1128	1925^{abc}	1.37	2.12^{a}	2.05	1.90^{ab}
SEM		*22.5*	*4.6*	*17.0*	*16.6*	*2.8*	*6.0*	*22.4*	*21.1*	*0.009*	*0.025*	*0.025*	*0.013*
Main effects													
sDDGS (0)		583^{b}	672	1998^{b}	3256^{b}	423^{b}	401^{a}	1002^{b}	1826^{a}	1.38	1.69^{b}	2.01	1.79
(200)		628^{a}	694	2196^{a}	3519^{a}	460^{a}	375^{b}	1096^{a}	1931^{b}	1.37	1.91^{a}	2.01	1.83
Enzyme	-	608	680	2095	3383	441	379	1040	1860	1.38	1.84	2.03	1.82
	+	603	687	2099	3389	443	397	1058	1897	1.36	1.76	2.00	1.79
No challenge		606	687	2078	3371	447	429^{a}	1060	1936^{a}	1.36	1.60^{b}	1.97	1.74^{b}
Challenge		605	679	2117	3401	436	347^{b}	1037	1821^{b}	1.39	1.99^{a}	2.05	1.87^{a}
Main effects and interactions		*Probability*											
Challenge		NS	NS	NS	NS	<0.05	<.0001	NS	<0.001	NS	<0.001	0.10	<0.001
sDDGS		<.0001	0.07	<0.001	<0.001	<0.001	<0.01	<0.01	<0.01	NS	<0.001	NS	NS
Enzymes		NS	NS	NS	NS	NS	0.07	NS	NS	NS	NS	NS	NS
Challenge × sDDGS		NS	NS	NS	NS	NS	<0.001	NS	NS	NS	<0.001	NS	0.06
Challenge × Enzymes		NS	0.07	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
sDDGS × Enzymes		NS	NS	<0.05	<0.01	NS	NS	0.09	0.07	NS	NS	NS	NS
sDDGS × Enzymes × Challenge		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value for each treatment represents the mean of 6 replicates

NS: Not significant, SEM: Standard error of mean

[a] Means within a column not sharing a superscript differ significantly at the $P<0.05$ for the treatment effects and at the P level shown for the main effects.

Table 6.3 Mortality and necrotic enteritis lesion scores in different regions of small intestine of chickens under necrotic enteritis challenge

Treatments	Enzymes	NE lesion scores[1]				Mortality (%)	
sDDGS (g/kg)		Duo	Jej	Ile	Small intestine[2]	d 14-21	d 0-35
0 (Unchallenged)	-	0.11	0	0	0.04	0	5.55
	+	0.17	0	0	0.06	0	7.38
200 (Unchallenged)	-	0.06	0	0	0.02	0	5.83
	+	0.11	0	0	0.04	0	5.75
0 (Challenged)	-	0.81	0.56	0.42	0.59	2.12	9.32
	+	0.81	0.50	0.39	0.56	3.50	13.72
200 (Challenged)	-	1.28	0.97	0.89	1.05	4.60	15.60
	+	0.94	0.78	0.83	0.85	5.00	16.05
SEM		0.034	0.028	0.034	0.021	1.31	2.41
Source of variation		----------*Probability*----------					
Challenge		<0.001	<0.001	<0.001	<0.001	<0.01	<0.01
sDDGS		0.07	<0.01	<0.001	<0.001	NS	NS
Enzymes		NS[4]	NS	NS	NS	NS	NS
Challenge × sDDGS		<0.01	<0.01	<0.001	<0.001	NS	NS
Challenge × Enzyme		0.10	NS	NS	NS	NS	NS
sDDGS × Enzymes		NS	NS	NS	NS	NS	NS
Challenge × sDDGS ×Enzymes		NS	NS	NS	NS	NS	NS

[1]Each value represents the mean of 18 birds per treatment (3 birds per replicate). Scores were given from 0 to 4 depending on the severity.
[2]Small intestine values represent the average of duodenal, jejunal and ileal scores
NS: Not significant, SEM: Standard error of mean
[a] Means within a column not sharing a superscript differ significantly (P<0.05).

6.3.2 Characterisation of ileal and caecal microflora

The effects of dietary treatments and *Eimeria* spp. challenge on ileal and caecal bacteria counts at d 13 are presented in Tables 6.4 and 6.5. Regardless of sDDGS inclusion and supplementation of enzymes, inoculation of the birds with *Eimeria* spp. resulted in lower lactobacilli, lactic acid bacteria and total anaerobic bacteria in the ileal contents, whilst coliform and *C. perfringens* remained unaffected at d 13. During this stage, neither enzymes nor DDGS altered the number of bacteria assessed in the present study. No significant interactions were observed between experimental factors in terms of bacterial counts in the ileum prior to *C. perfringens* inoculations. In the caecal contents of the same birds, sDDGS inclusion resulted in higher coliform counts than those which had received control diets, independent of challenge and enzyme addition. However, admixture of protease and xylanase markedly (P<0.05) suppressed the proliferation of coliforms in the caeca of the chickens at this time period. Lactose-negative enterobacteria were not detectable in the ileum and caeca

of the birds before the *C. perfringens* challenge. All other bacterial groups analysed in the caecal contents of the birds were not significantly altered either by dietary treatments or inoculation with *Eimeria* spp.

The bacterial counts in the ileal and caecal contents of chickens post-*C.p* challenge (d 17) are shown in Tables 6.6 and 6.7. Irrespective of dietary sDDGS and enzymes, coliforms, *C. perfringens*, lactobacilli, lactic acid bacteria and total anaerobic bacteria were more prominent in the ileum of the infected group than the uninfected group. However, the effect of sDDGS inclusion on bacterial groups analysed in the ileum of the birds at d 17 was not significant except for a reduction ($P<0.05$) in the number of total anaerobic bacteria, regardless of challenge and addition of enzymes. At the same time, while proliferation of lactobacilli tended ($P=0.07$) to be suppressed due to presence of sDDGS in the diets, supplementation of enzymes did not influence bacterial communities in the ileum of the birds except for a slight increase ($P=0.10$) in coliform counts. There were no significant interactions between main factors with regards to bacterial populations in the ileum.

As shown in Table 6.7, inoculation of the birds in the challenged group, regardless of enzymes and DDGS, influenced the gut microflora of the caecal contents by promoting the growth of total anaerobic bacteria ($P<0.001$) and lactic acid bacteria ($P<0.01$), as well as lactobacilli ($P<0.001$) at d 17. However, the number of lactose-negative bacteria was not significantly altered by any of the factors attributed to the treatments or disease challenge. At the same time, application of enzymes had no significant impact on caecal microflora irrespective to sDDGS and challenge. During the same period, it was found that inclusion of sDDGS increased the number of *C. perfringens* in the caeca, and the highest number of *C. perfringens* in the caecal content was in the infected birds offered diet containing 20 % sDDGS (Table 6.7). This was confirmed by significant interaction ($P<0.01$) between sDDGS and challenge arising from a marked impact of sDDGS ($P<0.01$) and challenge ($P<0.001$) on the proliferation of *C. perfringens* in the caecal content of challenged birds.

Table 6.4 Effects of sDDGS inclusion, enzyme supplementation and disease challenge on bacterial counts in the ileum of broiler chickens at d 13[1]

Treatments		Coliform bacteria	*Clostridium perfringens*	Lactobacilli	Lactic acid bacteria	Total anaerobic bacteria
sDDGS (g/kg)	Enzyme	---------- Log10 CFU/g digesta ----------				
0 (unchallenged)	-	3.85	3.44	7.53	7.98	7.66
	+	4.09	3.85	7.64	7.91	7.79
200 (unchallenged)	-	3.98	3.98	7.52	8.07	7.83
	+	3.43	3.45	7.75	8.32	7.75
0 (challenged)	-	3.74	3.47	6.73	7.48	7.60
	+	4.19	3.73	6.84	7.42	7.48
200 (challenged)	-	3.85	3.68	7.17	7.63	7.72
	+	3.33	3.60	6.89	7.17	7.13
SEM		*0.128*	*0.102*	*0.083*	*0.095*	*0.065*
Main effects						
sDDGS (0)		3.96	3.62	7.19	7.70	7.63
(200)		3.65	3.68	7.33	7.79	7.61
Enzyme	-	3.85	3.64	7.24	7.79	7.70
	+	3.76	3.66	7.27	7.71	7.54
No challenge		3.84	3.68	7.61[a]	8.07[a]	7.76[a]
Challenge		3.78	3.62	6.91[b]	7.42[b]	7.48[b]
Main effects and interactions		----------		*Probability*	----------	
Challenge		NS	NS	<0.001	<0.01	<0.05
sDDGS		NS	NS	NS	NS	NS
Enzyme		NS	NS	NS	NS	NS
Challenge × sDDGS		NS	NS	NS	NS	NS
Challenge × Enzyme		NS	NS	NS	NS	NS
sDDGS × Enzyme		NS	NS	NS	NS	NS
Challenge × sDDGS × Enzyme		NS	NS	NS	NS	NS

[1]Each value for each treatment represents the mean of 6 replicates

NS: Not significant, SEM: Standard error of mean

[a] Means within a column not sharing a superscript differ significantly at the $P<0.05$ for the treatment effects and at the P level shown for the main effects.

Table 6.5 Effects of sDDGS inclusion, enzyme supplementation and disease challenge on bacterial counts in the caeca of broiler chickens at d 13[1]

Treatments		Coliform bacteria	*Clostridium perfringens*	Lactobacilli	Lactic acid bacteria	Total anaerobic bacteria
sDDGS (g/kg)	Enzyme	---------- Log_{10} CFU/g digesta ----------				
0 (unchallenged)	-	5.37[a]	2.20	7.31	7.66	7.71
	+	4.34[b]	2.38	7.42	7.77	7.45
200 (unchallenged)	-	5.63[a]	3.00	7.52	7.77	7.69
	+	5.41[a]	2.95	7.24	7.32	7.39
0 (challenged)	-	5.06[a]	2.62	7.52	7.86	7.78
	+	5.02[ab]	2.69	6.90	7.30	7.29
200 (challenged)	-	5.33[a]	2.50	6.97	7.23	7.27
	+	5.10[a]	2.68	7.58	7.52	7.75
SEM		*0.084*	*0.198*	*0.079*	*0.078*	*0.063*
Main effects						
sDDGS (0)		4.95[b]	2.72	7.29	7.64	7.55
(200)		5.36[a]	2.03	7.32	7.46	7.52
Enzyme	-	5.34[a]	2.83	7.33	7.63	7.61
	+	4.96[b]	2.92	7.28	7.48	7.47
No challenge		5.19	2.13	7.37	7.62	7.55
Challenge		5.12	2.62	7.42	7.48	7.52
Main effects and interactions		---------- *Probability* ----------				
Challenge		NS	NS	NS	NS	NS
sDDGS		<0.05	NS	NS	NS	NS
Enzyme		<0.05	NS	NS	NS	NS
Challenge × sDDGS		NS	NS	NS	NS	NS
Challenge × Enzyme		NS	NS	NS	NS	NS
sDDGS × Enzyme		NS	NS	NS	NS	NS
Challenge × sDDGS × Enzyme		NS	NS	NS	NS	NS

[1]Each value for each treatment represents the mean of 6 replicates

NS: Not significant, SEM: Standard error of mean

[a] Means within a column not sharing a superscript differ significantly at the $P<0.05$ for the treatment effects and at the P level shown for the main effects.

Table 6.6 Effects of sDDGS inclusion and enzyme supplementation and disease challenge on bacterial counts in the ileum of broiler chickens at d 17

Treatments		Coliform bacteria	Lactose-negative Enterobacteria	*Clostridium perfringens*	Lactobacilli	Lactic acid bacteria	Total anaerobes
sDDGS (g/kg)	Enzyme	----------	----------	Log_{10} CFU/g digesta	----------	----------	----------
0 (unchallenged)	-	3.57	1.91	2.70^{c}	7.02	7.09	6.75
	+	4.19	1.00	3.02^{bc}	7.27	7.62	7.35
200 (unchallenged)	-	4.49	1.73	3.04^{bc}	7.04	7.67	6.68
	+	3.94	1.54	2.95^{bc}	6.14	6.79	6.62
0 (challenged)	-	4.56	1.29	3.87^{abc}	7.56	8.14	8.09
	+	5.05	2.73	4.12^{ab}	8.10	8.48	8.47
200 (challenged)	-	3.97	1.61	4.59^{a}	7.33	7.85	7.71
	+	5.26	3.18	4.13^{ab}	7.21	7.85	7.43
SEM		*0.140*	*0.197*	*0.141*	*0.150*	*0.135*	*0.115*
Main effects							
sDDGS (0)		4.34	1.73	3.78	7.48	7.83	7.66^{a}
(200)		4.41	2.01	3.89	6.93	7.54	7.11^{b}
Enzyme	-	4.14	1.63	3.77	7.23	7.68	7.31
	+	4.61	2.11	3.90	7.18	7.68	7.46
No challenge		4.04^{b}	1.54	3.19^{b}	6.86^{a}	7.29^{a}	6.85^{b}
Challenge		4.71^{a}	2.20	4.48^{a}	7.55^{b}	8.08^{b}	7.92^{a}
Main effects and interactions		----------	----------	*Probability*	----------	----------	----------
Challenge		<0.05	0.10	<0.001	<0.05	<0.01	<0.001
sDDGS		NS	NS	NS	0.07	NS	<0.05
Enzyme		0.10	NS	NS	NS	NS	NS
Challenge × sDDGS		NS	NS	NS	NS	NS	NS
Challenge × Enzyme		NS	<0.05	NS	NS	NS	NS
sDDGS × Enzyme		NS	NS	0.07	NS	NS	NS
Challenge × sDDGS × Enzyme		0.09	NS	NS	NS	NS	NS

[1]Each value for each treatment represents the mean of 6 replicates

NS:Not significant, SEM: Standard error of mean

[a,b] Means within a column not sharing a superscript differ significantly at the $P<0.05$ for the treatment effects and at the P level shown for the main effects.

Table 6.7 Effects of sDDGS inclusion, enzyme supplementation and disease challenge on bacterial counts in the caeca of broiler chickens at d 17

Treatments		Coliform bacteria	Lactose-negative Enterobacteria	*Clostridium perfringens*	Lactobacilli	Lactic acid bacteria	Total anaerobes
sDDGS (g/kg)	Enzyme	---------- Log_{10} CFU/g digesta ----------					
0 (unchallenged)	-	4.88^{c}	2.81	3.20^{d}	7.34^{bcd}	7.81^{b}	7.47^{c}
	+	5.04^{c}	2.45	2.99^{d}	7.18^{cd}	8.00^{ab}	7.56^{bc}
200 (unchallenged)	-	5.60^{a}	3.88	3.83^{bc}	7.06^{d}	7.73^{b}	7.46^{c}
	+	5.20^{abc}	2.45	3.49^{cd}	7.38^{bcd}	7.77^{b}	7.51^{bc}
0 (challenged)	-	5.19^{abc}	2.48	3.99^{bc}	8.04^{a}	8.34^{a}	8.28^{a}
	+	5.50^{ab}	3.13	4.06^{b}	7.84^{ab}	8.13^{ab}	7.90^{ab}
200 (challenged)	-	5.14^{bc}	2.79	5.04^{a}	7.74^{abc}	7.86^{b}	7.90^{ab}
	+	5.50^{ab}	2.46	5.16^{a}	8.08^{a}	8.40^{a}	8.27^{a}
SEM		*0.054*	*0.219*	*0.109*	*0.074*	*0.059*	*0.052*
Main effects							
sDDGS (0)		5.15	2.46	3.56^{b}	7.59	8.07	7.80
(200)		5.36	2.89	4.38^{a}	7.56	7.94	7.78
Enzyme	-	5.20	2.99	4.01	7.54	7.94	7.78
	+	5.31	2.37	3.92	7.62	8.07	7.81
No challenge		5.18	2.65	3.38^{b}	7.24^{b}	7.83^{b}	7.49^{b}
Challenge		5.33	2.71	4.56^{a}	7.92^{a}	8.18^{a}	8.09^{a}
Main effects and interactions		---------- *Probability* ----------					
Challenge		NS	NS	<0.001	<0.001	<0.01	<0.001
sDDGS		0.06	NS	<0.001	NS	NS	NS
Enzyme		NS	NS	NS	NS	NS	NS
Challenge × sDDGS		<0.05	NS	<0.05	NS	NS	NS
Challenge × Enzyme		<0.05	NS	NS	NS	NS	NS
sDDGS × Enzyme		NS	NS	NS	0.09	NS	NS
Challenge × sDDGS × Enzyme		NS	NS	NS	NS	0.06	0.06

[1]Each value for each treatment represents the mean of 6 replicates

NS: Not significant, SEM: Standard error of mean

[a] Means within a column not sharing a superscript differ significantly at the $P<0.05$ for the treatment effects and at the P level shown for the main effects.

Furthermore, the interaction between sDDGS and challenge (P<0.05) and also challenge and enzymes (P<0.05) was significant for the number of coliforms in the caecal contents at d 17.

6.3.3 Concentration of short chain fatty acids (SCFA) and pH of ileal and caecal content

The concentration of SCFA in ileal and caecal contents of the birds at d 13 and 17 are summarised in Tables 6.8, 6.9 and 6.10. At d 13, birds challenged with *Eimeria* spp. tended (P=0.09) to have a higher ileal lactic acid concentration, whilst formic acid tended (P=0.09) to be low. Regardless of challenge and supplementation with enzymes, inclusion of sDDGS decreased acetic acid (P<0.01), lactic acid (P<0.001) and total SCFA (P<0.001) concentration in the ileal contents prior to *C. perfringens* inoculations. At the same time, enzyme supplementation did not alter the concentration of SCFA in the ileal contents. In this regard, no interaction attributed to treatments or challenge was observed except between sDDGS and enzymes for the concentration of formic acid (P<0.05). The concentrations of SCFAs were more affected at d 17 when challenge increased acetic acid (P<0.01), lactic acid (P<0.001) and total SCFA in the ileum. Taking sDDGS as a separate factor, a lower (P<0.05) concentration of total SCFA was observed when sDDGS-containing diet was offered to the birds. Notably, there was a significant interaction between disease challenge and sDDGS with regards to total SCFA, especially without the enzyme supplements. A similar strong interaction (P<0.01) between disease challenge and sDDGS on the concentration of lactic acid in the ileum was detected at d 17. On the same day, birds offered diets supplemented with a cocktail of xylanase and protease had lower (P<0.001) ileal concentration of lactic acid and total SCFA, while there was no interaction between enzymes and sDDGS or challenge.

At d 13, an increase was noticed in caecal concentration of acetic acid (P<0.05), propionic acid (P<0.01), butyric acid (P<0.05) and total SCFA (P<0.05) in the birds receiving *Eimeria* spp. (Table 6.9). Besides, isovaleric acid tended (P<0.06) to increase while succinic acid was slightly reduced (P=0.07) in the caeca of the same birds. Irrespective of enzyme addition and *Eimeria* infection, incorporation of sDDGS into diets resulted in higher caecal concentrations of propionic (P<0.001), isobutyric (P<0.01), isovaleric (P<0.01), valeric (P<0.05) and lactic acids (P<0.01) at d 13. Before the *C. perfringens* challenge, supplementation of enzymes

markedly (P<0.001) reduced concentration of isobutyric and valeric acids, whilst concentrations of acetic acid and total SCFA were increased in the caecal contents of the chickens. A significant interaction of sDDGS and microbial enzyme supplementation was observed on the concentration of all SCFA determined in the caecal contents of the birds at d 13 except for butyric acid. The interaction between *Eimeria* challenge and sDDGS was also significant (P<0.001) for the total amount of SCFA in the caeca, with the highest value in the birds offered diet containing sDDGS supplemented with protease and xylanase.

In contrast, on d 13, the concentrations of SCFA in the caecal content were not significantly altered by disease challenge apart from a higher (P<0.01) concentration of succinic acid (Table 6.10). However, inclusion of sDDGS, independently, increased the concentration of SCFAs in the caeca of the birds of the same age except for lactic acid, which was reduced. Birds receiving diets supplemented with enzymes also showed higher (P<0.05) concentrations of succinic acid and tended (P=0.06) to have a higher concentration of SCFA in the caeca after *C. perfringens* inoculations. At this age, the interaction between sDDGS and enzymes was also significant for the concentrations of lactic (P<0.05), isovaleric (P<0.05) and isobutyric acids (P<0.01).

As shown in Figure 6.1, the pH of ileal contents of the birds at d 13 and 17 were significantly (P<0.01) lower in the birds challenged with *Eimeria* spp. and *C. perfringens*. Inclusion of sDDGS significantly (P<0.05) increased the pH in the ileum of the birds before and after the *C. perfringens* challenge. At d 13, caecal pH was only affected by sDDGS which led to a reduction (P<0.01) compared to the birds given control diets. Addition of enzymes did not alter the pH of ileal and caecal contents.

Table 6.8 Effects of sDDGS inclusion, enzyme supplementation and disease challenge on short chain fatty acids concentrations in the ileum of broiler chickens at d 13 and d 17

Treatments		Day 13 (μmol/g digesta)				Day 17 (μmol/g digesta)			
sDDGS (g/kg)	Enzyme	Formic	Acetic	Lactic	Total SCFA	Formic	Acetic	Lactic	Total SCFA
0 (unchallenged)	-	0.08^{bc}	1.7^{ab}	44.7^{a}	47.2	0.1	1.8^{b}	50.4^{bc}	52.6^{bc}
	+	0.15^{abc}	2.1^{a}	38.0^{ab}	41.0	0.2	1.5^{b}	28.5^{cd}	30.4^{de}
200 (unchallenged)	-	0.31^{a}	1.1^{c}	15.6^{c}	17.3	0.1	1.8^{b}	18.7^{d}	21.2^{e}
	+	0.20^{ab}	0.9^{c}	24.4^{bc}	36.5	0.1	2.0^{b}	13.9^{d}	16.1^{e}
0 (challenged)	-	0.01^{c}	1.3^{bc}	40.7^{ab}	42.6	0.2	4.9^{ab}	64.2^{ab}	71.5^{ab}
	+	0.08^{bc}	1.7^{ab}	49.6^{a}	52.2	0.8	7.6^{a}	43.9^{bc}	58.5^{bc}
200 (challenged)	-	0.24^{ab}	1.3^{bc}	31.6^{abc}	33.5	0.1	3.7^{b}	80.2^{a}	85.7^{a}
	+	0.13^{bc}	1.0^{c}	32.9^{abc}	34.4	0.2	2.9^{b}	43.1^{bc}	47.2^{cd}
SEM		*0.022*	*0.09*	*2.53*	*3.49*	*0.07*	*1.15*	*3.68*	*3.95*
Main effects									
sDDGS (0)		0.08^{b}	1.7^{a}	43.3^{a}	45.7^{a}	0.3	3.9	46.7	53.3^{a}
(200)		0.22^{a}	1.0^{b}	26.1^{b}	30.4^{b}	0.1	2.6	39.0	42.6^{b}
Enzyme	-	0.15	1.3	33.2	35.2	0.1	3.0	53.4^{a}	57.8^{a}
	+	0.14	1.4	36.3	41.0	0.3	3.5	32.3^{b}	38.1^{b}
No challenge		0.18	1.4	30.7	35.5	0.1	1.8^{b}	27.9^{b}	30.1^{b}
Challenge		0.12	1.3	38.7	40.7	0.3	4.8^{a}	57.8^{a}	65.7^{a}
Main effects and interactions		----------*Probability*----------							
Challenge		0.09	NS	0.10	NS	NS	0.01	<0.001	<0.001
sDDGS		<0.01	<0.001	<0.001	<0.01	NS	NS	NS	<0.05
Enzyme		NS	NS	NS	NS	0.10	NS	<0.001	<0.001
Challenge × sDDGS		NS	NS	NS	NS	NS	0.09	<0.01	<0.05
Challenge × Enzyme		NS	NS	NS	NS	NS	NS	NS	NS
sDDGS × Enzyme		<0.05	0.07	NS	NS	NS	NS	NS	NS
Challenge × sDDGS × Enzyme		NS	NS	NS	NS	NS	NS	NS	<0.05

[1]Each value for each treatment represents the mean of 6 replicates

NS: Not significant, SEM: Standard error of means

[a] Means within a column not sharing a superscript differ significantly at the $P<0.05$ for the treatment effects and at the P level shown for the main effects.

Table 6.9 Effects of sDDGS inclusion, enzyme supplementation and disease challenge on short chain fatty acids concentrations (µmol/g) in the caeca of broiler chickens at d 13

Treatments		Acetic	Propionic	Iso-But	Butyric	Iso-Vale	Valeric	Lactic	Succinic	Total SCFA
sDDGS (g/kg)	Enzyme	µmol/g digesta								
0 (unchallenged)	-	87.7^{ab}	4.4^{b}	0.6^{ab}	19.6	0.2	1.0^{a}	0.55^{bc}	9.7^{c}	123.9^{b}
	+	83.6^{b}	3.5^{cd}	0.7^{a}	18.7	0.2	1.0^{a}	0.44^{bc}	12.1^{c}	120.6^{b}
200 (unchallenged)	-	80.7^{b}	2.5^{e}	0.3^{c}	16.0	0.1	0.5^{cd}	0.39^{c}	22.6^{ab}	122.9^{b}
	+	82.7^{b}	2.8^{de}	0.3^{c}	18.2	0.1	0.4^{d}	0.32^{c}	15.9^{abc}	121.1^{b}
0 (challenged)	-	66.6^{c}	3.7^{cd}	0.6^{ab}	13.6	0.2	0.9^{ab}	0.67^{ab}	23.7^{a}	109.6^{c}
	+	82.9^{b}	3.9^{bc}	0.7^{a}	19.1	0.2	1.0^{a}	0.39^{c}	10.9^{c}	119.2^{bc}
200 (challenged)	-	84.7^{b}	3.2^{cde}	0.5^{b}	16.2	0.1	0.7^{bc}	0.55^{bc}	12.5^{c}	118.6^{bc}
	+	96.0^{a}	6.1^{a}	0.6^{ab}	20.4	0.3	1.0^{a}	0.84^{a}	14.9^{bc}	141.1^{a}
SEM		*1.28*	*0.11*	*0.02*	*0.65*	*0.02*	*0.03*	*0.033*	*1.02*	*1.25*
Main effects										
sDDGS (0)		80.2^{b}	3.8	0.7^{a}	17.8	0.2	1.0^{a}	0.51	14.1	118.3^{b}
(200)		86.2^{a}	3.7	0.4^{b}	17.7	0.2	0.7^{b}	0.52	16.5	125.9^{a}
Enzyme	-	79.9^{b}	3.4^{b}	0.5	16.4^{b}	0.1	0.8	0.54	17.1	118.8^{b}
	+	86.5^{a}	4.1^{a}	0.6	19.1^{a}	0.2	0.9	0.50	13.5	125.5^{a}
No challenge		83.6	3.3^{b}	0.5^{b}	18.1	0.1^{b}	0.8^{b}	0.42^{b}	15.1	122.1
Challenge		82.8	4.2^{a}	0.6^{a}	17.3	0.2^{a}	0.9^{a}	0.61^{a}	15.5	122.1
Main effects and interactions		*Probability*								
Challenge		<0.05	<0.01	NS	<0.05	0.06	NS	NS	0.08	<0.05
sDDGS		NS	<0.001	<0.001	NS	<0.01	<0.05	<0.01	NS	NS
Enzyme		<0.05	NS	<0.001	NS	NS	<0.001	NS	NS	<0.01
Challenge × sDDGS		<0.01	<0.001	NS	NS	NS	<0.05	NS	NS	<0.001
Challenge × Enzyme		NS	<0.001	NS	NS	NS	NS	<0.05	NS	NS
sDDGS × Enzyme		<0.001	<0.001	<0.001	NS	<0.01	<0.01	<0.05	<0.01	<0.01
Challenge × sDDGS × Enzyme		NS	NS	NS	NS	NS	NS	<0.05	<0.01	NS

[1]Each value for each treatment represents the mean of 6 replicates

NS: Not significant, SEM: Standard error of means

[a] Means within a column not sharing a superscript differ significantly at the $P<0.05$ for the treatment effects and at the P level shown for the main effects.

Table 6.10 Effects of sDDGS inclusion, enzyme supplementation and disease challenge on short chain fatty acids concentrations in the caeca of broiler chickens at d 17.

Treatments		Acetic	Propionic	Iso-But	Butyric	Iso-Vale	Valeric	Lactic	Succinic	Total SCFA
sDDGS (g/kg)	Enzyme	µmol/g digesta								
0 (unchallenged)	-	72.0	3.7^{bc}	0.6^{cd}	16.4	0.2^{cd}	0.7^{b}	1.9^{ab}	6.5^{cd}	104.1
	+	81.5	3.4^{bc}	0.4^{d}	14.9	0.1^{d}	0.6^{b}	1.3^{a}	15.8^{ab}	120.9
200 (unchallenged)	-	83.4	2.2^{c}	0.3^{d}	19.8	0.1^{d}	0.6^{b}	0.4^{bc}	20.5^{a}	127.3
	+	75.2	3.0^{bc}	0.4^{d}	18.3	0.1^{d}	0.7^{b}	0.7^{bc}	17.4^{ab}	115.9
0 (challenged)	-	79.6	7.4^{a}	1.0^{ab}	21.2	0.5^{ab}	1.4^{a}	0.1^{c}	6.2^{d}	117.4
	+	82.1	6.0^{ab}	0.8^{bc}	18.7	0.4^{bc}	1.1^{a}	0.0^{c}	11.4^{bcd}	120.5
200 (challenged)	-	85.8	7.2^{a}	1.3^{a}	21.2	0.6^{a}	1.4^{a}	0.1^{c}	4.4^{d}	121.8
	+	88.3	8.1^{a}	1.3^{a}	20.5	0.7^{a}	1.4^{a}	0.1^{c}	13.9^{abc}	134.3
SEM		*1.67*	*0.38*	*0.05*	*0.72*	*0.03*	*0.04*	*0.23*	*0.923*	*2.34*
Main effects										
sDDGS (0)		78.8	5.1	0.7	17.8	0.3	1.0	2.07^{a}	10.0^{b}	115.8
(200)		83.2	5.1	0.8	20.0	0.4	1.0	0.33^{b}	14.0^{a}	124.8
Enzyme	-	80.2	5.1	0.8	20.0	0.3	1.0	1.13	9.0^{b}	117.7
	+	81.8	5.1	0.7	18.1	0.3	1.0	1.27	14.6^{a}	122.9
No challenge		78.0	3.1^{b}	0.4^{b}	17.4^{b}	0.1^{b}	0.7^{b}	2.34^{a}	15.0^{a}	117.1
Challenge		83.9	7.2^{a}	1.1^{a}	20.4^{a}	0.5^{a}	1.4^{a}	0.06^{b}	9.0 ^{b}	123.5
Main effects and interactions		*Probability*								
Challenge		NS	NS	NS	NS	NS	NS	NS	<0.01	NS
sDDGS		0.08	<0.001	<0.001	<0.05	<0.001	<0.001	<0.01	<0.01	NS
Enzyme		NS	NS	NS	NS	0.10	NS	<0.05	<0.05	0.06
Challenge × sDDGS		NS	NS	NS	NS	NS	NS	NS	NS	NS
Challenge × Enzyme		NS	NS	0.09	NS	0.07	NS	NS	NS	NS
sDDGS × Enzyme		NS	NS	<0.01	NS	<0.05	NS	<0.05	<0.05	NS
Challenge × sDDGS × Enzyme		NS	NS	NS	NS	NS	NS	NS	<0.05	NS

[1]Each value for each treatment represents the mean of 6 replicates

NS: Not significant, SEM: Standard error of means

[a] Means within a column not sharing a superscript differ significantly at the $P<0.05$ for the treatment effects and at the P level shown for the main effects.

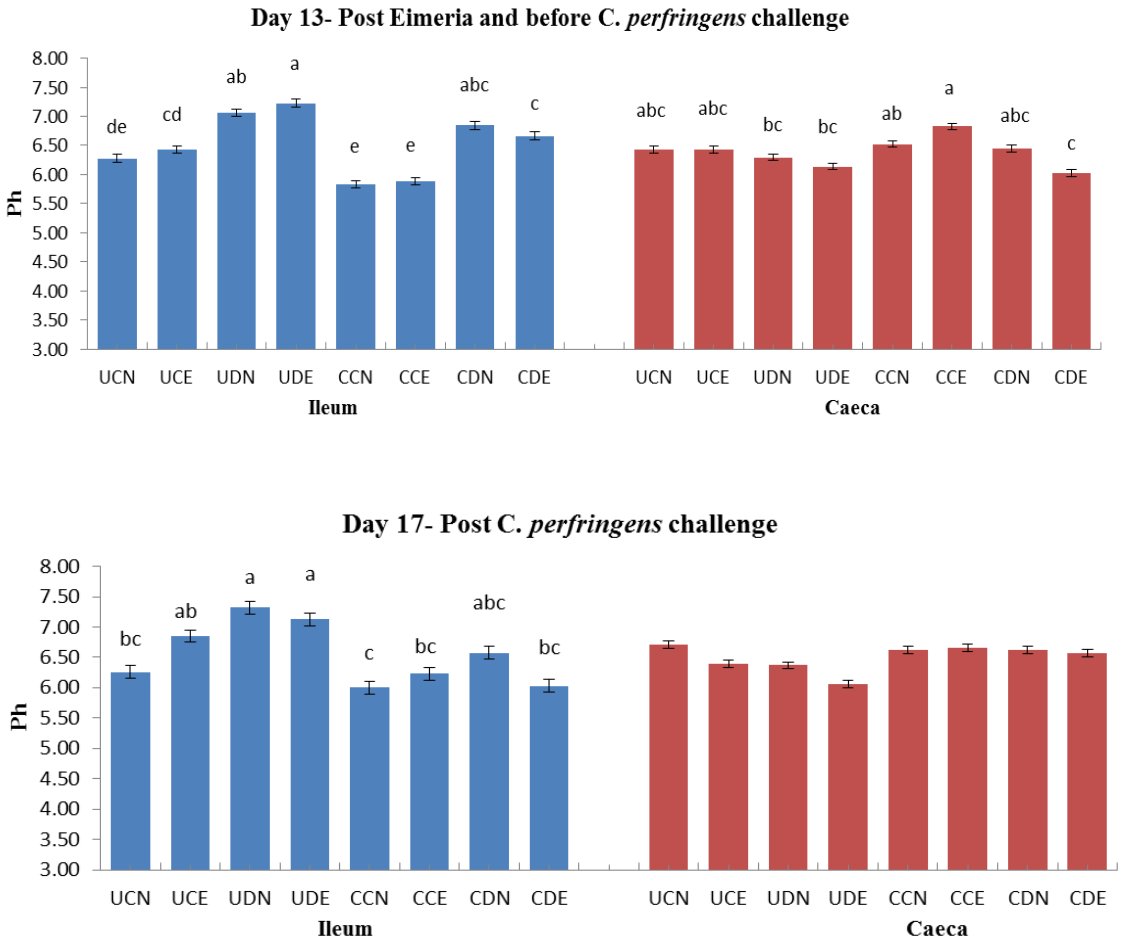

Figure 6.1 pH of ileal and caecal contents of chickens on d 13 and 17 (pre- and post-*C. perfringens* challenge)

Unchallenged control and no enzymes (UCN), Unchallenged control and enzymes (UCE), Unchallenged DDGS and no enzymes (UDN), Unchallenged DDGS and enzymes (UDE), Challenged control and no enzymes (CCN), Challenged control and enzymes (CCE), Challenged DDGS and no enzymes (CDN), Challenged DDGS and enzymes (CDE).

6.3.4 Immune response of the birds

The total serum concentration of immunoglobulins (IgA, IgG and IgM) at days 13, 21 and 35 are shown in Table 6.11. The concentration of IgA at d 21 (P<0.001) and d 35 (P<0.05) was

Table 6.11 Effects of sDDGS inclusion, enzyme supplementation and disease challenge on total serum concentration of immunoglobulins (IgA, IgG and IgM) in broiler chickens at d 13, 21 and 35.

Treatments		IgA (mg/mL)			IgG (mg/mL)			IgM (mg/mL)		
sDDGS (g/kg)	Enzyme	d13	d21	d35	d13	d21	d35	d13	d21	d35
0 (unchallenged)	-	0.18^{c}	0.19^{d}	0.39	0.76	1.83^{d}	5.17	0.17	0.14^{c}	0.34^{bc}
	+	0.22^{c}	0.43^{bc}	0.42	0.99	3.84^{bc}	4.96	0.22	0.20^{c}	0.33^{bc}
200 (unchallenged)	-	0.22^{c}	0.42^{bc}	0.54	0.80	4.09^{bc}	5.24	0.16	0.25^{bc}	0.42^{bc}
	+	0.49^{a}	0.36^{cd}	0.51	1.08	3.06^{bcd}	4.93	0.21	0.14^{c}	0.24^{c}
0 (challenged)	-	0.27^{bc}	0.77^{a}	0.81	1.13	4.59^{ab}	6.71	0.23	0.54^{a}	0.85^{a}
	+	0.25^{bc}	0.60^{ab}	0.59	0.69	4.40^{b}	6.55	0.38	0.34^{abc}	0.65^{ab}
200 (challenged)	-	0.40^{ab}	0.46^{bc}	0.51	1.24	2.20^{cd}	5.29	0.30	0.27^{bc}	0.23^{c}
	+	0.29^{bc}	0.30^{cd}	0.58	0.97	6.41^{a}	6.21	0.25	0.48^{ab}	0.50^{bc}
SEM		*0.019*	*0.027*	*0.033*	*0.054*	*0.246*	*0.332*	*0.021*	*0.032*	*0.041*
Main effects										
sDDGS (0)		0.23^{b}	0.50^{a}	0.55	0.89^{b}	3.70	5.85	0.25	0.31	0.54^{a}
(200)		0.35^{a}	0.39^{b}	0.54	1.02^{a}	3.94	5.42	0.23	0.28	0.35^{b}
Enzyme	-	0.27	0.46	0.56	0.98	3.18^{b}	5.60	0.21	0.30	0.43
	+	0.31	0.42	0.53	0.93	4.43^{a}	5.66	0.27	0.29	0.46
No challenge		0.30	0.35^{b}	0.47^{a}	0.91	3.20^{b}	5.08	0.19^{b}	0.18^{b}	0.33^{b}
Challenge		0.29	0.53^{a}	0.62^{b}	1.00	4.40^{a}	6.19	0.29^{a}	0.41^{a}	0.56^{a}
Main effects and interactions		----------*Probability*----------								
Challenge		NS	<0.001	<0.05	NS	<0.05	NS	<0.05	<0.001	<0.01
sDDGS		<0.01	<0.05	NS	<0.05	NS	NS	NS	NS	<0.05
Enzyme		NS	NS	NS	NS	<0.01	NS	NS	NS	NS
Challenge × sDDGS		NS	<0.001	<0.05	NS	NS	NS	NS	NS	<0.05
Challenge × Enzyme		<0.01	<0.05	NS	NS	NS	NS	NS	NS	NS
DDGS × Enzyme		NS	NS	NS	NS	NS	NS	NS	NS	NS
Challenge × sDDGS × Enzyme		<0.06	NS	NS	NS	<0.001	NS	NS	<0.05	0.06

[1]Each value for each treatment represents the mean of 6 replicates

NS: Not significant, SEM: Standard error of means

[a] Means within a column not sharing a superscript differ significantly at the $P<0.05$ for the treatment effects and at the P level shown for the main effects.

elevated in the challenged birds, but remained unchanged at d 13. Furthermore, infected birds had higher total IgG at d 21 and d 35, and higher IgM at all three of the time evaluations. Irrespective of challenge and enzyme, incorporation of sDDGS to the diets improved (P<0.01) the IgA and IgG titre at d 13, but the d 21 assessment showed a decrease (P<0.05). Birds fed diets containing sDDGS also had lower (P<0.05) IgM at d 35. No effects of treatments were observed for the total immunoglobulins except for an increase (P<0.01) in IgG at d 21 as a result of enzyme supplementation.

There was an interaction of challenge x sDDGS at d 21 (P<0.001) for IgA, and at d 35 (P<0.05) for IgM where the serum IgA and IgM response was lower in the infected birds fed diets containing sDDGS. The same response for IgA was observed for the interaction (P<0.05) between challenge and enzyme, but at d 17 and d 21.

6.4 DISCUSSION

6.4.1 Growth response, lesion scores and mortality

The challenge model used in the current experiment impaired growth performance and induced necrotic-like lesions in the gastrointestinal tract of broilers despite minimal, but significant, mortality observed in the period of inoculations with *C. perfringens.* However, inoculation of the birds with *Eimeria* spp. appeared to have less effect in terms of the magnitude of growth impairment, likely because of the low virulence of vaccine strains that were used in the model (Kocher *et al.*, 2004; Wu *et al.*, 2010). The higher FI and BWG in the birds receiving DDGS for most of the study period is in accordance with the observations in preceding chapters (Chapters 4 and 5).

There is a paucity of data regarding the impact of DDGS on the development of enteric disease; thus, it is difficult to make comparison with other reports. In the literature, however, Whitney *et al.* (2006b) reported that addition of 10% DDGS to the diet reduced the proportion of lesions associated with a *Lawsonia interacellularis* challenge in growing pigs. This result conflicts with the pattern of lesion scores obtained in the current experiment, where lesions were indeed exacerbated in birds fed diet containing DDGS. Differences in species and varying amounts of DDGS inclusion as well as the nature of different diseases may explain this discrepancy between the two experiments. Nevertheless, the inability of

DDGS to prevent necrotic enteritis in the current study is in agreement with results obtained by Perez *et al.* (2011) in a study in which DDGS were included at up to 20% in diets for broilers infected with *E. acervulina.* It is of importance to note that, in the grower phase of feeding (last two weeks) of the present study, the adverse effect of DDGS and additional interaction with challenge was diminished and eventually birds receiving DDGS showed similar performance to the control group of birds. This may be attributed to the presence of insoluble fibre, which has been shown to increase cellular turnover in the intestine; therefore such an effect in the distal portion of the intestine may adversely affect the ability of microorganisms to colonize mucosal cells, particularly in the post-challenge period (Jin *et al.*, 1994). However, further investigation needs to be carried out to elucidate such impact for different ingredients such as DDGS.

The lack of effect of enzyme supplementation in post-*Eimeria* inoculation period (before *C. perfringens*) is in agreement with results reported by Walk *et al.* (2011) who found that a combination of xylanase, protease and phytase failed to alleviate associated reduction in growth performance of the birds exposed to a live coccidia oocyte vaccine.

Published data are lacking and contradictory regarding the effect of enzymes on broiler performance and the mode of action under necrotic enteritis challenge. Jackson *et al.* (2003) found that addition of β-mannanase reduced lesion scores and improved performance in the birds whose growth had previously been retarded by *Eimeria* spp. and C. *perfringens* challenge. The authors attributed such improvement to the stimulation of the immune system and also degradation of mannose. A tendency for the effect of enzyme on BWG in the third week was related to approximately 4.5 % enhancement as the difference observed between non-enzyme supplemented treatments and those fed a combination of xylanase and protease. Some mitigating evidence appears to have arisen from the magnitude of the response to supplemental protease and xylanase which was more pronounced for the diets containing 20 % DDGS, particularly in the challenged group with approximately 12.1 and 2.3 % improvement in BWG and FCR in the whole study period, respectively. Due to the predisposing influence of wheat and barley to necrotic enteritis, an attempt was made to keep the amount of wheat and barley at a similar level in the basal experimental diets in the current experiment, and sDDGS largely substituted sorghum and soybean meal. Therefore, it is most likely that lower protein and amino acid digestibility (observed in Chapter 5) made this diet favourable for enzymes to act on as it has been shown that response to the enzymes is more apparent in nutritionally marginal diets (Cowieson, 2010). This observation concurs with

earlier research reporting mitigation in severity of NE in birds receiving a combination of enzymes.

6.4.2 Intestinal microbial profiles

Similar to the findings of the current experiment, Parker *et al.* (2007) reported a reduction in bacterial number of *Eimeria*-infected broilers. However, Wu *et al.* (2010) found no significant influence of *Eimeria* spp. on microbial profile in a necrotic enteritis challenge model. This would suggest that changes in the bacterial profiles may be more dependent to the diet composition, particularly protein content or feed additives. However, no effect of dietary treatments or enzyme supplementation was found on the ileal microbial profile of broilers prior to *C. perfringens* inoculation. With regards to DDGS, Loar *et al.* (2012) also found no significant effect of DDGS up to 30 % on the proliferation of *C. perfringens* in the caeca of broilers. This is in close agreement with the observation in the current study. In contrast, Perez *et al.* (2011) demonstrated a shift in bacterial populations associated with the caecal mucosa in broilers infected with *Eimeria* spp. and fed increasing levels of DDGS. Nevertheless, in the present study, the focus was on digesta, which probably explains the differences between these observations.

Noteworthy are the known effects of high protein content (Drew *et al.*, 2004) and certain amino acids, for example, methionine (Muhammed *et al.*, 1975; Dahiya *et al.*, 2007b) and glycine (Dahiya *et al.*, 2005), on the proliferation of *C. perfringens.* In the present trial, the interaction between the challenge and diet led to increase in number of *C. perfringens* in the caeca (d 17) and may be associated with a low digestibility of nutrients in the diet containing sDDGS. Although protein and amino acid digestibility were not measured in the current experiment, results of previous experiments (Chapters 4 and 5) revealed that high levels of sDDGS (same material) impaired the digestibility of protein and most amino acids in broilers. Therefore, it is probable that poor digestibility of protein in the DDGS-diet may have resulted in an increase in concentration of protein in the lower intestine, thus encouraging the proliferation of pathogenic bacteria, like *C. perfringens*. However, it was indeed apparent from the result that the effect of DDGS was more pronounced in the caecal digesta than ileal content. It is believed that *Lactobacillus* and *Entrobacteria* species affect gut health. While *Lactobacillus* spp. are regarded as a major component of the microbial barrier to infection, *Entrobacteriaceae* often impair gut health as they include some pathogens like *Escherichia*

coli (Ewing and Cole 1994). Therefore, lower counts of *Lactobacillus* compared to *Entrobacteriaceae* disadvantages gut health. In the current experiment, by incorporating DDGS in the diet, regardless of other factors, there was a strong tendency towards suppression of *Lactobacilli*, which may have impaired performance of that group of birds. A likely explanation may lie in the presence of NSP, which are thought not only to accelerate the proliferation of *Clostridia,* but also simultaneously restrain the growth of other beneficial bacteria in the intestine of chickens, such as *Lactobacillus* spp. (Annett *et al.*, 2002; McDevitt *et al.*, 2006).

6.4.3 Gut fermentation and pH

It is evident from the results that, in challenged birds, total ileal concentration of SCFA increased (before and after *C. perfringens* challenge) in birds fed sDDGS-enriched diet, while the reverse was noted in the unchallenged group. It was expected that an increase in dietary fibre content due to sDDGS would elevate the synthesis of SCFA. However, the fermentation is greatly affected by microbial activity and the population of certain bacteria, and this may have contributed, particularly to the concentration of SCFA in infected birds. The reason for the decrease in fermentation as a result of feeding sDDGS in unchallenged birds is unknown, although it matches with the increased pH observed in the ileal content of those birds. The data confirmed the caeca as the major site of fermentation, with higher concentrations of SCFA than in the ileum. The concentrations of fermentation products were further elevated in the caeca after *Eimeria* inoculation, although the reason for this change in fermentation is unclear. Despite the lack of change in total SCFA, the increase in some individual SCFA in the caeca of the birds (both days) concurs with previous observations (Chapter 4). It has been shown that presence of lactic acid may result in an unfavourable environment for the pathogenic bacteria (Kim *et al.*, 1978; Pollman *et al.*, 1980) and may also promote colonisation of some beneficial bacteria in the gastrointestinal tract of non-ruminant animals (Wells *et al.*, 2005; Pierce *et al.*, 2007). Therefore, a decrease in lactic acid concentration in the challenge birds fed DDGS both in ileum and caeca in d17 is in line with the poor performance of the corresponding birds.

6.4.4 Immunological response

The elevated concentration of total IgA in the infected birds that received the diet containing sDDGS and enzyme suggests a beneficial effect of the by-product or enzyme. However, such

an effect was not accompanied by a reduction in *C. perfringens* count in the intestinal lumen. It is proposed that the elevation in the level of IgA could be linked to the possible increase in B and T lymphocytes, but such interpretation cannot be proven until further investigations are carried out on cell-mediated immune response of challenge birds fed similar diets.

It is also apparent from the results that birds fed DDGS-containing diets responded differently in terms of non-specific IgA and IgG total concentrations. Before inoculation with *C. perfringens*, the higher concentrations of IgA and IgG in the birds fed diets containing DDGS are in agreement with results obtained on healthy grower pigs (Weber and Kerr, 2011). This could be linked to a possible influence of yeast *β*-glucan present in DDGS which has been shown to induce a positive effect on total serum immunoglobulin (Sauerwein *et al.*, 2007; Lim *et al.*, 2009). However, such investigations have not been conducted under challenge conditions. Indeed, the results revealed that the elevated concentration of IgA in birds on DDGS diet was reversed after *C. perfringens* inoculations. In this regard, a nutrient cost will be incurred by the host associated with development of immune response due to diversion of nutrients from growth process to the development and function of immune system (Mountzouris *et al.*, 2010). Such nutrient expense may become exacerbated in the case of feeding a low digestible material (like DDGS) to broiler chickens.

6.5 CONCLUSION

It can be deduced from the results of this study that high inclusion of sDDGS may increase the susceptibility to NE by modulating the proliferation of *C. perfringens* in the intestinal tract. This effect was also accompanied by interaction of dietary DDGS and disease challenge, as well as suppression in immune competence of birds shown by a decrease in total response of immunoglobulin in broilers when birds were inoculated with *C. perfringens*. However, the recovery of infected birds fed on diets containing DDGS after challenge requires further research to elucidate the potential of DDGS inclusion in the diet at a lower level under enteric diseases. This study may represent the first to assess changes in microbial profiles of broiler chickens fed DDGS under subclinical or clinical NE. Therefore, further investigations to elucidate such results are warranted. The tendency towards higher BWG in the birds fed a combination of enzymes and a high inclusion of sDDGS under NE challenge deserves further investigation to ascertain any possible mitigation effect.

CHAPTER 7

EVALUATION OF NET UTILISATION OF ENERGY BY BROILER CHICKENS FED DIETS CONTAINING DISTILLERS' DRIED GRAINS WITH SOLUBLES AND MICROBIAL ENZYMES

Abstract

The present study was conducted to elucidate the impact of distillers' dried grains with solubles (DDGS) and microbial enzymes on energy utilisation of broiler chickens. Two levels of DDGS (0 and 30 %) with or without an enzyme cocktail were used in a 2 × 2 factorial experiment involving a total of 240 male Ross 308 broiler chicks. Each treatment was replicated 6 times with 10 birds per replicate. Birds were given starter diets from d 0 to d 10 and subsequently grower diets until d 28. At d 17, 8 birds per treatment (2 birds per chamber) were transferred to 16 closed-circuit calorimeters for the assessment of heat production from d 21 to 24. Simultaneously, the comparative slaughter (CS) technique was applied to the birds from d 18 to 28 in a total of 24 cages to evaluate the body composition, heat production and net energy of production (NEp). Feeding the DDGS-containing diet impaired ($P<0.001$) feed efficiency in the starter and grower phase of feeding. Metabolisable energy intake of the birds remained unaffected by experimental treatment. However, birds given the diet containing DDGS consumed more O_2 ($P<0.01$), had a lower RQ ($P<0.05$), and produced more heat ($P<0.01$) than the control birds when assessed by indirect calorimetry (IC). With either method, NE of the diet and the efficiency of ME for NE was significantly ($P<0.05$) poorer for the DDGS-diet, regardless of enzyme supplementation. Addition of enzymes was ineffective in improving the ratio of NE:ME. Net energy of production (NEp) of the birds fed the control diet was superior ($P<0.001$) to that of birds fed DDGS. Supplementation with enzyme markedly improved ($P<0.01$) NEp of the birds from d 18 to 28. The analyses of body composition showed a decrease ($P<0.05$) in the fat deposition and revealed a tendency ($P=0.06$) to an increased protein deposition when a high level of DDGS was fed to the birds. To conclude, NE and NEp of the diet were highly sensitive to the inclusion of DDGS in the diet, while ME was not significantly affected by dietary treatments.

7.1 INTRODUCTION

The development of a more precise estimate of available energy in raw materials and therefore diet for poultry is gaining importance as feed energy prices have constantly increased over the past few decades. Although the evaluation of energy value of feed for poultry has been predominantly based on ME contents, the net energy (NE) system represents a potentially more precise method to assess available energy for non-ruminant animals as it accounts for the amount of energy lost as heat. The advantage of a net energy system corresponds to the fact that the energy values of diet and maintenance are independent of the feed characteristics (Noblet *et al.*, 2010a). Several investigations (De Groote, 1974; Pirgozliev and Rose, 1999; Carré *et al.*, 2002) have been carried out in the past in an attempt to evaluate the NE value of diets for poultry but the adaptation of such a system is still practically impaired. The major shortcomings of the ME system comprise overestimation of the energy value of fibrous and high protein feedstuff and underestimation of starch or fat-rich ingredients (Noblet *et al.*, 1994). In this regard, it has been shown in the pig industry that, in an NE system, fibrous material can be included at a higher level and such diets are practically lower in crude protein, which can result in substantial economic benefits (Payne and Zijlstra, 2007).

The nutritive value of distillers' dried grains with solubles (DDGS) as a fibre-rich ingredient and in poultry diets (Lumpkins *et al.*, 2004; Thacker and Widyaratne, 2007; Swiatkiewicz and Koreleski, 2008) and ME content of this material (Cozannet *et al.*, 2010; Adeola and Zhai, 2012), as well as the effect of the application of enzymes on the ME value of broiler diets (Min *et al.*, 2009b) have been documented in the literature. However, information regarding the influence of such by-products on the net energy value of broiler diets is lacking. In addition, the lack of differentiation between diets of poultry in terms of NE value and the efficiency of ME for NE (ME:NE ratio) has been reported in the literature (Carré *et al.*, 2002).

The effect of supplemental enzymes, particularly carbohydrases, on ME of diets for broilers is inconsistent. In some cases, no alteration in ME was observed, although the improvement of broiler productivity as a result of enzyme addition may be apparent (Hong *et al.*, 2002; Wu *et al.*, 2004). Recently, it has been proposed that net energy of production (NEp) can be a better estimate of energy utilisation in broilers, when exogenous enzymes, in particular phytase, are added to the diet. This has been confirmed with a high correlation between BW

and NEp (Pirgozliev *et al.*, 2011). Therefore, the potential of exogenous enzyme application in poultry diets would be promising when NE of the diet is evaluated. The net value of the diet can be determined using various techniques. The comparative slaughter technique (CS), carbon-nitrogen method or indirect calorimetric (IC) measurements have been employed in a range of studies to evaluate the effect of different diets and feed additives in poultry. The aim of the present study was to investigate the impact of a high level of DDGS inclusion and enzyme supplementation on net energy. The IC and CS were used to further assess the effects on heat production, net energy value of the diets and body composition in the carcasses.

7.2 MATERIALS AND METHODS

7.2.1 Bird management and diet preparation

A total of 240 male Ross 308 broiler chicks were used in a 2×2 factorial design having 6 replicates per treatment in a total of 24 experimental brooder cages. Four extra cages were also allocated to an extra 40 chickens receiving 4 experimental diets until day 17 when birds were transferred to calorimetric chambers. Two basal experimental diets were formulated in such a way that 300 g/kg sorghum DDGS was incorporated in a corn-wheat based diet. The diets were with or without an enzyme supplement. The enzyme cocktail comprised of xylanase, protease, amylase and glucanase activities. Xylanase (Ronozyme WX), protease (Ronozyme ProAct) and amylase and glucanase (Ronozyme A) were added at the recommended levels of 0.25, 0.2 and 0.25 g/kg diet, respectively. Thus, experimental diets included a control diet with or without enzymes and a diet having 30 % DDGS with or without enzymes, making 4 dietary treatments. All diets were formulated to be isoenergetic, isonitrogenous and meet or exceed nutrient specifications and digestible amino acid requirements recommended for Ross 308 (2007). The composition of the diets is presented in Table 7.1.

Birds were given starter diets from d 0 until d 10 when they are transferred to grower diets. All diets were pelleted before being fed to the birds. The measurements of energy utilisation in calorimetric chambers were carried out from 21 to 24d which was in the middle of 18 to 28d period when the comparative slaughter technique was applied to the birds from the same batch of birds, simultaneously.

Table 7.1 Ingredients and nutrient composition of experimental diets

Ingredients (g/kg)	Starter		Grower	
	Control	sDDGS	Control	sDDGS
Maize	251.8	200.0	302.6	257.1
Wheat	283.0	197.0	277.5	185.0
Soybean Meal 48%	323.8	170.0	273.7	134.0
sDDGS	0.0	300.0	0.0	300.0
Canola Oil	32.0	43.0	39.5	50.0
Soy concentrate 68%	60.0	34.7	60.0	25.0
Dicalcium phosphate	19.2	18.7	17.6	13.5
Limestone	13.2	13.2	11.8	13.8
Sodium chloride	4.5	2.3	4.5	1.5
Vitamin and mineral premix[1]	2.0	2.0	2.0	2.0
Sodium bicarbonate	2.0	2.0	2.0	2.0
DL-Methionine	2.5	2.8	2.7	2.8
L-Lysine HCl	0.8	6.1	0.8	5.8
Choline Cl -70%	0.0	0.0	0.3	0.0
L-Arginine	0.0	1.8	0.0	1.6
L-Threonine	0.2	1.4	0.0	0.9
TiO_2	5.0	5.0	5.0	5.0
Nutrients calculated				
M.E. (MJ/kg)	12.56	12.56	12.97	12.96
Protein (g/kg)	239.9	239.4	221	221
Crude fibre	27.4	44.4	26.4	43.6
Calcium	10.4	10.2	9.1	9.1
Total P	7.7	8.1	7.2	7.0
Available P	5.2	5.3	4.5	4.5
Choline	15.0	17.5	15.7	16.8
Arg	16.4[2] (14.5)[3]	14.5 (13)	14.8 (13.1)	12.7 (11.5)
Ile	10.8 (9.1)	9.2 (8.2)	9.8 (8.5)	8.2 (7.5)
Lys	14.3 (13.1)	13.6 (13.0)	13.0 (11.4)	12.0 (11.3)
Met	6.1 (4.7)	6.0 (4.7)	6.0 (4.8)	5.8 (4.8)
Met + Cys	10.0 (9.3)	9.8 (9.1)	9.2 (8.6)	9.1 (8.5)
Thr	9.5 (8.3)	9.5 (8.3)	8.6 (7.5)	8.3 (7.5)

[1] Contained vitamins and minerals in milligrams per kilogram of diet as follows: vitamin A (as all-trans retinol), 12,000 IU; cholecalciferol, 3,500 IU; vitamin E (as d-α-tocopherol), 44.7 IU; vitamin B12, 0.2 mg; biotin, 0.1 mg; niacin, 50 mg; vitamin K3, 2 mg; pantothenic acid, 12 mg, folic acid, 2 mg; thiamine, 2 mg; riboflavin, 6 mg; pyridoxine hydrochloride, 5 mg; D- calcium panthothenate, 12 mg; Mn, 80 mg; Fe, 60 mg; Cu, 8 mg; I, 1 mg; Co, 0.3 mg; and Mo, 1 mg.
[2]Values of amino acids based on total amino acid [3]Values of amino acids based on digestible amino acids

Temperature was set at 33-34 ºC on the first day of the experiment and then gradually decreased by 1 ºC every second day until 24 ºC was reached by d 21. A lighting program of 18 h light and 6 h darkness was maintained throughout the trial except the first day when

birds had 24 hours of light. Birds had access to feed and water *ad libitum*, from water and feeding troughs attached to each cage.

7.2.2 Indirect calorimetric measurement

A total of 32 male broiler chickens were selected from the same batch of birds receiving experimental treatments up to 17 d with a body weight close to the average of the respective experimental cages. Birds were subsequently assigned to 16 closed-circuit respiratory chambers set up in a climate-controlled house. Each of 4 experimental treatments was replicated 4 times accommodating 2 birds per chamber. Birds were given 4-day adaptation period with *ad libitum* access to feed and water prior to commencing the actual measurements on d 21.

7.2.2.1 Description of respiratory chambers and operational procedure

All 16 calorimetric chambers were similar in design described by Farrell (1972), but were larger in size (100 cm long × 76 cm high × 70 cm wide), and equipped with a wire-mesh cage 89 cm long × 60 cm high × 60.5 cm wide (Figure 7.1). Water was used to seal the chamber according to the modifications made by Farrell (1972). The pressure was controlled by an electronic sensor mounted on each chamber and connected to a solenoid valve. Temperature and humidity of each chamber were constantly monitored using an electronic thermometer and humidity sensor (Figure 7.1). Air was circulated with an air pump calibrated to pump at 24 L/min. Chamber air was driven through a screw-capped plastic bottle containing 2 L of potassium hydroxide (KOH). The moisture of each chamber was absorbed by equipping a polycarbonate train containing approximately 3 kg of dried silica gel connected immediately after the bottle containing KOH to return the air to the chamber. The concentration of KOH was adjusted to 32% for all chambers to trap carbon dioxide (CO_2) exhaled by the birds in sealing chambers. Oxygen (O_2) was provided by equipping each chamber with a 490 L cylinder fitted with a regulator and a reducing valve.

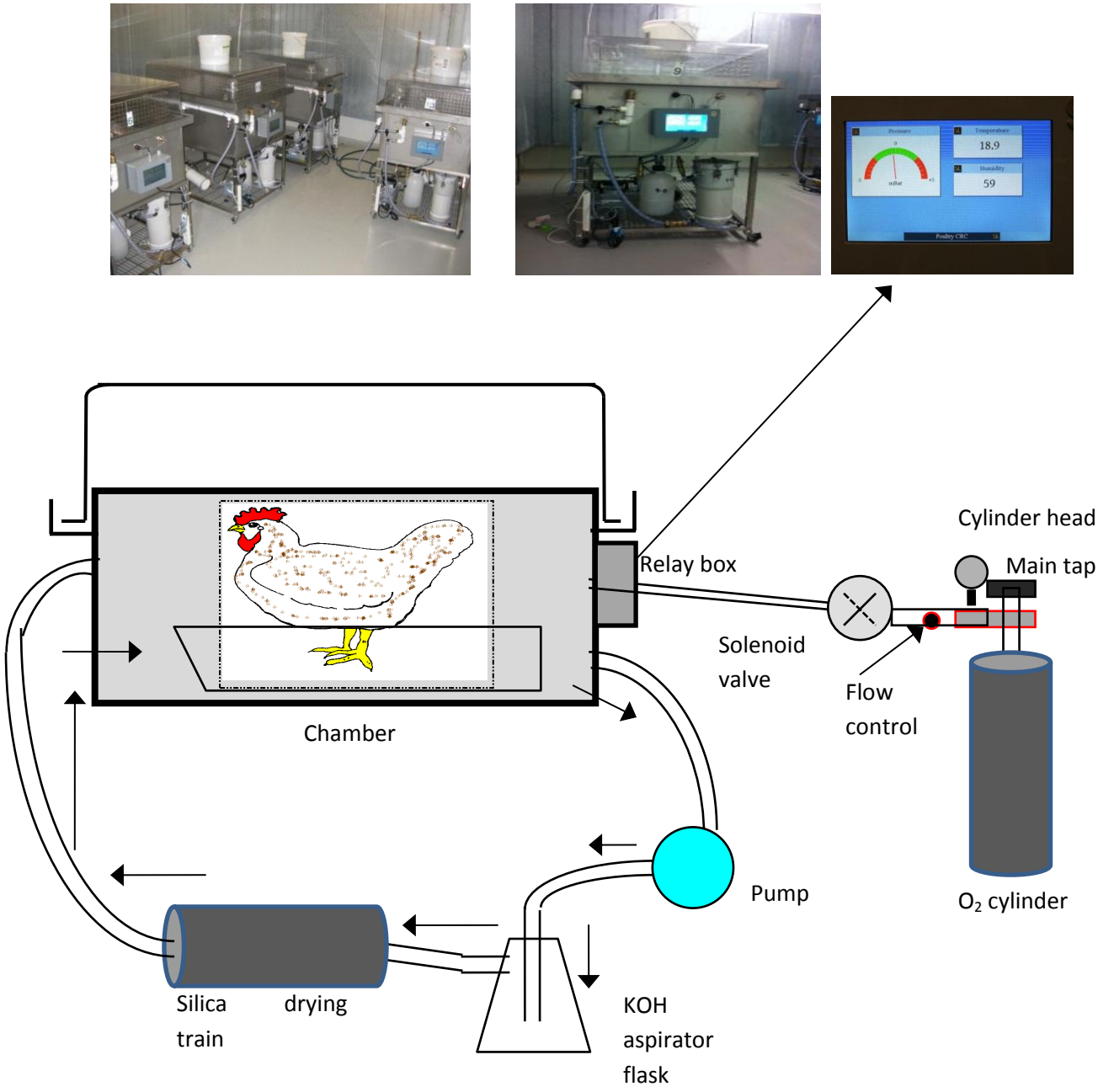

Figure 7.1 Schematic diagram of close-circuit calorimetry (Adapted from Yang, 2008)

7.2.2.2 AME determination (total collection)

The AME was determined by collecting all excreta during the 3 days of measurements in chambers. Feed consumption was accurately recorded throughout the experiment. Spilled feed and feathers were collected from excreta, and all excreta collected were subsequently oven-dried at 80 ºC for 24 h. The GE energy content of experimental diets and excreta from each chamber were determined using an adiabatic bomb calorimeter (IKA® WERKE, C7000,

GMBH and CO., Staufen, Germany) with benzoic acid as the standard. The ME of diets (kJ/g) was calculated according to the following equation:

$$\text{ME (MJ/Kg diet)} = \frac{(\text{Feed intake} \times \text{Diet GE}) - (\text{Excreta output} \times \text{Excreta GE})}{\text{Feed intake}}$$

7.2.2.3 Oxygen consumption and carbon dioxide recoveries

The O_2 consumption was calculated as the difference in weight of the oxygen cylinder at the beginning and end of each run. Subsamples of KOH from each chamber were taken after all the solutions from each KOH bottle was made up to 2 L. Collected KOH samples were kept at 4 ºC until analysed for CO_2 recoveries. The recovery of CO_2 was performed according to the method described by Annison and White (1961) and Swain (1980) based on a barium chloride ($BaCl_2$) precipitation technique. Briefly, 1 mL of KOH solution was accurately pipetted into a dried and pre-weighed 15-mL centrifuge tube in duplicate. Subsequently, 1.5 mL of NH_4Cl was added to each tube. The solution was gently swirled and mixed thoroughly. After the addition of 5 mL $BaCl_2$ to the tubes, the mixture was centrifuged for 15 min at 3000*x*g. The supernatant from each tube was carefully decanted and the carbonate pellet was then resuspended in 5 ml distilled water followed by centrifugation for 30 min at 3500 rpm. The supernatant was subsequently decanted and the tubes were dried overnight at 105 ºC. Finally, tubes were cooled in a desiccator and accurately weighed to record the $BaCO_3$ recovered from 1 mL aliquot of KOH solution. The CO_2 recovery was then calculated by multiplying the weight of $BaCO_3$ (in 2 L KOH) by 0.2229 (the fraction of molecular weight of CO_2 to the molecular weight of $BaCO_3$).

7.2.2.4 Heat production and estimation of basal metabolism

The degree of oxidation of diets corresponds to the O_2 consumed and the amount of CO_2 produced by birds. Therefore, those values from each chamber were used to calculate heat production (HP) according to the Brouwer equation (1965) without correction for urinary nitrogen. Heat production was measured for 3 days in sealed chambers, but was suspended for about 2-3 hours each day to allow for collection of excreta, replenishing feed and water, and replacement of KOH bottles and silica gel trains with new sets. Observations of HP were made on an hourly basis and then converted to a 24 h basis taking the time of readjusting the system into account. The respiratory quotient (RQ) of the 3-day run was also calculated as a

ratio between the volumes of CO_2 produced to the volume of O_2 consumed by birds. In order to calculate heat increment, the estimation of fasting heat production (FHP) is essential. Thus, the fasting heat production was estimated using the value of 450 kJ/$BW^{0.70}$ per day proportional to BW of the birds corresponding to the asymptotic HP (at zero activity) over a 24 h fasting as proposed by Noblet *et al.* (2010b).

7.2.2.5 Net energy value of the diets

Net energy intake and NE value of the diets were calculated as described by Noblet *et al.* (1994). The fasting heat production value plus the energy retained in the birds gave NE intake. Therefore, retained energy (RE) as kJ/d was calculated by deduction of HP (kJ/d) from metabolisable energy intake (MEI) (kJ/d). Net energy value of the diet was then calculated as kJ/kg DM as follow:

NE = (RE+FHP) / DMI

where NE is net energy (calorimetric measurements) as kJ/kg DM, RE is retained energy (kJ/d), FHP is fasting heat production (kJ/d) and DMI is dry matter intake (kg).

7.2.3 Comparative slaughter technique

On day 18, the birds in experimental cages were given approximately 6 hours fasting by withdrawal of feed. After weighing birds, two birds from each replicate (12 per treatment) with weight close to the cage average were chosen, humanely euthanased by cervical dislocation, and immediately frozen until laboratory analyses were performed. The remaining birds were reared until day 28 when the same procedure was carried out, followed by slaughtering another 2 birds per cage. In order to determine ME, excreta sample collection from each cage was performed in the last 4 days of the experiment from d 24 to 28.

Two chickens per replicate, collected at d 18 or 28, were processed together. After chopping birds, the whole intact chicken was coarse-ground, thoroughly mixed with a blender until a homogenised subsample was able to be taken. To calculate DM of carcasses, the wet subsamples were accurately weighed before and after freeze-drying and finally ground and kept in an air-tight container prior to further analyses. Fat, protein and energy content of

samples were determined according to the methods described in Section 3.2.2. The concentration of TiO_2 in the diet sample and excreta was determined using the method of Short *et al.* (1996) as described in Section 4.2.4.1.

Calculations

A series of calculation were carried out according to Olukosi *et al.* (2008) and Pirgozliev *et al.* (2011):

ME (MJ/kg) was calculated for the period of 10 days from d 18 to d 28 using the following formula:

$ME = GE_d - [GE_e \times (Ti_d/Ti_e)]$

where GE_d is gross energy (MJ/kg) in diet; GE_e is the gross energy (MJ/kg) in excreta, Ti_d is the concentration of titanium in the diets; and Ti_e is the concentration of titanium in the excreta.

To calculate total energy retained in the carcasses (RE), the following calculations were performed:

(A) Initial GE content of carcase at d 18 (KJ) = carcase GE (KJ/g) × body weight of bird at d 18 (g)
(B) Final gross energy content of carcase at d 28 (kJ) = carcase GE (KJ/g) × body weight of bird at d 28

RE (kJ) = (B) – (A)

Dietary net energy value for production was then calculated using the following formula:

NEp (kJ/g diet) = RE / Feed intake

ME intake (MEI) was calculated by multiplying the ME (kJ/g) by the amount of feed (g) consumed by each bird. Heat production (HP), including heat increment and fasting heat production, was subsequently calculated by deduction of RE from ME intake:

HP (kJ) = (MEI – RE).

To compare the NE value of the diet by two methods, FHP of the birds was estimated in a similar way for the birds in chambers using the value of 450 kJ/$BW^{0.70}$ (Noblet *et al.* 2010a,b).

Dietary NE corrected for FHP was subsequently calculated using the same formula as previously mentioned in Section 7.2.2.

The following formulas were used to calculate energy retained as fat (RE_f) and protein (RE_p):

RE_f (kJ) = (Carcase fat content d 28 (g) - Carcase fat content d 18 (g)) × 38.2 kJ/g

RE_p (kJ) = (Carcase protein content d 28 (g) - Carcase protein content d 18 (g)) × 23.6 kJ/g

where 38.2 and 23.6 kJ/g are the energy value per gram of fat and protein according to Larbier and Leclercq (1992).

The efficiency of ME use for energy, lipid and protein retention was calculated using the following formulas:

Efficiency of ME use for energy retention (K_{RE}) = NEp/MEI

Efficiency of ME use for fat retention (K_{REf}) = RE_f/MEI

Efficiency of ME use for protein retention (K_{REp}) = RE_p/MEI

7.2.4 Animal ethics

The Animal Ethics Committee of the University of New England approved all the experimental procedures of this experiment (AEC 10/075).

7.2.5 Statistical analyses

All data were subjected to a two-way ANOVA as a 2×2 factorial arrangement of treatments using General Linear Model procedures of SAS to test the main effects of diets, enzyme supplementation and their interaction. All data were checked for normal distribution before any analyses were performed. For all variables, when a significant difference was detected,

means were separated using least square means option of SAS at $P \leq 0.05$. All results are presented for both the main and treatment effect means.

7.3 RESULTS

7.3.1 Growth response and bird performance

The performance data are presented in three periods (Table 7.2). The starter period was 0 to 10 d; the grower period was from d11 to 18; and the third period was from day 18 to 28 d during which measurements by comparative slaughter technique and indirect calorimetry were taken concurrently. Feed consumption increased (P<0.01) in the birds fed DDGS in 0-10d. Dietary treatments did not affect BWG of the birds during the starter phase of feeding. The same was revealed by feeding grower diets excluding days 10 to 18 when birds on the control diet showed higher (P<0.001) BWG. However, regardless of DDGS inclusion, birds gained more weight (P<0.05) when starter diets were supplemented with an enzyme cocktail. Feed conversion ratio was markedly impaired (P<0.01) in the whole period of study DDGS was incorporated in the diets. Supplementation of enzymes was only effective in the first 10 days of the study during which the FCR was significantly improved (P<0.05) independent of diet effect. The mortality was not affected by experimental diets in this trial.

7.3.2 Respiratory quotient (RQ) and heat production (HP) from indirect calorimetric measurements

The data for the RQ and heat production measured in calorimetric chambers are presented in Table 7.3. The consumption of O_2 increased (P<0.01) in the birds fed DDGS-containing diets while there was no significant effect of experimental diets on the volumes of CO_2 produced by the birds. The relevant RQ decreased (P<0.05) in the birds given DDGS-containing diets and markedly increased (P<0.05) as enzymes were added to the diets.

Table 7.2 Effects of supplementation of an enzyme cocktail[1] and a high level of DDGS inclusion on the performance of broiler chickens

Treatments	Enzymes	Feed intake (g/b)			Body weight gain (g/b)			FCR		
		d0-10	d11-17	d18-28	d0-10	d11-17	d18-28	d0-10	d11-17	d18-28
Control	-	266.1	696.9	1370.2	195.3	472.1	865.2	1.36	1.48	1.58
	+	267.7	717.2	1313.6	208.3	500.8	860.5	1.29	1.44	1.54
DDGS	-	291.8	729.6	1364.8	196.4	443.1	817.4	1.49	1.65	1.67
	+	289.9	694.9	1376.1	205.4	432.0	841.5	1.41	1.61	1.64
SEM		*3.29*	*8.17*	*22.05*	*2.64*	*5.99*	*17.62*	*0.017*	*0.023*	*0.018*
Main effects[3]										
Diet	Control	266.8[b]	707.1	1341.9	201.8	486.4[a]	862.8	1.32[b]	1.46[b]	1.56[b]
	DDGS	290.8[a]	712.2	1370.4	200.9	437.5[b]	829.5	1.45[a]	1.63[a]	1.65[a]
Enzyme	-	278.9	713.2	1367.5	195.8	457.5	841.3	1.43[a]	1.57	1.63
	+	278.8	706.1	1344.8	206.8	466.4	850.9	1.35[b]	1.52	1.59
Source of variation		----------			*Probability* ----------					
Diet		<0.001	NS	NS	NS	<0.001	NS	<0.001	<0.001	<0.05
Enzyme		NS	NS	NS	<0.05	NS	NS	<0.05	NS	NS
Diet × Enzyme		NS	NS	NS	NS	NS	NS	NS	NS	NS

[1]Enzyme combination included xylanase, protease, amylase and glucanase.

[2]Each value represents the mean of 6 replicates (cages) for each treatment group

NS: Not significant, SEM: Standard error of means

[a] Means within the same column not sharing a superscript differ significantly at the *P* level shown.

Table 7.3 Effects of supplementation of an enzyme cocktail[1] and a high level of DDGS inclusion on respiratory quotient (RQ) and heat production (HP) of broiler chickens from indirect calorimetric measurements

Treatments	Enzyme	V_{O2} (L/b/d)	V_{CO2} (L/b/d)	RQ	HP (KJ/b/d)	HP (KJ/kg $BW^{0.70}$/d)	FHP estimate (KJ/kg $BW^{0.70}$/d)
Control	-	45.96	47.89	1.04	983.8	914.4	521.0
	+	43.52	47.11	1.08	940.5	866.2	526.9
DDGS	-	46.75	47.32	1.02	993.8	941.9	512.7
	+	46.84	48.52	1.04	1001.3	930.8	524.1
SEM		*0.243*	*0.323*	*0.006*	*5.11*	*6.21*	*4.79*
Main effects[3]							
Diet	Control	44.73[b]	47.49	1.06[a]	962.1[b]	890.3[b]	523.9
	DDGS	46.79[a]	47.92	1.02[b]	997.5[a]	936.3[a]	518.4
Enzyme	-	46.35	47.60	1.03[b]	988.7	928.1	516.9
	+	45.18	47.81	1.06[a]	970.9	898.5	525.5
Source of variation		---------- *Probability* ----------					
Diet		<0.01	NS	<0.05	<0.05	<0.01	NS
Enzyme		0.07	NS	<0.05	NS	0.07	NS
Diet × Enzyme		<0.05	NS	NS	0.06	NS	NS

[1]Enzyme combination included xylanase, protease, amylase and glucanase.

[2]Each value represents the mean of 4 replicates (chamber) for each treatment group

NS: Not significant, SEM: Standard error of means

[a] Means within the same column not sharing a superscript differ significantly at the *P* level shown.

Accordingly, heat production measured by gaseous exchange was influenced by the experimental treatments in a way that birds on DDGS-containing diets released more heat ($P<0.05$) when compared to the bird that received the control diet. In addition, enzyme supplementation tended to result in less ($P=0.07$) heat production compared to the birds fed enzyme-free diets when expressed as a proportion of BW.

A similar estimation of FHP was observed proportionate to BW of broilers. For these variables, interaction of diet and enzyme supplementation was deemed to be not significant except for O_2 consumption of the birds, which tended to decrease ($P=0.07$) with enzyme supplementation only in the birds given the control diet.

7.3.3 Metabolisable energy and net energy values of diets from indirect calorimetric measurements

As shown in Table 7.4, although metabolisable energy determined by the total collection method in the chambers was lower (P<0.001) in birds receiving DDGS-diet, there was no effect on ME intake for the experimental groups. No impact of enzyme addition was observed on either ME of dietary treatments or ME intake of the birds. The value of NE for the control diet was markedly (P<0.001) superior to the diet containing DDGS, but enzyme supplementation did not reveal a significant impact on NE. In addition, the efficiency of ME for the NE was significantly (P<0.05) poorer for the DDGS-diet regardless of enzyme supplementation, which was deemed to be ineffective to improve the ratio of NE to ME.

7.3.4 Heat production and net energy for production and diet measured by comparative slaughter technique

As presented in Table 7.4, ME and ME intake remained unaffected from 18 to 28d, the period in which the measurements were carried out. Using CS, neither diet nor enzyme supplementation altered the HP of the birds. However, as a proportion of BW, birds on DDGS-diet released more heat (P<0.01) compared to those fed the control diet with no effect of enzyme observed on HP from d 18 to d 28.

The net energy value of the diet (P<0.001) and NE:ME ratio (P<0.01) measured by CS were lower in the birds fed DDGS. The NE of the diet was higher (P<0.05) in the birds fed diet supplemented with enzymes (Table 7.4). Broilers given control diet retained more (P<0.01) energy than those fed diet containing DDGS (Table 7.5). Similarly, diets supplemented with the enzymes led to a higher (P<0.01) RE compared to the birds receiving enzyme-free diets, regardless of DDGS. Net energy for production (NEp) was adversely affected (P<0.001) as DDGS was incorporated into the diet, while enzymes markedly counteracted (P<0.01) this effect, exhibiting 0.37 unit improvement, independent of diet effect.

Table 7.4 Heat production, dietary metabolisable energy, net energy and efficiency of ME for NE measured by comparative slaughter technique or indirect calorimetric method

Treatments	Enzyme	MEm (KJ/g)	MEt (KJ/g)	MEI (KJ/b/d)		HP (KJ/kgBW$^{0.70}$)		NE (KJ/g)		NE:ME	NE:ME
Method		CS	IC	CS	IC	CS	IC	CS	IC	CS	IC
Control	-	12.57	12.95	1680.8	1552.4	860.5	914.4	9.17	9.07	0.77	0.70
	+	12.79	13.01	1700.7	1504.1	834.7	866.2	9.55	9.42	0.78	0.73
DDGS	-	12.21	12.38	1666.7	1502.8	938.2	941.9	8.40	8.42	0.72	0.68
	+	12.62	12.37	1713.8	1524.8	916.3	930.8	8.90	8.51	0.74	0.68
SEM		*0.098*	*0.055*	*19.46*	*30.65*	*14.45*	*6.21*	*0.086*	*0.077*	*0.007*	*0.006*
Main effects											
Diet	Control	12.67	12.98^{a}	1690.7	1528.2	847.6^{b}	890.3^{b}	9.36^{a}	9.24^{a}	0.77^{a}	0.71^{a}
	DDGS	12.41	12.40^{b}	1690.2	1513.8	927.3^{a}	936.3^{a}	8.65^{b}	8.46^{b}	0.73^{b}	0.68^{b}
Enzyme	-	12.40	12.66	1673.7	1527.6	899.3	928.1	8.79^{b}	8.74	0.75	0.69
	+	12.70	12.72	1707.2	1514.5	875.5	898.5	9.23^{a}	8.96	0.76	0.70
Source of variation		----------*Probability*----------									
Diet		NS	<0.001	NS	NS	<0.01	<0.01	<0.001	<0.001	<0.01	<0.05
Enzyme		NS	NS	NS	NS	NS	0.07	<0.05	NS	NS	NS
Diet × Enzyme		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

IC: Indirect calorimetry

CS: Comparative slaughter technique

MEt: Metabolisable energy measured by total collection

MEm: Metabolisable energy measured by marker (TiO_2)

MEI : metabolisable energy intake

NE: Corrected for fasting heat production

NS: Not significant, SEM: Standard error of means

7.3.5 Body composition and the efficiency of ME for the energy deposited as fat and protein

The results of body composition are presented in Table 7.5. Deposited protein and the value of energy as protein in the body were not significantly altered. However, birds given the control diet tended (P=0.06) to deposit less protein in the carcasses. In addition, deposition of fat and energy value as fat were markedly lower (P<0.05) in the birds receiving the diets containing DDGS. Enzyme supplementation had no effect on these parameters. Furthermore, the ratio between the values of RE_p and RE_f was significantly higher (P<0.05) in the birds fed DDGS, reflecting the tendency (P=0.06) to retain more protein than fat in the carcasses of those groups of birds.

Table 7.5 Effects of supplementation of an enzyme cocktail[1] and a high level of DDGS inclusion on protein and fat retention, energy and the energy retained in form of fat and protein in broiler chickens from d 18 to d 28 measured by comparative slaughter technique

Treatments	Enzymes	Retained protein (g/b)	Retained fat (g/b)	RE (KJ/b)	NEp (KJ/g)	RE_p (KJ/b)	RE_f (KJ/b)	RE_p/RE_f
Control	-	141.7	103.5	726.5	5.44	334.5	395.4	0.85
	+	142.8	106.1	767.3	5.77	337.1	405.4	0.85
DDGS	-	149.3	92.6	668.4	4.90	352.3	353.9	1.00
	+	149.8	96.1	718.7	5.30	353.5	367.0	0.97
SEM		*1.29*	*2.59*	*7.78*	*0.060*	*3.05*	*9.92*	*0.025*
Main effects								
Diet	Control	142.2	104.8[a]	746.9[a]	5.60[a]	335.7	400.4[a]	0.85[b]
	DDGS	149.5	94.3[b]	693.5[b]	5.09[b]	352.9	360.4[b]	0.98[a]
Enzyme	-	145.5	101.1	697.4[a]	5.16[a]	345.3	374.6	0.92
	+	146.3	98.1	742.9[b]	5.53[b]	343.4	386.2	0.91
Source of variation		---------- *Probability* ----------						
Diet		0.06	<0.05	<0.01	<0.001	0.06	<0.05	<0.05
Enzyme		NS	NS	<0.01	<0.01	NS	NS	NS
Diet × Enzyme		NS	NS	NS	NS	NS	NS	NS

[1]Enzyme combination included xylanase, protease, amylase and glucanase.

[2]Each value represents the mean of 6 replicates (cage) for each treatment group

NS: Not significant, SEM: Standard error of means

[a] Means within the same column not sharing a superscript differ significantly at the P level shown.

Table 7.6 shows the efficiency of MEI for energy, protein and fat retention. The efficiency values of use of MEI for energy and fat retention were significantly depressed (P<0.05) by feeding DDGS-containing diet. The K_{REp} tended (P=0.06) to increase in broilers given the diet with DDGS. Enzyme addition had no impact on the efficiency of MEI for RE, RE_p and RE_f, and there was no interaction between enzyme supplementation and diets.

Table 7.6 Effects of supplementation of an enzyme cocktail[1] and a high level of DDGS inclusion on the efficiency of ME utilisation for energy, fat and protein retention in broiler chickens from d 18 to d 28 measured by comparative slaughter technique

Treatments	Enzymes	K_{RE}	K_{REf}	K_{REp}
Control	-	0.433	0.235	0.200
	+	0.452	0.239	0.198
DDGS	-	0.410	0.213	0.211
	+	0.420	0.214	0.207
SEM		*0.0053*	*0.0057*	*0.0021*
Main effects				
Diet	Control	0.442[a]	0.236[a]	0.199
	DDGS	0.414[b]	0.213[b]	0.208
Enzyme	-	0.421	0.224	0.205
	+	0.435	0.226	0.202
Source of variation		---------- *Probability* ----------		
Diet		<0.05	<0.05	0.06
Enzyme		NS	NS	NS
Diet × Enzyme		NS	NS	NS

[1]Enzyme combination included xylanase, protease, amylase and glucanase.

[2]Each value represents the mean of 6 replicates (cage) for each treatment group

NS: Not significant, SEM: Standard error of means

[a] Means within the same column not sharing a superscript differ significantly at the P level shown.

7.4 DISCUSSION

7.4.1 Growth response of birds and metabolisable energy

High inclusion of DDGS has been shown to adversely influence feed efficiency in broilers (Lumpkins *et al.*, 2004; Loar *et al.*, 2010), and this is apparent from the results of the current experiment. This could be linked to higher fibre content, thus a possible reduction in nutrient digestibility and, more importantly, energy expenditure that is investigated in this study. Moreover, in accordance with the results of previous experiments (See Chapters 4 and 5), the effect of enzyme supplementation was only significant in early life, leading to improvement

of feed efficiency. In fact, during the grower period in which energy utilisation was assessed were conducted, only a partial, 4-point, improvement was noted for the FCR of the birds.

In the current experiment dietary AME was determined by two different methods, namely, total collection and using an indigestible marker. Despite the adverse effect of DDGS-containing diet on the AME calculated from total collection methods in respiration chambers using 2 birds, no influence was noted when expressed by the ratio of marker in diet and excreta samples. The systematic error for the analysis, bird variation and the variation for the detection of marker (TiO_2) may be responsible for these discrepancies. Indeed, the ME intake for the birds measured using two different techniques was not affected by treatments that therefore are comparable between the two. However, regardless of treatments, ME intake appeared to be higher in the metabolic cages which could be linked to the higher feed consumption and the length of period (10d vs. 3d). For the main effects, given the fact that ME intake of the diets has been comparable, the sensitivity of ME to explain energy utilisation is questionable due to significant differences noted for the FCR of the birds fed diets with or without DDGS. This suggests a major drawback for the evaluation of dietary energy by ME when a high fibrous material such as DDGS is included in broiler diets.

7.4.2 Heat production of the birds (indirect calorimetric or comparative slaughter method)

Birds fed the DDGS-containing diet released more heat compared to those without DDGS. Considering main effect, in general, the measurements were consistent between the two techniques (IC or CS). However, the estimates of HP measured by CS appeared to be lower. This could be associated with the higher number of birds in the cages compared to just two birds in calorimetric assessments, the higher ME intake of the birds as well as the longer period of measurement for CS (10 days). Stocking density was higher (x kg/m^2) in metabolic cages than chambers providing less space to move which probably explains at least, in part, the lower HP observed by CS. Furthermore, the length of period and environmental factors may have contributed to such differences. There are no reported data on the impact of DDGS inclusion on heat production in broiler chickens. However, it is known that energy expenditure includes the cost for the maintenance of muscle, fat and bone tissue as well as metabolically active organs such as the liver, gut, respiratory and reproductive system (Spratt

et al., 1990; Pirgozliev *et al.*, 2011). Spratt *et al.* (1990) elucidated that the liver, gastrointestinal tract and reproductive system may account for 30% of total energy expenditure of a hen, whereas these vital organs account for only 4% of the whole BW. Hence, it is suggested that any possible alteration in the weight of these organs may result in different heat production. In the current experiment, the large amount of DDGS incorporated in the diet (30%) may have contributed to the development of visceral organs, in particular, the gastrointestinal tract. Such an effect on the weight of different regions of the small intestine and visceral organs was elucidated in the previous experiments with broiler chickens, testing the same DDGS material (Chapters 4 and 5).

Fibre content of the diet may also contribute to differences in heat production in broiler chickens. Jørgensen *et al.* (1996) recorded an increase in heat production of broiler chickens by inclusion of pea fibre. They interpreted this to be a result of physiochemical properties of NSP from pea which could strongly impact the energy expenditure through gut development and the weight of visceral organs. It has also been demonstrated that by increasing the NSP content in the diet, energy losses as heat increase in addition to substantial loss of energy as VFA in the excreta of broiler chickens (Choct, 1999). Therefore, it could be interpreted that the NSP content of diet not only impedes the digestive process, but also negatively impacts net energy utilisation by increasing heat production. Given the fact that DDGS contain a larger proportion of NSP than do cereal grains (Widyaratne and Zijlstra, 2007; Choct and Petersen, 2009), a similar explanation may be applied for the higher heat production in broilers fed a high level of DDGS.

A tendency to decrease heat production as a result of enzyme inclusion was only revealed in the data assessed by IC. Such an effect was, in fact, more pronounced in the birds that received the control diet, which could be due to differences in the availability of substrate for the different enzymes used in the current experiment. Compared to the DDGS-containing diet, the control diet comprised a substantially higher amount of wheat and corn, therefore, greater soluble NSP and starch content for xylanase and amylase to act on, respectively.

7.4.3 Net energy value of the diets, production and the efficiency of ME for NE

In the present study, the net energy values of the diets measured by IC are comparable with the figures reported in broiler chickens by Noblet and co-workers (Noblet *et al.*, 2003; Noblet

et al., 2007). It is evident from the results that the NE and NEp values of the diets were compromised when DDGS was incorporated in the diet of broilers. This consistently corresponds to the higher HP observed in birds fed DDGS. The adverse effect of DDGS on the NE value of the diet was expected due to greater quantity of degradation of dietary fibre in the hindgut. This results in the production and absorption of fermentation products. This fermentation process results in a lower metabolic efficiency when energy is evaluated in the small intestine (Noblet *et al.*, 1989). In contrast to other animal species, poultry do not digest a significant amount of fibre, therefore the contribution of fibre to the differentiation between NE and ME is limited (Carré *et al.*, 1995).

In general, the net efficiency of utilization of energy is expressed as the NE:ME ratio (Sarmiento-Franco *et al.*, 2000). In poultry, the effect of diet on NE value and NE:ME is inconsistent. However, the majority of reports have not shown a great effect of diet composition on the efficiency of using ME for NE in poultry (Noblet *et al.*, 2010a). To my knowledge, this study is the first to evaluate the NE value of diets containing DDGS in broilers, and this makes the discussion of results very difficult. Nevertheless, Noblet *et al.* (2010b) found no change in the NE:ME ratio when a high level of NDF was present in broiler diets. The same authors also reported that HI, HP and the coefficient for the efficiency of ME utilisation for NE by dietary CP were unaffected. However, depending on the physicochemical properties of feed ingredients the results may differ. For instance, Carré *et al.* (2002) reported a substantial decrease in the NE:ME ratio by incorporating fermentable sugars in broiler diets. In fact, this low ratio was accounted for by the energy losses through the fermentation process. However, the result of the current experiment supports the advantage of NE over ME in explaining the energy utilisation of the birds by having a significant effect on the ratio of NE:ME. As previously mentioned, the large proportion of DDGS included in diet may have influenced the fermentation process by increasing the production of fermentation end-products. This effect has already been confirmed in a previous experiment investigating the same material in broiler diets (Chapter 4). Thus, the increase in energy losses may, at least in part, provide an interpretation for the lower values observed in the efficiency of ME for NE in the birds fed diets containing DDGS.

Results of the current experiment indicate that the NEp was very sensitive to the effect of enzyme supplementation, while no impact of enzyme on ME was observed. A possible improvement in nutrient and energy availability may be responsible for the enhancement in energy utilisation, as the available nutrients would be deposited in the carcass in the form of

protein and fat, thereby resulting in an increased retention of energy in the body. This observation is in line with those reported by Olukosi *et al.* who found that a combination of xylanase, amylase, protease and phytase improved NEp in broiler chickens. However, in the current experiment, enzyme supplementation failed to alter the NE value of the diet or the efficiency of ME for NE when assessed by IC. This, at least in part, may be due to a different magnitude of response for the two diets which in fact was more pronounced in the birds fed the control diet. As a result of enzyme addition, a non-significant (3.85%) improvement in the NE of the diet was obtained in the birds fed no DDGS in contrast to the birds receiving diet containing DDGS. This is in line with the values of HP observed in the same birds assessed by IC. Surprisingly, despite the effect of enzyme supplementation on NEp and NE (corrected for FHP) using CS techniques, there was no such influence on ME intake or BW of the birds. However, the partial effect of enzymes on HP, nutrient utilisation or even individual bird variation may be responsible for the detection of higher NEp in the birds fed enzyme supplements.

7.4.4 Deposition of energy in form of fat and protein

Body composition has some relationship with ME intake and therefore energy retained in the body as documented in the literature (Haakansson and Svensson, 1984; Boekholt *et al.*, 1994; Lopez *et al.*, 2007). Generally, in broiler chickens ranging from 1 to 42 d, around 35 to 40% of ME intake is deposited as fat and protein in the body (Lopez and Leeson, 2005; Lopez *et al.*, 2007). The results of the current experiment are close to these estimates, showing an average of 22% and 20% of ME intake for the retention of fat and protein, respectively. Similar to the results obtained by Olukosi *et al,* (2008) the efficiency of ME utilisation for the retention of fat and protein were lower than the values reported by Larbier and Lerclercq (1992) and Lawrence and Fowler (1997) who reported an average of 0.40 and 0.60 for the efficiency of protein deposition and an average of 0.44 and 0.80 as the energetic efficiency for fat deposition. Indeed, in the current experiment and study by Olukosi *et al.* (2008), the efficiency of fat and protein was calculated according to the total ME intake, whereas the values reported by the aforementioned authors were only for the ME intake above maintenance requirements, and this is responsible for these discrepancies. However, the energetic efficiency for fat retention was higher than protein, which concurs with data from the literature (Boekholt *et al.*, 1994; Lopez *et al.*, 2007). Notably, in the present study, the

energy retained as fat was higher than the proportion of the energy retained in the form of protein. This is in contrast with the results of other authors who studied the retention of fat and protein in broilers from 1 to 21d (Sanz *et al.*, 2000; Bregendahl *et al.*, 2002; Olukosi *et al.*, 2008). In the current study, the retention of energy in broiler chickens was evaluated from 18 to 28d at which stage fat deposition has probably come close to protein deposition in whole carcasses. It has been shown that deposition of fat increases with age as BW increases, whereas protein deposition in the body remains fairly constant (Leeson and Summers, 1997). Therefore, with the higher fat content of the body, the energy retained as fat is expected to be higher in the current trial, as observed.

Interestingly, feeding a diet containing sDDGS with higher fibre content tended to change body composition towards the deposition of more protein in the body compared to fat deposition, and this was reflected in the higher RE_p:RE_f ratio. A likely explanation may be the higher fibre intake in the birds receiving DDGS, which may have changed the partitioning of deposited energy in favour of protein. This concurs with another report on the effect of dietary fibre on partitioning the energy in broiler chickens (Jørgensen *et al.*, 1996). Furthermore, the lower NE value of the DDGS-containing diet may indicate a restriction in the energy content of the diet that correspondingly fat is metabolised while protein is mostly deposited as shown by Boekholt *et al.* (1994). It is also important to mention that muscle contributes more to HP than does fat tissue (Close, 1990). Indeed, the ATP required for the deposition of 1 kcal of protein is substantially higher than that required for the same quantity of fat. Taking this into account, the higher efficiency of ME for fat deposition in the birds receiving the control diet compared to the birds fed DDGS-containing diet may partially explain the lower HP of those birds. Despite the lack of effect on protein and fat deposition, the energy retained was indeed higher when enzymes were added to the diets. No explanation is available for this lack of enzyme effect on fat and protein retention. However, the variation between samples may also be responsible for the failure to detect any significant differences between experimental treatments for the enzyme supplementation.

7.5 CONCLUSION

The results of the current experiment clearly demonstrated the adverse effect of including DDGS on NE value of broiler diets coupled with an increase in HP by the birds. The

differences between NE:ME for the two basal diets suggests a higher sensitivity of NE in the utilisation of energy by broiler chickens when a high level of DDGS is included in the diets. Nevertheless, the scope for the high inclusion of DDGS in broiler diets seems promising if a proper NE system is implemented for poultry. However, the superiority of an NE system to ME in poultry is still to be determined and is highly dependent on the determination of NE value of different feed ingredients and a wide range of diets which require further studies. The results for the detection of a significant effect of dietary treatment using comparative slaughter technique and indirect calorimetry were relatively consistent; however, different observations for the HP and thereby retained energy point to a need for further examination to elucidate the source of variation between the two techniques when the same treatments are applied to the birds.

CHAPTER 8
GENERAL DISCUSSION AND CONCLUSION

8.1 INTRODUCTION

Large quantities of DDGS have become available for use in animal feed as more grains are used for biofuel production. However, there are problems associated with including high levels of DDGS in non-ruminant animal diets. The major limitations to greater utilisation of DDGS in diets include variability of the material, high fibre content, nutrient imbalances and bulk density. Effective use of DDGS in poultry diets can result from increased knowledge of nutritional and physiological response of the bird to the feeding of this by-product. Firstly, the potential of different exogenous enzymes and their interaction with the dietary ingredients such as DDGS is not fully understood. This requires investigation on the digestive function and the response to various different exogenous enzymes when DDGS are included in diets, particularly at high levels. Such levels may worsen bird performance due to the presence of large amounts of NSP and lower nutrient digestibility compared to the parental grain. Much research has focussed on nutrient contents, in particular amino acid levels and their digestibility and largely ignored other aspects of DDGS in terms of its impact on animal performance. For instance, there has been a lack of research on the impact of inclusion of DDGS on intestinal microflora, gut health, overall disease development and immune response in broiler chickens. Therefore, after comprehensive analyses of the DDGS from different batches (Chapter 3), four major experiments (Chapters 4, 5, 6 and 7) were conducted in the present dissertation to investigate the impact of DDGS and different microbial enzymes on performance, gut development, microflora and nutrient utilisation in broilers, and the interaction of the feed factors under necrotic enteritis challenge.

The following sections discuss the major findings from the individual studies and elucidate their interrelationship. From this discussion, it will be possible to identify the gaps in research on DDGS and chart the way forward for better utilisation of the product.

8.2 CHARACTERISTICS OF sDDGS AND GROWTH RESPONSE OF BIRDS

Compared to other work in the literature, a lower variability was observed between batches of sDDGS assessed in the current study. However, there was a degree of variability in nutrients such as amino acids (Lys and Met) and, some minerals. Most of the NSP present in sDDGS was found to be insoluble, with less that 5 % soluble NSP (Chapter 3). An increase in feed consumption as a result of high level of inclusion of DDGS was apparent in the first two studies when compared to a maize and soybean meal- or soya concentrate-based diet as the control (Chapters 4 and 5). This was accompanied by an increase in BWG in the grower phase (Chapter 4) and also from 1 to 21d in Chapter 5. These findings, by and large, are in agreement with the literature (Olukosi *et al.*, 2010; Oryschak *et al.*, 2010a; Shim *et al.*, 2011), although a few studies did not report any increase in feed intake or BWG (Thacker and Widyaratne, 2007; Loar *et al.*, 2010). The increased feed intake and subsequent growth rate of the birds was perhaps simply the reflection of the birds eating more to overcome the dilution effect of the NSP in order to satisfy their energy and nutrient needs. Another explanation is that as the birds consumed more NSP, which were insoluble by nature, it led to the development of the small intestine as well as the gizzard, to facilitate an increased intake of feed. However, a significant depression in FCR of the birds fed DDGS was consistently observed in all phases of growth (Chapters 4, 5, 6 and 7).

Notably, the response of healthy birds to the diet containing sDDGS was found to be substantially different, in terms of BWG and FCR, from the response of broilers challenged with *Eimeria spp.* and *Clostridium perfringens.* This indicates a predisposing influence of high level of sDDGS to the outbreaks of NE (Chapter 6). This could be linked to the lower protein, amino acid digestibility of such diets, and more importantly, the elevated level of NSP present in DDGS.

The positive impact of xylanase on FCR was most pronounced at a high level of sDDGS inclusion (Chapters 4 and 5) while protease appeared to be most effective on the BWG of the birds fed diets containing sDDGS (15 and 30 %) as shown in Chapter 5. Despite the lack of significant synergy between xylanase and protease, an improvement in FCR of the birds fed diets containing both enzymes indicates possible benefits of combining the two enzymes for broilers fed DDGS. In addition, the combination of the two enzymes appeared to alleviate the

effects of necrotic enteritis on bird performance (Chapter 6). Furthermore, enzyme supplementation was more effective during the early stage of growth (Chapter 7).

8.3 GUT DEVELOPMENT AND DIGESTIVE FUNCTION

It was observed that birds fed diets containing sDDGS developed heavier proventriculus and gizzard at an early age than birds on the control diets (Chapters 4 and 5). This may be due to the presence of large amounts of insoluble NSP, being structural material and able to stimulate gizzard development (Svihus, 2011; Sacranie *et al.*, 2012). Such effect was indeed observed in the intestinal tract which was heavier in the birds fed sDDGS compared with control birds. Prior to this study, there does not seem to be any data on the effect of DDGS on intestinal development. However, it has been well documented that feeding fibrous materials to birds can stimulate the development of the upper GIT (Gonzalez-Alvarado *et al.*, 2007; Gonzalez-Alvarado *et al.*, 2008; Jimenez-Moreno *et al.*, 2009). This effect is likely due to the resistance of the fibrous and coarse material in sDDGS to grinding and possibly a concomitant mechanical stimulation of the gut tissues. Nevertheless, the impact of such material may differ, depending on the intestinal region, as shown in Chapter 5. Supplementation of protease individually or in combination with xylanase had no influence on the gut development either in terms of weight or length of intestine. However, xylanase reduced the length of the jejunum, ileum and small intestine which concur with other reports (Brenes *et al.*, 1993; Lazaro *et al.*, 2004).

Terminal digestion of extracellularly available substrates, such as disaccharides and peptides are performed by intestinal enzymes (Iji, 1998). Therefore, any negative impact on activities of digestive enzymes, including maltase and sucrase (Chapter 4) may provide evidence for the lower nutrient digestibility and impaired feed efficiency of the birds fed diets containing large amounts of sDDGS (Chapters 4, 5, 6 and 7). The high fibre (NSP) content and the increased fermentation (Chapter 4) may explain the compromised intestinal enzyme activity. It appears that diets containing DDGS may provide substrate for fermentation in the lower GIT to increase the concentration of SCFA. This increased fermentation may have some ramifications on providing energy for the birds so as to explain how birds perform better on DDGS (Olukosi *et al.*, 2010).

8.4 NUTREINT UTILISATION

Feed efficiency was impaired as the sDDGS level rose to 30 %. This was directly associated with poor nutrient, especially DM and CP, digestibility, as recorded in the first feeding trials (Chapters 4 and 5). Sorghum is the main grain from which DDGS is produced in Australia, therefore the nature of protein in sorghum, may be responsible for the poor protein quality. Nutrients such as protein and starch are encapsulated in a tightly packed cell wall matrix in sorghum, in addition to the fact that the main sorghum proteins, kafirin, are highly resistant to proteolysis, as indicated in a review by Selle *et al.* (2010). Correspondingly, the poor amino acid digestibility observed in the birds which received sDDGS (Chapter 5) supported the findings of other workers (Bandegan *et al.*, 2009; Kim *et al.*, 2010). Additionally, the high NSP content of sDDGS may be a culprit contributing to impaired nutrient utilisation (Adeola *et al.*, 2010). Indeed, a large amount of undigested NSP, in particular, insoluble faction was found in the ileum of the birds that received DDGS (Chapters 4 and 5), which may have contributed to poor feed efficiency in the bird fed on diets containing sDDGS.

Despite the relatively low fraction of soluble NSP in sDDGS (Chapter 3), the viscosity of ileal content of birds on the sDDGS diets was still higher than those fed diets free of sDDGS (Chapters 4 and 5). Such an increase in intestinal viscosity obviously has impeded nutrient utilisation because the application of xylanase and protease ameliorated it, as shown in Chapters 4 and 5. However, supplementation of xylanase and protease individually or in combination in the first two experiments did not lead to significant improvement in CP or DM digestibility of the birds receiving sDDGS. Similar findings have recently been reported by Kalmendal and Tauson (2012) in broilers fed a wheat-soybean meal-based diet although others (Freitas *et al.*, 2011) have reported a positive protein digestibility response to exogenous protease. Nevertheless, the current studies are the first in which the effect of xylanase and protease in diets containing DDGS was investigated. Response to diets may differ, depending on the nature of ingredients and possibly the amount of substrates available for enzymatic digestion. Notably, the positive impact of protease on digestibility of some amino acids was in good agreement with a recent report by Angel *et al.* (2011). This was particularly apparent in the birds fed high levels of sDDGS (Chapter 5). Overall, the improvement observed in BW of the birds that received protease without a significant effect on DM and CP digestibility may point to the need to investigate the mode of action of protease further.

As expected, xylanase could effectively degrade a proportion of total NSP and insoluble fraction as shown in Chapters 4 and 5. This was particularly pronounced for the arabinoxylans, as dominant NSP type present in the diets containing sDDGS. This may explain the improvement in feed efficiency of the birds, which was indeed significant in the birds fed large amounts of sDDGS. It is evident from the composition of NSP in the ileum (Chapter 5) of the birds that supplementation with protease may counteract the effect of xylanase as the concentration of xylose and arabinose, and to the lesser extend total insoluble NSP, was higher when xylanase and protease were added together. As previously mentioned in Chapter 5, it is proposed that exogenous protease possibly degrades a fraction of xylanase, consequently affects the activity of xylanase (Naveed *et al.*, 1998; Ghazi *et al.*, 2003). This was consistently reflected in the concentration of available free sugars in the lower intestine where large amounts of free sugars from undigested NSP were found in the birds fed sDDGS with a combination of xylanase and protease (Chapter 5).

8.5 GUT MICROBIAL PROFILES AND HEALTH

The negative impact of high inclusion of sDDGS on gut health was revealed by higher necrotic enteritis lesion scores observed in the birds challenged with *Eimeria spp.* and *C. perfringens* (Chapter 6). Despite the paucity of relevant data in the literature, these results are congruent to the lack of preventive impact of DDGS reported in broilers infected by *E. acervulina* (Perez *et al.*, 2011). However, the result contradicts the positive influence of DDGS on gut health observed in growing pigs (Whitney *et al.*, 2006a). Such observation is further supported by the profiles of bacteria in response to the inclusion of sDDGS which was indeed different in healthy and challenged birds. In unchallenged birds, feeding a diet containing sDDGS did not influence microbial profile, in particular *C. perfringens* number in the ileum, which agrees with other reports in the literature (Loar *et al.*, 2010; Loar *et al.*, 2012). Conversely, in the caecal content of birds fed DDGS and infected with *C. perfringens*, the population of this organism was significantly increased, which also coincided with impaired feed efficiency of the same group of birds. This shows that the nature of diet may directly or indirectly affect the proliferation of *C. perfringens*, thereby subsequently, precipitating the onset of clostridial intestinal disease (Branton *et al.*, 1997). The higher content of fibre in the diets containing DDGS (Chapters 4, 5 and 6) may have accelerated the colonisation of *C. perfringens* under the disease challenge imposed on the

birds. The results are in line with the levels of serum immunoglobulins observed in Chapter 6.

As previously shown, inclusion of sDDGS in broiler diets led to a significant compromise in protein and amino acid digestibility (Chapters 4 and 5) which may influence the proliferation of *C. perfringens* in the lower intestine through provision of a large amount of undigested protein for this proteolytic bacterium. For most of the enumerated bacteria, there was no effect of enzyme supplementation (Chapter 6), in line with the lack of significant effects of enzymes on bird performance at the peak of disease.

8.6 NET ENERGY UTILISATION

Although metabolisable energy has been widely accepted for the estimation of energy value of feed ingredients and diet of poultry, net energy is a more accurate estimate due to the fact that heat production is accounted for. The importance of such precise estimate of energy for a fibrous ingredient like DDGS was proven by the difference between the net energy values of the diets with or without sDDGS (Chapter 7). Indeed, broilers fed a high level of sDDGS (30 %) produced substantially more heat than the control. The significant difference for the net efficiency of utilisation of energy expressed by the ratio of NE:ME (Sarmiento-Franco *et al.*, 2000) showed that the actual energy value of the diets can vary even when ME value were comparable.

For the DDGS, the effect on heat production may be associated with an increased fermentation in the lower intestine (Chapters 4 and 6) and also more developed GIT (Chapters 4 and 5), both of which require more energy and therefore heat. It can be interpreted that the NSP content of DDGS may impact net energy more than it does metabolisable energy. The increased gut viscosity caused by the feeding of large amounts of DDGS (Chapter 5), means that the gut would have to work harder than usual to deal with a highly viscous environment (Choct, 1999) and thereby more energy is demanded. Possibly, the increased proliferation of fermentative organisms in the intestine of birds fed DDGS (Chapter 6) may also further elucidate the energy loss by those birds. The impact of exogenous enzymes on NEp of the birds agreed with several studies, for example, Olukosi *et al.* (2008) and Pirgozliev *et al.* (2011). However, such measurements, for the enzyme effect, would have been more meaningful if it was undertaken in the early age. Carcass analyses

revealed a tendency for a higher rate of protein deposition compared to fat, which could also be another justification for the more heat produced in the birds fed diets containing sDDGS.

8.7 CONCLUSIONS AND RECOMMENDATIONS

The current studies clearly demonstrate that inclusion of sDDGS in diets for broiler chickens is a viable option if the material is available and economically justified. However, the adverse impact of high levels of dietary inclusion of sDDGS on feed efficiency, digestive function and development of necrotic enteritis underline the need for precaution. Further investigation is warranted to elucidate factors affecting protein digestibility of sDDGS. The measurements of tannin and kafirin contents of the material may possibly give an indication of the extent of protein availability from sDDGS. Noteworthy, due to difference type of grains from which DDGS are driven, the response of the birds may vary.

The negative influence on digestive enzymes (sucrase and maltase) further explains the mechanisms behind the compromised performance of broilers fed sDDGS at 20 % and beyond. Therefore, it can be generally conceded that inclusion of DDGS in broiler diets should be restricted to 10-15 %, particularly without enzyme supplementation. Notably, the application of xylanase and protease individually or in combination appears to be beneficial which, to some degree, reduces the negative impact of DDGS on FCR. Further investigations for the interactive effect of enzymes, more specifically with DDGS, are required, to quantify the scope for improvement in the nutritive value of diets containing DDGS. In this regard, biotechnology may be able to develop carbohydrases with the ability to degrade substantial amounts of insoluble NSP in DDGS, particularly from sorghum. It was evident that the BWG of broilers was significantly improved when protease was supplemented to the diets, in spite of no substantial change in the digestibility of most nutrients. More research is needed to explain the mode of action of protease, ostensibly in terms of digestive enzyme activities, endogenous nutrient losses, amino acids in particular, and energy utilisation.

Although the role of DDGS in predisposing birds to necrotic enteritis was apparent from the current study, future research into the effect of DDGS on local immunity of the gut, histopathology, mucosal bacterial colonisation and the performance of broilers under different prevalent poultry diseases is also warranted. Further research will also be required to

identify the net energy value of DDGS, preferably using regression analysis in an experiment using different levels of such ingredients in semi-purified diets. Such investigation will be strengthened by simultaneous use of different techniques, for example, comparative slaughter and indirect calorimetry in the measurement of energy use.

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