ABSTRACT

BARASCH, ILANA BETH. The Evaluation of a Novel Heat-Stable Xylanase Supplemented to Broiler Chicken Diets. (Under the direction of Jesse L. Grimes and Jimmy D. Garlich).

The purpose of this research was to evaluate the efficacy of a new (novel) xylanase as a feed enzyme for broiler chickens. As the usage of dietary feed enzymes has continued to gain popularity in the poultry industry, numerous new enzymes are indentified as potential products. However, each new enzyme must be evaluated for its efficacy in the bird itself. The goal of this research was to evaluate a new mono-component xylanase and its efficacy through feed manufacturing, supplementation to the bird, and quantify the birds' response. In total, seven trials were conducted, two pilot trials and five subsequent trials.

Two trials were conducted using a commercial carbohydrase product and served as a pilot study. These two trials were conducted with the purpose of evaluating a known product and aid in designing the optimal way to evaluate the new xylanase. From the results in these trials it was determined that in addition to growth performance, digesta viscosity and nitrogen-corrected apparent metabolizable energy (AME_n) would be suitable parameters to evaluate the new xylanase.

Initial evaluation of the xylanase was conducted in the starter period (0 to 3 weeks of age), raising birds in battery cages, and supplementing the xylanase to wheat-based diets. A trial was first conducted to evaluate optimal inclusion level of the xylanase in mash diets for broiler chickens in the starter period. The response to the xylanase was evident through a measurable reduction in digesta viscosity, however no growth performance response was observed. A xylanase inclusion level was selected based on bird response data to increasing xylanase inclusion levels. With the selected inclusion level, bird response was evaluated when xylanase was supplemented in reduced energy diets that had been pelleted. Both

improvements in live performance and uplifts in AME_n were observed with xylanase supplementation. Feed conversion ratio (FCR) was improved most at the lowest level of dietary energy when xylanase was supplemented. With the low dietary energy level (2,770 kcal/kg), xylanase was included in increasing concentrations in both mash and pelleted (presented to birds as a crumble) diets in a side-by-side comparison. Improvements in FCR and body weight gain (BWG) were observed with xylanase supplementation, however these responses were only observed in the pelleted (crumbled) diets, not in the mash diets.

The following two trials were conducted in litter floor pens and birds were raised to market age. Xylanase was supplemented in reduced-energy wheat-based diets in combination with a dietary phytase, an enzyme commonly included in broiler diets. Inclusion of both enzymes individually resulted in improvements in bird performance and AME_n throughout the production period in reduced energy diets. However, further improvements were observed when enzymes were supplemented to diets not reduced in energy. When the xylanase and phytase were included in combination, great uplifts in AME_n were observed than when either enzyme was included individually. Finally, xylanase was supplemented to reduced-energy corn-soybean meal diets. Xylanase supplementation improved BWG and FCR in the first 14 days, but was not observed by market age. As in the previous trial, improvements due to xylanase supplementation were greater in diets not reduced in energy. While not evident in growth performance, improvements due to xylanase were observed in AME_n evaluated at market age (42 days).

In conclusion, this novel xylanase has potential as a dietary enzyme supplement for broiler chickens. This enzyme presented the heat-stability to withstand the pelleting process (85^0C) as well as measurable responses in live performance, reduction in digesta viscosity,

and improvements in AME_n.

The Evaluation of a Novel Heat-Stable Xylanase Supplemented to Broiler Chicken Diets

by
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INTRODUCTION

Feed costs have always been a major portion of the cost for animal production. However, between 1999 and 2008 the overall cost of feed doubled resulting in feed costs being up 70 to 80% of production costs (Donohue and Cunningham, 2009). This was in part due to an increased proportion of corn going to ethanol production rather than animal feed, as well a global increase in the demand for feed grains and fuel (Masey O'Neill *et al*., 2012; Donohue and Cunningham, 2009). In response to the rising cost of feed ingredients, poultry and swine producers in the United States increased inclusion of alternative ingredients, such as wheat or dried distillers grains with solubles (DDGS), to the traditional corn-soybean meal based diets (Adeola and Cowieson, 2011; Yanez *et al*., 2011). Increased production of DDGS, a co-product of ethanol production, became a new source of protein and energy for non-ruminant diets (Wang *et al*., 2007; Lumpkins *et al*., 2004).

There are numerous feed additives that have been investigated for improving the digestibility and feeding value of these traditional and alternative ingredients to poultry. These include antimicrobials, organic acids, botanicals, probiotic, prebiotics and exogenous enzymes (Houshmand *et al*., 2011; Rosen, 2010; Yang *et al*., 2009). Although there has been much research into the various options for feed additives, the focus of the current review is on exogenous enzymes. Enzymes have been commercially available since the 1980's when carbohydrase enzymes entered the market. Since then, the value of the enzyme market increased to over 700 to 800 million USD (Bao *et al*., 2013). Whereas antimicrobials may raise concerns for some consumers, enzymes are proteins that can be metabolized by the animals and do not cause not concern to consumers (Pariza and Cook, 2010; Ferket, 1993).

In order to reduce the development of antibiotic-resistant microbes, which are a potential threat to both animal and human health, the poultry and livestock industry is trying to replace the rise of antibiotics in feed with alternative methods of promoting good animal health. Likely the solution will be a combination of multiple strategies through feed additives, diet formulation strategies, genetic selection of plants and livestock, and environmental management. The inclusion of exogenous enzymes in feed will play a key role in this solution due to its increasingly low cost of inclusion, its potential beneficial effects on environmental conditions and bird performance, and the flexibility it provides in using lower cost ingredients in diets.

LITERATURE REVIEW

Enzymes and digestion

Enzymes are selective biological catalysts. An enzyme is a protein folded into a specific three-dimensional conformation; maintaining the conformation is important for its ability to act as a catalyst (Sheehan, 2010). The catalyst will bind a reactant, known as a substrate, forming a complex. The catalyst (enzyme) lowers the activation energy, or the amount of energy needed for the reaction to take place, and products will dissociate. An enzyme must maintain a stable conformation, but still have some flexibility in its structure to allow it to change its conformation to form a complex with its substrate (Turner *et al*., 2007; Danson *et al*., 1996). Following the reaction, the enzyme will return to its original form and be ready to catalyze another reaction. Enzymes have varying degrees of specificity; some act on a group of related substrates, others are highly specific to the substrates on which they act (Horton *et al*., 2006). Enzymes can be identified as endo- or exoenzymes, describing whether the degradative action occurs at central linkages (endo-) or at linkages on the terminal ends (exo-) of the molecule. Enzymes are also classified by their mode of action, for example: hydrolases, isomerases, ligases, transferases, lyases and oxioreductases (Horton *et al.,* 2006). Most enzymes involved in digestion are classified as hydrolases; hydrolases cleave their substrate using a water molecule (Pond *et al*., 2005).

A variety of enzymes are synthesized endogenously in an animal's body and are essential for the animal to digest the nutrients of the feed they consume. Animals cannot absorb nutrients from feed unless they are broken down into individual components. For example, in an animal's gastrointestinal tract, carbohydrates such as polysaccharides must be

broken down into monosaccharides by way of digestive enzymes before they can be absorbed. The main digestive enzymes produced in the pancreas and gastrointestinal tract hydrolyze carbohydrates (amylolytic), lipids (lipolytic), or proteins (proteolytic) (Pond *et al*., 2005; Turk *et al*., 1982). Although animals naturally produce digestive enzymes, they may lack enzymes for some feed components or may be ineffective due to anti-nutritional factors (ANF) (Bedford and Schulze, 1998; Campbell and Bedford, 1992). For example, animals do not produce enzymes that hydrolyze complex carbohydrates such as cellulose or hemicellulose. Any hydrolysis of these complex carbohydrates may be done by microbial enzymatic activity (Pond *et al*., 2005; Silva and Smithard, 2002), which usually takes place in the distal portions of the gastrointestinal tract of monogastrics.

In commercial animal production, exogenous enzymes are commonly included in feed as additives (Adeola and Cowieson, 2011; Bedford, 2000). Through industrial fermentation of either a fungal or bacterial source, specific enzymes can be isolated and packaged into a usable form (Masey O'Neill *et al*., 2014a; Adeola and Cowieson, 2011). Exogenous feed enzymes can then be mixed into a designed ration and consumed by the animal, acting on appropriate substrates as it enters the gastrointestinal tract with the rest of the consumed feed. Enzymes can be included to supplement endogenous production of an enzyme, such as α-amylase, or enzymes that are not produced endogenously such as xylanase or β-glucanase (Bedford and Schulze, 1998; Campbell and Bedford, 1992).

Efficacy of feed enzymes

The efficacy of an enzyme and its ability to transform reactants into products is the enzyme activity. Enzyme activity describes the quantity of substrate catalyzed to a product and is dependent on five main factors: pH, temperature, substrate specificity and concentration, enzyme concentration, and the presence of inhibitors. These factors affect the stability of the protein structure of the enzyme and therefore its efficacy as a catalyst for a reaction (Fields, 2001; Fagain, 1995). All enzymes have optimal conditions in which they perform most efficiently. This varies between enzymes, but due to some degree of structural flexibility, most will still catalyze reactions within a range rather than at a set point. Moisture content is also an important factor in enzyme activity because most enzymes require an aqueous environment to be active (Ravindran, 2013; Fagain, 1995; Campbell and Bedford, 1992; Pawlik *et al*., 1990). Maintaining efficacy of a dietary exogenous enzyme can be challenging, as it will encounter harsh conditions through feed manufacturing as well as the gastrointestinal tract environment; therefore, all factors that impact enzyme efficacy must also be considered in terms of these interactions (Igbasan *et al*., 2000; Campbell and Bedford, 1992).

One of the factors that can influence enzyme activity is the pH of the surrounding environment (Sabatier and Fish, 1996; Fagain, 1995). Changes in pH can affect the binding of the enzyme to the substrate, catalytic activity of the enzyme, the ionization of the substrate, and the three-dimensional protein structure of the enzyme (Danson *et al*., 1996; Shoichet *et al*., 1995). Whereas enzymes are proteins that are folded in specific conformations, much of this folding and conformations is due to the sequence of amino acid

residues that form the primary structure of the protein. When the protein is folded in its secondary and tertiary structures, there is hydrogen bonding and other weak interactions between amino acid residues that form and hold the structure intact (Feller, 2010; Horton *et al*., 2006). Some amino acids, such as the basic amino acids lysine, arginine, serine, cysteine, and histidine, have functional groups with charges that can be altered in various pH conditions. Changes in pH can alter these charges and consequently their binding capability (Horton *et al*., 2006; Danson *et al.*, 1996). A change in binding within the tertiary structure can lead to denaturation and the enzyme loses functionality (Fields, 2001). Most enzymes have a range for optimal activity and outside that range activity decreases until it is non-existent (Horton *et al*., 2006). In animals, gastric pH is low, or acidic, which maintains an unfavorable environment for foreign pathogens as well as maintains an environment favorable for endogenous digestive enzymes (Wyatt *et al*., 2008). However, enzymes in the small intestine function best at pH close to neutral or slightly acidic. Since a range of pH values will be encountered in the digestive tract of a bird from 3 to 4 in the crop, proventriculus, and gizzard to an almost neutral 6 to 7 in the small intestine, optimal pH must be a consideration when including exogenous enzymes in the feed. Changes in pH in the gut can also alter the solubility of the substrate. For example, phytate is soluble in lower pH such as is present in the crop, but insoluble in the small intestine; phytase must work on phytate early in the digestive tract where it is most soluble (Angel *et al*., 2002).

A common cause of protein denaturation and loss of enzymatic activity is exposure to high temperatures (Horton *et al*., 2006; Fields, 2001). While there may be a range in pH where only the tertiary structure is altered, but the internal structure remains intact, a small

change in temperature can cause disruptions to both secondary and tertiary protein structures (Feller, 2010; Horton *et al*., 2006; Fagain, 1995). Most proteins are stable at temperatures up to 50 to 60^0C, however there are thermophilic organisms which thrive in much higher temperatures (Horton *et al*., 2006; Chesson, 1993). These thermophiles produce enzymes and other cellular components that are stable at higher temperatures and can withstand highly acidic or alkaline environments (Haki and Rakshit, 2003; Danson *et al*., 1996). Thermostability can be a concern when supplementing exogenous feed enzymes into animal diets. Commercial animal diets can be exposed to temperatures high enough to denature most proteins during feed processing such as conditioning, pelleting, extrusion, or expansion. During the manufacturing of animal feed the addition of moisture (steam) and heat are utilized to gelatinize starch in the feed, improving the digestibility of starch by the animal (Svihus *et al*., 2005). Enzymes can be selected that are intrinsically more thermostable (Turner *et al*., 2007) or methods can be implemented to improve thermostability, such as applying a protective coating or by genetically altering amino acids residues of the enzyme (Gilbert and Cooney, 2010; Rao *et al*., 1998).

Activity of the enzyme is also dependent on the concentration of the substrate. A substrate is a reactant that an enzyme reacts with to form products. In order to have an enzymatic reaction there must be substrate present and it must be in a form capable of reacting with the enzyme. The concentration of the substrate and its corresponding enzyme will be a strong factor in determining the rate of the reaction. Most enzyme conformations are designed to have a hydrophobic interior and hydrophilic surface. Within the surface are also clefts, or indentations, that will recognize and bind to specific molecules or compounds

which allows the enzyme to have specificity. One of these clefts is the active site that typically contains certain amino acid residues that will react with a specific substrate, at which time the enzyme will complex with the substrate, altering the conformation of the enzyme so it can not react with another substrate. When the reaction has been catalyzed, the products formed will be dissociated and the enzyme will return to its original conformation. Although, increasing concentrations of the substrate will increase the rate of product formed, there is a limit where the rate of reaction will reach a maximum, regardless of further increases in substrate concentration. In terms of digestion, the dietary components or feed ingredients provided to an animal are the source of the substrates for enzymes in the animal's gastrointestinal tract. A variety of digestive enzymes are required in order to break down the components of the feed. In addition to the digestive enzymes produced endogenously, exogenous enzymes can be supplemented in animal feed to increase overall digestion. However, when supplementing exogenous enzymes, it is important to consider what enzymes will be most effective depending on what substrates are provided in the diet.

Another factor to consider in an enzymes' activity is the concentration of the enzyme. If the substrate is present in excess, the response of increasing the amount of product formed should be a linear relationship. For example, if the concentration of the enzyme is doubled, the rate of reaction would also double. However, when substrate concentration is no longer in excess, it becomes limiting, and the rate of reaction will slow until it plateaus, at which point additional enzyme will not increase the rate of reaction. In commercial enzyme products, there is usually availability of a product in a variety of concentrations (Adeola and Cowieson, 2011) to allow flexibility for inclusion levels. To determine the appropriate

enzyme concentration to include in animal diets, dose response studies must be conducted on each enzyme product since enzymes differ from each other in substrate specificity. Not all enzymes and substrates are the same and enzymes from different sources can have different substrate specificities (Choct, 2006), just as the substrate provided from different sources of grain can also affect enzyme efficacy (Crouch *et al*., 1997).

Even in a scenario where there is adequate enzyme and substrate, enzyme activity can still be impacted by the presence of inhibitors. An enzyme inhibitor is a compound that can bind to an enzyme and interfere with its activity either by preventing complex formation between enzyme and substrate or by inhibiting the reaction of product formation (Horton *et al*., 2006). Inhibitors are typically small molecules that can bind reversibly or irreversibly to an enzyme. In nature inhibitors exist because they can be used by a cell as a mode of regulating metabolism. Some inhibitors occur naturally in ingredients used in animal feeds, such as trypsin inhibitors (Bedford and Schulze, 1998), amylase inhibitors (Classen, 1996), and xylanase inhibitors (Elliott *et al*., 2003); however, most are inactivated by heat during processing or ingredients or feed (Juge *et al.,* 2004; Rouau and Surget, 1998). Enzyme inhibition can also occur in the form of steric hindrance (Rose *et al*., 2010). Steric hindrance is based on the protein structure of the enzyme itself and refers to how accessible the active site of the enzyme is for the substrate. If the substrate cannot reach the active site, the enzyme cannot easily or efficiently catalyze a reaction.

Although in the past several years there have been great advancements in the analytical technology for evaluating, identifying, and quantifying enzymes, the evaluations of enzyme activity continues to rely on *in vitro* assays. Enzyme activity is assayed by

measuring the disappearance of a defined substrate or the generation of a known product from a biochemical reaction over a set time period (McAllister *et al*., 2001; Sabatier and Fish, 1996). Enzyme activity is expressed as units of activity, which describes the quantity of enzyme required to produce a quantity of product under set conditions such as time, temperature, pH and buffer concentrations. This type of analysis is useful for maintaining quality control or evaluating enzyme stability (Sheehan, 2010; Chesson, 1993), but is not always an accurate prediction of an animals' response to feed supplemented with the enzyme (Sabatier and Fish, 1996). There are unknown and uncontrollable factors present *in vivo* such as inhibitors, variations in pH, endogenous enzymes, inconsistent levels of substrate, and changing rate of movement of digesta contents. This could make it challenging to estimate activity level in the gastrointestinal tract of an animal, especially due to short food-passage time in birds. For this reason, regardless of *in vitro* results, an enzyme product must always be tested *in vivo* in conditions similar to commercial production to determine response.

History of enzymes use in animal production

Progression of development of enzyme inclusion in animal feeds

Enzymes from microorganisms have been known to be involved in processes such as baking, brewing, alcohol production, and cheese production since ancient Greece (Haki and Rashit, 2003). However, it was not until 1874 when a Danish chemist named Christian Hansen was able to extract rennet from dried calves stomachs with a saline solution. Rennet is a complex of enzymes synthesized in the stomachs of ruminants that contains the proteases chymosin (rennin) and pepsin, and a lipase capable of curdling the casein component of milk

improving digestibility of the mother's milk to the young. This was the first enzyme preparation of relatively high purity used for industrial purposes to become widely used in cheese production because it can separate the solid from liquid fractions of milk (Hatti-Kaul, 2007). Various other industries such as textiles, ethanol production, laboratory assays and dietary supplements have since utilized enzymes (Polizeli *et al*., 2005). Currently, in animal production nutrition, exogenous enzymes can be added to the feed to aid the animal in breaking down fiber, protein, starch, and phytate-bound phosphorus.

The use of exogenous enzymes as feed additives first began in order to improve the efficiency of diet utilization. It was understood that ruminants could digest the fiber components better than monogastric animals due to the enzymatic capacity from the microbial population in the rumen (Hastings, 1946). In the early to mid 1900's, researchers began investigating the inclusion of exogenous enzymes in animal diets to improve performance (Jensen *et al*., 1957; Hastings, 1946; Clickner and Follwell, 1926; Hervey, 1925). This research was conducted on a limited scale due to small quantities of crude product, limited number of animals, and limited capability of statistical analysis. Even with an animal's endogenous digestive enzymes and gut microflora, there is still about 15 to 25% of the diet that monogastrics, such as pigs and poultry, cannot digest (Ravindran, 2013; Barletta, 2010) and 35 to 50% of the forage ruminants cannot digest (Beauchemin *et al*., 2004; Van Soest, 1994). Early research was conducted with supplemental exogenous amylase to improve starch digestion in poultry diets (Adeola and Cowieson, 2011; Annison, 1993; Hesselman *et al*., 1981). In the 1980's researchers demonstrated that birds are capable of adequately producing amylase to digest the starch portion of the diets (Moran, 1985;

Moran, 1982) and the focus shifted to investigate what was improving performance if it was not starch digestibility. Later, it was suggested that some of these early crude enzyme preparations also contained β-glucanase activity (Choct, 2006; Campbell and Bedford, 1992).

In the 1960's, researchers started to take interest in the inclusion of fiber-degrading exogenous feed enzymes for lambs and beef cattle that could improve forage cell wall digestibility since plant cell walls can make up to 70% of the dry matter for typical forage (Beauchemin *et al*., 2004). The efficiency of meat or milk production for a ruminant animal is limited by the digestibility of the forage they consume, as forage constitutes the majority of their diet. Observed responses were variable and the mode of action was not investigated (McAllister *et al*., 2001). Researchers had the perception that the enzymatic capacity of the rumen could not be further improved by the addition of dietary enzymes, or that exogenous enzymes would be degraded by rumen proteolysis (Adesogan, 2005). Additionally, production of exogenous enzymes was too expensive to be considered feasible for use in animal feed. During the 1960's there was also interest in developing phytase feed enzymes for swine and poultry in response to the large amount of phytate limiting the calcium and phosphorus (P) availability in the diets (Selle *et al*., 2010; Nelson *et al*., 1968).

In the 1980's commercial feed enzymes were successfully introduced into the European poultry industry. The first products were targeted toward fiber-degrading enzymes, developed to better manage birds on wheat, barley, and rye, mainly in Europe, where those ingredients were commonly used (Barletta, 2010; Pariza and Cook, 2010; Leeson *et al*., 2000). The inclusion of feed enzymes to break down ANF, such as non-starch polysaccharides, in the wheat and barley-based diets resulted in improved litter quality,

reduced feed costs due to improved nutrient utilization, improved bird uniformity, and reduced variation in bird performance between flocks (Barletta, 2010; Jackson, 2010; Bedford, 2000; Leeson *et al*., 2000). The inclusion of the fiber-degrading enzymes, xylanase and β-glucanase, reduced the nutritional variation in feed ingredients, resulting in more uniform production. Improved utilization and reduction in variation due to the spread of β-glucanase inclusion also led to a spread in the usage of barley in poultry feeds (Campbell and Bedford, 1992). By the late 1980's commercial carbohydrases had expanded to include amylase, protease, and mannanase, but were more targeted towards corn-soy diets.

Improving digestion and absorption of nutrients can also have environmental benefits, such as reducing the volume of waste produced (Bedford, 1995) as well as decreasing the amount of P and nitrogen excreted. This became important in the 1990's when soil nutrient levels, especially P, became of greater concern. Rainwater runoff carries P and nitrogen to streams and lakes. These nutrients greatly increase algae growth. During this time, there were legislative measures put in place for both the poultry and swine industries in an effort to minimize the negative impact of animal production on the environment (Greiner and Konietzney, 2010). This brought along the introduction of exogenous phytase enzymes to the commercial market in 1991 (Selle and Ravindran, 2007; Angel *et al.,* 2002; Campbell and Bedford, 1992). The inclusion of exogenous phytase allowed for better utilization of phytate-bound P in the diets, reducing the undigested fraction being excreted as well as reducing the amounts of inorganic P that had to be added to the diets. Lower inclusion levels of inorganic P also had an added economic benefit to this trend as prices of mineral phosphates increased greatly. However, there was concern due to the high cost of the

phytase additives that they may be too expensive to have an application in animal feed (Chesson, 1993).

Exogenous enzymes have been commercially used in swine and poultry rations since the 1980's but have been expanded to maximize utilization of less costly raw feed materials as the prices of corn, soy, fat and mineral phosphates soared in late 2007 (Bedford and Partridge, 2010). By 2000, enzyme inclusion in poultry diets was almost universal in Europe (Bedford, 2000). Starting in the late 1990's, there was another push towards the development of carbohydrase enzymes as corn prices continually increased (Crouch *et al*., 1997). In countries such as the United States, which predominantly used corn in poultry and swine diets, this caused a shift to utilizing wheat or byproducts such as DDGS (Mathews and McConnell, 2009; Leeson *et al*., 2000). Although carbohydrase inclusion had become standard for wheat or barley based diets, there was increased interest in benefits from including carbohydrases to corn based diets (Masey O'Neill *et al*., 2012; Barletta, 2010; Zanella *et al*., 1999). During this surge of research and development, an increasing number of commercial products were produced that had improved thermostability, allowing products to be added to the diets prior to pelleting. This improved the usability of some additives that were now available in dry and liquid forms, allowing flexibility during feed manufacturing depending on the set up in the feed mills.

Due to a great reduction in the cost of fermentation and more precise identification of enzymes in a preparation, in the late 1990's researchers reevaluated the role of feed enzymes in the ruminant diets, and for the first time for dairy cows (Beauchemin *et al*., 2003). The supplementation of exogenous fibroyltic enzymes, such as xylanases and cellulases, has been

shown to improve animal performance due to improved ruminal fiber digestion, increasing digestible energy intake (Beauchemin *et al*., 2003). However, observed animal responses to enzyme supplementation has been variable (Beauchemin and Holtshausen, 2010). One major challenge that faces the evaluation of exogenous feed enzymes for ruminant animals is the lack of an adequate bioassay to assess the value or response of the animal to the enzyme product (McAllister *et al*., 2001). In addition to not being able to evaluate a response, the modes of action are still relatively unknown due to the complexity of fiber digestion in ruminants (Beauchemin and Holtshausen, 2010). There are many techniques employed for protecting feed enzymes from degradation in a gastric or ruminal environment. These techniques include coating or encapsulating the enzyme with an acid-insoluble polymer or resin (Beauchemin *et al*., 1998). There are some indications that the application of feed enzymes to ruminants in liquid form could increase adsorption of the enzymes to the feed, which could improve their viability in the rumen by avoiding some proteolysis (Beauchemin *et al*., 1998).

Some of the current driving force in enzyme research is due to the consumer pressure to remove antibiotic growth promoters. While a concern related to antibiotic use in poultry increasing antibiotic resistant bacteria has been around since the 1970's (Dibner and Richards, 2005; Levy *et al*., 1976), public concern has continued to increase and there are additional legislative restrictions on the horizon. Without the usage of antibiotics to reduce microbial load on the birds, the industry is searching for alternative methods to manage gut health. A possible alternative is the inclusion of exogenous enzyme products. The popularity of enzyme usage is due not only to the positive effects on animal performance, but

also to the lack of potential harmful effects on consumers (Gunawardana, 2009; Ferket, 1993). Enzymes are proteins that are synthesized naturally, so their inclusion does not present health concerns to consumers as some other feed additives might. Enzymes will either be digested or excreted by the animal, so there is no fear of depositing residues in meat or eggs (Barletta, 2010; Pariza and Cook, 2010).

Changes in enzyme technology

The technology for identifying and producing exogenous enzyme products has improved greatly over the past fifteen years. In a review by Bedford (2000), the author discussed three key areas in which significant improvements needed to be made for animal feed enzymes: substrate identification, thermotolerance, and impacts of product formation during enzymatic hydrolysis. Even having achieved great improvements in each of these areas, the technology for selecting and producing enzymes for use in feed is still developing. When exogenous feed enzymes were first introduced, production was expensive and products were not well defined (Choct, 2006; McAllister *et al*., 2001; Pawlik *et al*., 1990; Edney *et al*., 1989). Assays for verifying enzyme activity, especially using 'in feed' assays, resulted in variable and inconsistent results (Sheehan, 2010; Angel *et al*., 2002; Cosson *et al*. 1999) making it difficult to quantifiably measure activity. Some of the variability was due to a lack of standardization, which created confusion to those trying to conduct assays (Cosson *et al*., 1999). Sampling, sample preparation methods, and assays for analysis, especially for carbohydrases have improved (Sheehan, 2010).

Early enzyme products also were not thermostable; therefore, they could only be included in non-pelleted feed, or added post-pellet. Early enzyme products could be applied to the feed in dry form if feeding non-pelleted feed, or could be applied in liquid form post-pelleting. Most of these enzymes were produced from mesophilic microorganisms, which are classified as having an optimal growing temperature between 15 and 50 degrees Celsius (Taylor and Vaisman, 2010; Karshikoff and Ladenstein, 2001). Mesophilic enzymes were often not well suited for harsh conditions of feed manufacturing (Demirjian *et al*., 2001). Although the enzymes are relatively stable in dry conditions, the enzymes are activated when they are hydrated in the digestive tract (Campbell and Bedford, 1992). The exposure to moisture that is necessary for activity can also be detrimental since this causes the enzyme to lose some stability in its structure, especially during feed manufacturing. Exposure to not only the moisture of steam during conditioning, but also heat, can cause an enzyme to lose stability and be degraded. To overcome this challenge, researchers searched for more heat-stable enzymes. During the 1990's technology started to change and a trend started of investigating microorganisms with inherent thermostability (Turner *et al*., 2007; Demirjian *et al*., 2001). Thermophilic microorganisms, which have optimal growing temperatures between 50 and 80 degrees Celsius (Taylor and Vaisman, 2010; Karshikoff and Ladenstein, 2001), have similar structures and mechanism to their mesophilic counterparts, but are active at different optimal temperatures (Karshikoff and Ladenstein, 2001). However, thermophiles in general were more difficult to culture and produced lower yields than mesophilic organisms (Turner *et al*., 2007).

By the early 2000's the cost of production had greatly reduced, which resulted in reduced cost of the products to consumers and increased the number of companies in production investigating new enzymes and new technology. Genetic engineering techniques have enabled manufactures to produce higher quantities of the desired enzymes at costs low enough that they can be including in commercial diets (Ravindran, 2013). Manufacturers no longer have to isolate the enzyme from the source, such as rennet from the stomach of a suckling calf; using recombinant DNA technology the enzyme can be produced in microorganisms. A selected enzyme can be cloned and then expressed in other host microorganisms (Turner *et al*., 2007), allowing for more efficient enzyme production and recovery (Pariza and Cook, 2010). Microorganisms are an excellent source of enzymes because of their broad biochemical diversity and their susceptibility to genetic manipulation (Rao *et al*., 1998).

Most poultry and swine diets in the United States are exposed to heat during feed manufacturing, whether for food safety reasons or for the advantages gained by pelleting feed. For this reason it is advantageous to produce feed additives that are thermostable enough to withstand the heat and moisture of conditioning, pelleting, or extruding, so they can be included in the feed mixing prior to pelleting. Genetic engineering has allowed for great advancement in identifying new enzymes as well as making alterations to improve those identified (Turner *et al*., 2007). Thermophilic organisms contain and produce cellular components, such as enzymes, that are stable in high temperature and can withstand highly acidic or alkaline conditions (Taylor and Vaisman, 2010; Haki and Rakshit, 2003; Wang and Shih, 1999). There are also other methods available for increasing the thermostability of an

enzyme. One method is to protect the enzyme with a coating following granulation of the enzyme (Gilbert and Cooney, 2010). Enzymes may be coated with a variety of materials including sugars, gums, proteins, lipids, or synthetic polymers (Gibbs *et al*., 1999). The specifics of coating method or coating material will vary between manufacturers and other propriety information. In general the idea is to coat the enzyme with a material durable enough to withstand feed manufacturing, but that will dissolve in the upper part of the GI tract of the animal consuming the product so the enzyme is able to work. Granulation or coating can also improve the stability of the product extending shelf life and preventing interactions with other ingredients in a premix (Sheehan, 2010). Another method is to create a more thermostable variant of the enzyme through genetic manipulation, or site-directed mutagenesis (Gilbert and Cooney, 2010). This method includes substituting surface amino acids in the enzyme with more hydrophobic amino acids, or increasing the number of a specific amino acid capable of forming cross bonds within the molecule.

Genetic engineering technology has also altered how enzyme products can be produced and sold to consumers. Masey O'Neill *et al*. (2014a) describe enzyme products as falling under three categories: single-component products, blended, and cocktails. Single- or mono-component enzymes have one main activity and may or may not have small amounts of other enzymatic side activities. Technology has allowed for selected enzyme activities to be expressed in high quantities and may have been genetically engineered to improve thermostability. In a blended product, several mono-component enzyme products are produced and then blended together. This allows the manufacturers to better control the desired activity levels of the product and allows for creating customized products. The third

category of enzyme products includes enzyme cocktails or combination products. These products result from a single fermentation that will include a wide array of main and side activities. This is more similar to the traditional crude enzyme preparations that have been used for decades.

There is discrepancy about which type of products may be more advantageous. There is the school of thought that a mono-component enzyme may be advantageous because it provides high amounts of one activity that can be supplemented to a diet with one main targeted substrate; for example, supplementing a β-glucanase to a diet containing a high proportion of barley. Some researchers have reported a mono-component enzyme to improve performance (Francesch *et al.,* 2012), in some cases better than a cocktail or combination product (Yu *et al*., 2007).

It is possible to mitigate the viscosity of the intestinal contents (chyme) caused by soluble non-starch polysaccharides (NSP) with a single enzyme since the polymers don't have to be fully degraded for viscosity to reduce (Chesson, 1993; GrootWassink *et al*., 1989). However, it may be advantageous to have multiple activities that are complementary such as xylanase and β-glucanase (Pettersson and Aman, 1989), or xylanase and phytase (Cowieson and Bedford, 2009). Although the primary substrate in barley is β-glucan, the presence of pentosans in the cell wall shows the importance of including both endo-xylanase and endo-β-glucanase activity (Classen, 1996) and may be more beneficial to include together for optimal reduction of digesta viscosity (Campbell and Bedford, 1992).

While there is opportunity to improve animal performance with a single enzyme supplementation, there is also the possibility to further improve performance by adding

multiple enzymes at once; this can be done by way of multi-enzyme products, or enzyme cocktails. Theoretically, using multiple enzymes increases the variety of substrates the enzyme product can work on due to the diversity of enzyme activities present (McAllister *et al*., 2001; Chesson, 1993; GrootWassink *et al*., 1989). A cocktail or combination product may be especially advantageous when trying to mitigate the effects of NSP. Since most NSP are heteropolymers they have complex structures that may require multiple enzymes for the NSP to be hydrolyzed (Polizeli *et al*., 2005). However, the results observed when using these cocktails have been inconsistent. Zanella *et al*. (1999) demonstrated that supplementing lower energy broiler diets with an enzyme cocktail of amylase, protease, and xylanase resulted in the same growth performance as birds fed a basal corn-soybean diet. However, Irish and Balnave (1993) reported that a multi-enzyme product designed to work on soybean meal did not improve growth performance. The main challenge with using an enzyme cocktail is it is difficult to identify the respective contributions or actions of each enzyme component (Masey O'Neill *et al*., 2014a; Angel *et al*., 2010; McAllister *et al*., 2001).

The enzyme solution may be different for each situation. It will be dependent on the type of diets being supplemented, what substrates are provided in the diets, and how well the enzyme product matches with the provided substrate. In some diets it may be clear which one or two substrates are being targeted and one can include well-defined products. In more complicated diets that contain many ingredients, including multiple by-product ingredients, there may be a more diverse set of substrates, such as cell walls and cooked proteins, and may benefit more from a product containing a broad range of activities (Campbell and Bedford, 1992). When targeting multiple substrates in one diet, it is important to remember

that one will not observe full additivity from the combination of enzymes. This is due to the fact that once an enzyme has hydrolyzed its substrate, there may be less total substrate remaining to be hydrolyzed by other enzymes (Adeola and Cowieson, 2011; Cowieson, 2010; Selle *et al*., 2010).

Use of exogenous enzymes use in poultry production

There are numerous enzyme-containing feed additives available on the market today. The classes of the most commonly used exogenous enzyme in poultry production are phytase and the carbohydrases xylanase and β-glucanase (Adeola and Cowieson, 2011; Sheehan, 2010; Bedford, 2000). Other classes of enzymes used in animal feed are proteases, α-amylase, mannanse, and α-galactosidase, but their use is not as widespread. Exogenous enzymes have been demonstrated to improve the digestibility of starch, amino acids, fat, and minerals (Bedford, 2000).

Cowieson (2010) identified two main mechanisms by which exogenous enzymes can improve profitability in poultry production: 1) enhance the digestibility of dietary nutrients and 2) reduce nutrient requirements of the animal. Incorporating exogenous enzymes into diets might not improve the digestibility of a good quality feed ingredient; however, it can improve nutrient digestibility from lower quality ingredients (Bedford, 2000). For example, ANF, such as NSP present can reduce nutrient digestibility of a diet; by supplementing exogenous enzymes to counteract the ANF, overall nutrient digestibility of a diet can be improved. In addition, improving the feeding value of lower quality ingredients can help reduce the variability in animal response due to variation in specific shipments of ingredients

due to many factors such as genetics, local growing conditions, and seasons of the year (Campbell *et al*., 1989). This provides opportunities to reduce costs by purchasing less digestible, lower cost, or less popular feed ingredients. As typical ingredients in the United States' poultry and swine industries continue to increase in cost and decrease in availability, alternative ingredients will increase in importance (Leeson *et al*., 2000).

Traditionally, the poultry industry in the United States has formulated feeds based on corn and soybean meal. Over the past several years, the cost of corn has increased dramatically, mainly as a result of corn-based ethanol production (Donohue and Cunningham, 2009). In addition to corn-based ethanol production, bio-diesel utilizing animal fat and vegetable oils has also gained popularity. This resulted in increased competition and cost for an important energy source used in poultry feed (Gashaw and Teshita, 2014; Donohue and Cunningham, 2009). Exogenous enzymes are not usually included in corn-soybean based diets because these diets are considered highly digestible (Cowieson, 2005; Odetallah *et al*., 2003). However, because the expense of feed accounts for such a great proportion of production, there is increased interest to improve nutrient utilization from all dietary ingredients. For example, the addition of enzymes could further improve starch digestion from corn (Masey O'Neill *et al*., 2014a; Cowieson, 2005), as well as improve protein utilization of under- or over-heated soybean meal (Kocher *et al*., 2002; Zanella *et al*., 1999). There may not be a highly measurable improvement compared to those seen in wheat and barely based diets, but even small improvements could have great economic value when put in the scale of a large company. Cowieson (2010) suggests for a standard corn-soy diet with average digestibility there is a loss of about 440 kcal/kg of

energy from undigested starch, protein, and fat at the ileal level. This 400 to 450 kcal/kg of undigested energy is what is potentially available for improved digestibility by use of exogenous enzymes.

There are two main ways to include enzymes in a feed formulation: "on top" of the feed or assigned matrix value. When adding an enzyme on top of a feed, no changes or nutrient reductions are made to the feed formulation. This may not directly reduce the cost of feed, but it may mitigate variability between crops of certain grains and subsequently have other performance benefits, such as drier litter or more uniform growth. Reducing the variability in nutrient content of bio-availability allows for lower margins of safety when formulating micronutrients, which can reduce over-inclusions of micronutrients resulting in less excretion and reduced feed costs (Pariza and Cook, 2010). By assigning a matrix value to the enzyme, such as the expected energy increase or increased available phosphorous value achieved when the enzyme is added, changes can be made to the feed formulation to account for those nutrient up-lifts. This allows the opportunity to reduce the inclusion of a costly ingredient such as fat or inorganic P (Cowieson and Masey O'Neill, 2013; Adeola and Cowieson, 2011).

Phytase

Exogenous phytases are one of the most common exogenous enzymes added to poultry and swine rations (Cowieson, 2010). The inclusion of phytase to improve P digestibility developed as a solution to reduce P excretion due to environmental concerns. Approximately two-thirds of the P in plant material is bound as phytate (*myo*-inositol

hexaphosphate), or phytic acid, the main storage form for P in plant seeds (Simons *et al*., 1990). Although monogastrics have endogenous phytase activity in their small intestine, their ability to hydrolyze phytate is limited due to an insufficient level of phytase activity and low microbial population in the upper part of the digestive tract (Griener and Konietzny, 2010; Selle *et al*., 2010) especially in young birds.

Phytases (*myo*-inositol hexaphosphate hydrolases) hydrolyze the ester bonds between the inositol ring and the phosphate groups making up the phytate, or phytic acid molecule, resulting in a stepwise dephosphorylation of the molecule. The phosphate groups of the phytate give the molecule a negative charge which allows it to complex with positively-charged molecules, such as cations, rendering the components less digestible (Angel *et al*., 2002). As the phosphate groups are released by the phytase, increasing the P available to the animals, other minerals, proteins, and starch bound by the phytate molecule are released as well (Cowieson *et al*., 2004; Shelton *et al*., 2004; Angel *et al*., 2002).

The contributions to energy, amino acids, P, and calcium improvements from phytase inclusion are easily quantifiable, making it an attractive additive for poultry and swine producers (Cowieson, 2010). Improved nutrient digestibility with the inclusion of phytase, allows the enzyme to be given a matrix value for feed formulation and provides the opportunity to reduce expensive ingredients in the diet, such as inorganic P. Although the response to phytase is relatively consistent, there may be variation in response between feed ingredients due to endogenous phytase activity in grain (Bedford, 2000). Phytases appear to better degrade phytate to release bound P in pigs compared to broiler chickens, but the 'extra-

phosphoric effects' of the enzyme are more pronounced in broiler chickens (Selle *et al*., 2010).

Protease

Proteins, or more specifically the amino acids comprising the proteins, have an important metabolic role. Amino acids are the building blocks for proteins, for example enzymes, and are major components of cell membranes, and provide structure such as to muscle, hair, feathers, skin, and hooves (Pond *et al*., 2005). Inadequate protein and specific amino acids is possibly the most common nutrient deficiency since most energy sources in the diets are low in protein (Pond *et al*., 2005). Poultry diets in the United States commonly contain soybean meal as a main protein source, but may also include other vegetable-based or animal-based protein sources. The protein of properly processed soybean meal is well digested by poultry (Odetallah *et al*., 2003; Kocher *et al*., 2002). However, protein sources such as animal by-product meal may contain more complex proteins that the bird cannot easily digest, such as collagen or elastin (Pond *et al*., 2005).

Proteases can be supplemented to poultry diets to reduce nitrogen excretion by improving protein utilization, subsequently reducing the dietary protein requirement (Yu *et al*., 2007). Similar to the environmental concerns associated with high levels of P excretion, there are also concerns associated with high levels of nitrogen excretion (Oxenboll *et al*., 2011; Tamminga *et al*., 1995). High levels of nitrogen excretion can result in husbandry and welfare concerns such as high levels of atmospheric ammonia and litter moisture (Ritz *et al*.,

2004). This can be a result of factors such as indigestible protein sources or ANF, such as protease (trypsin) inhibitors and lectins.

Proteases cleave the peptide bonds at certain amino acids residues and have varying degrees of specificity. Some proteases have a broad range of substrates (Odetallah *et al*., 2003), while others have a more narrow range of substrates (Bastawde, 1992). Proteases break down those proteins that are mainly used as storage proteins in various plant materials and protein-like anti-nutrients in vegetable proteins. There are high concentrations of storage proteins in seeds, especially those from legumes such as soy. These storage proteins provide a nitrogen source for the plant during development and can bind to starch. When the proteases break down storage proteins, bound, energy-rich starch that can be digested by the animal is also released (Barletta, 2010). Proteases can also improve protein digestibility by reducing the levels of trypsin inhibitors and lectins found in raw plant proteins (Barletta, 2010).

Protease addition to poultry diets has yielded variable results. Researchers have reported improved broiler performance with the addition of protease to a sub-optimum crude protein level, such as improved feed efficiency and greater body weight gain (Yu *et al*., 2007; Odatellah *et al*., 2003), as well as greater breast meat yield (Wang *et al*., 2006; Odetallah *et al*., 2005). While sometimes provided as a single enzyme (Odatellah *et al*., 2003), proteases are often included in cocktail products. The mode of action *in vivo* is unknown (Angel *et al*., 2010), and since most proteases are in enzyme cocktails it is difficult to identify the action of protease alone (Isaksen *et al*., 2010).

Carbohydrase

Carbohydrase enzymes were originally introduced in diets containing "viscous grains" such as wheat and barley to reduce to anti-nutritive effects associated with them (Choct and Annison, 1992; Bedford and Classen, 1992). While phytases have been demonstrated to be beneficial to the poultry industry and are included in most commercial diets (Cowieson, 2010), the inclusion of carbohydrases is not as wide spread. The lack of popularity of the carbohydrases compared to the phytases is due to the difficulty of measuring and predicting the response from the enzymes. This is due to a combination of factors, including the substrates available, which vary due to the cereal grain and diet formulation (Masey O'Neill *et al*., 2014a). The addition of carbohydrases can improve apparent metabolizable energy (AME) value of a digest through improvements in fat and starch digestibility. Carbohydrases will be discussed in further detail in the following section.

Carbohydrates and carbohydrases

Carbohydrates

Commercial diets for monogastrics, such as poultry and swine, are based on a cereal grain providing the majority of the energy in the diets in the form of carbohydrates (Classen, 1996). Carbohydrates, consisting mainly of starch and cellulose, are the major components in plant tissues, constituting about 50 to 80% of the dry matter of forages and cereals (Van Soest, 1994). Carbohydrates are classified based on the number of carbon atoms per molecule of carbohydrate, the types of carbon-carbon bonds, and the number of molecules of

sugar in the compound. A monosaccharide has a single molecule of sugar, a disaccharide has two molecules, an oligosaccharide has three to ten molecules, and a polysaccharide has more than ten sugar molecules (Pond *et al*., 2005). These sugar molecules are connected by either α- or β-glycosidic linkages. Two categories of monosaccharides of interest are hexoses and pentoses. Hexoses, such as glucose, are monosaccharides that contain a six-carbon backbone, while pentoses, such as xylose and arabinose contain a five-carbon backbone.

In animal nutrition, carbohydrates can be broadly classified into two categories: starch and fiber. Starch molecules are polysaccharides made up of glucose monomers joined together by α-1,4-glycosidic linkages. These polysaccharide chains can be found in either the linear form of amylose or the branched form of amylopectin. In the presence of the digestive enzyme α-amylase, starch can be hydrolyzed to mono- (glucose) and disaccharides (maltose) so the animal is able to absorb the nutrient and utilize it as an energy source. Birds are able to synthesize and secrete adequate levels of amylase to digest starch provided in the diet and are able to increase the rate of this process if necessary to adapt to diets higher in starch (Moran, 1982). The second classification for carbohydrates is fiber, or non-starch polysaccharides (NSP). The category of NSP includes several complex carbohydrates found in plant cell walls and can either be soluble or insoluble. Insoluble NSP, such as cellulose, are made up of glucose monomers linked by β-1,4-glycosidic linkages which render them resistant to breakdown unless in the presence of cellulase, a β-glycosidase. Animals do not synthesize cellulase endogenously; however, cellulase can be synthesized by the microbes in the rumen or lower gut of non-ruminants, allowing for some breakdown of these molecules (Pawlik *et al*., 1900). Soluble NSP, such as hemicellulose (pentosans) and β-glucans, are

found mostly in the cell wall of cereal grains. Because xylose and arabinose are both five-carbon sugars, or pentose sugars, arabinoxylans are often referred to as pentosans (Paloheimo *et al*., 2010).

Some of the less soluble forms, such as starch, serve as energy reserves in roots, tubers, and seeds. Starch is found in the endosperm of cereal grains and is held in place by other carbohydrates that make up the cell walls such as cellulose (insoluble) and non-cellulose (soluble) polysaccharides. The relatively insoluble portions, such as cellulose and hemicellulose, are most important for providing structural support in living plants.

Non-Starch Polysaccharides

Within the category of non-starch polysaccharides (NSP), there are both insoluble and soluble NSP. The insoluble NSP, such as cellulose, in the cell walls of cereal grains are found in low levels and are thought to have little negative impact on nutritive value of the grain (Smits and Annison, 1996; Bedford, 1995). Most of carbohydrates in the cell wall are made up of heteropolymers, polysaccharides that contain more than one variety of sugar residues, such as β-glucans and arabinoxylans (pentosans) (Classen, 1996). The differences in the structure of the polysaccharide can change its properties such as solubility, capacity for binding water and susceptibility to enzymatic degradation (Bedford and Schulze, 1998; Bedford, 1995; Bastawde, 1992). β-glucans, linear polymers of glucose with β-(1,3) (1,4)-glycosidic linkages, are primarily hydrolyzed by endo-β-glucanase (Classen, 1996). β-glucans will vary in length and the exact patterns on substituents on the glucan backbone. Arabinoxylans consist of a backbone of β-1,4-D-xylose residues, and will have a variety of

different substituents, such as arabinose residues positioned along the backbone (Campbell and Bedford, 1992). The amount of substituents and the degree of branching on the xylan will depend on the grain source of the NSP (Rose *et al*., 2010). Arabinoxylans are primary hydrolyzed by endo-1,4-β-xylanase activity, which cleaves (1,4)-linkages of the xylan backbone (Classen, 1996; Smits and Annison, 1996). The total amount and proportion of different polysaccharides will vary among grains (Classen, 1996; Smits and Annison, 1996).

Mechanisms of impaired nutrient digestion

The soluble fraction of the NSP in the grain may impair nutrient digestion in monogastrics, and is often manifested as reduced performance, such as growth and feed efficiency (Choct *et al*., 1996; Choct and Annison, 1992). There are several mechanisms by which certain grains act as ANF. The two main mechanisms that NSP impair nutrient digestion are increasing digesta viscosity and acting as a physical barrier for enzymes (Masey O'Neill *et al*., 2014a; Classen 1996; Bedford and Classen, 1992; Hesselman and Aman, 1986).

One way NSP impair nutrient digestion is by binding with water present in the intestines causing them to hydrate, swell in volume, and increase the viscosity of the digesta contents (Smits and Annison, 1996; Wang *et al*. 1992; Campbell *et al*., 1989). As the concentration of NSP increases, the solubilized NSP interact or become entangled with each other forming gels (Jacob and Pescatore, 2012; Bedford, 1995). An increase in digesta viscosity reduces the ability of the gut contents to mix, an action which is critical for micelle formation and the absorption of fat and fat-soluble nutrients (Santos *et al*., 2004; Wallace and

Chesson, 1995; Edney *et al*., 1989). Increased gut viscosity can also slow digesta (chyme) gut passage rate as well as limit the accessibility of the digestive enzymes to their substrates (nutrients) (Campbell and Bedford, 1992). The increased bulk of the digesta due to increased viscosity reduces the diffusion rate of the nutrients to the mucosal surface and limits the interaction between enzyme and substrate (Ikegami *et al*., 1990; Hesselman and Aman, 1986).

The reduction in gut passage rate due to the viscous digesta has also been suggested to increase mucus secretion produced by goblet cells (Bedford and Cowieson, 2012; Choct *et al*., 1996; Smits and Annison, 1996; Classen, 1996) which contributes to the unstirred water layer of the mucosal lining (Moran, 1985; Smithson *et al*., 1981). For nutrients to be absorbed by the animal, the nutrients must cross the aqueous barrier formed by the unstirred water layer. An increased thickness of the unstirred water layer further inhibits the rate nutrient uptake due to the reduced ability of nutrients, especially fat or fat-soluble, to cross the water to reach the mucosa (Classen, 1996; Smits and Annison, 1996; Johnson and Gee, 1981).

Increased viscosity due to NSP can also have an effect on the integrity of the intestinal morphology itself. This can result in decreased villous height and surface areas (Teirlynch *et al*., 2009) as well as increased proliferation rates of enterocytes (Smits and Annison, 1996). Increased proliferation rates of enterocytes can decrease activity of specific epithelial surface enzymes. Not only does this negatively affect the uptake of nutrients, it also increases the maintenance cost of the animal (Parsaie *et al*., 2007; Zhang *et al*., 2005).

Similarly to how the increased digesta viscosity can inhibit or slow digestion, the cell walls of the feedstuffs themselves can act as a physical barrier, reducing the animal's ability to utilize starch and protein in the cells of the endosperm (Hesselman and Aman, 1986). When these cell walls are broken down, the starch and protein within the cell will be more accessible to enzymes, improving digestibility. This barrier effect can reduce total starch digestion or delay starch digestion until the distal portion of the small intestine (Classen, 1996), where nutrients released are not available for digestion. This also can result in microflora migrating to more anterior portions of the small intestine where is will compete with the host for nutrients (Bedford, 1995).

The reduced nutrient digestibility results in more undigested nutrients being present in the lower gut, which provides energy to microflora in the gut and can increase the population of undesirable, or pathogenic, microflora (Gehring *et al*., 2013; Choct *et al*., 1996; Campbell and Bedford, 1992). This situation is complicated by the reduced passage rate of the digesta which can decrease oxygen tension in the small intestine and provide a more stable environment where fermentative microflora can become established (Gao *et al*., 2008; Wagner and Thomas, 1978). This can allow gut microflora to multiply and produce a greater volume of volatile fatty acids, lactic acid, and a decrease in the pH value. (Gao *et al*., 2008; Ricke *et al*., 1982). This shift in the microbial population and growth can cause deconjugation of bile acid, which is necessary for fat digestibility (Bedford, 2000; Smits and Annison, 1996; Bedford, 1995; Campbell *et al*., 1983).

NSP in cereal grains

Corn is a common and large component of poultry diets, especially in the United States (U.S.) and is considered a highly digestible feedstuff. Historically, NSP have not been a major concern with dietary inclusion of corn, although corn does contain some arabinoxylans, as well as very low levels of β-glucans (Smits and Annison, 1996). In the past, 'viscous' cereal grains such as wheat (Annison, 1993), barley (Hesselman *et al*., 1981), and rye (Antoniou and Marquardt, 1981), were included in poultry diets in limited quantities due to their high concentrations of soluble NSP that act as ANF. In the past two decades as ethanol production expanded, dried distillers grains (DDGS) have become a more common alternative ingredient in U. S. poultry diets (Lumpkins *et al*., 2004). DDGS are a co-product of ethanol production from the starch, a process that results in all remaining nutrients in the co-product (DDGS) being concentrated about three times, including the NSP content (Peron and Partridge, 2010).

Wheat can be a good alternative to corn in poultry diets. Although there can be a lower energy value associated with wheat, wheat has higher crude protein content and higher lysine concentration than corn (Cowieson, 2005; Wang *et al*., 2005; Crouch *et al*., 1997). However, great variability in apparent metabolizable energy (AME) values between wheat crops, presents a challenge when feeding this cereal to poultry (Leeson *et al*., 2000; Annison, 1993; Chesson 1993). The variability in metabolizable energy is correlated with the starch digestibility rather than the protein and starch content of the grain (Choct *et al*., 1999).

Although the anti-nutritional effects are similar to the other viscous grains, the main NSP in barley are β-glucans (Jacob and Pescatore, 2010; Hesselman *et al*., 1981). As with

rye, feeding barley also presents a management challenge of wet litter due to sticky dropping as a result of undigested β-glucans (Classen, 1996); this is perhaps even a bigger concern than the high fiber content of the barley (Yu *et al*., 1998). Arabinoxylans are also the main NSP in rye, typically in higher concentrations than found in wheat (Pawlik *et al*., 1990). Effects of β-glucans in barley are similar to rye, but rye is more severe (Campbell and Bedford, 1992). Inclusion of rye as a cereal grain in poultry diets has been related to problems such as viscous feces (sticky droppings), that adhere to the feathers and feet of the bird, and poor growth and feed conversion in younger birds (Misir and Marquardt, 1978). Rye inclusion can also result in increased viscosity of the digesta contents; however, it has been suggested that perhaps the shift in microbial population and its consequences are more of the issue with rye than the digesta viscosity (Campbell *et al*., 1983). Therefore, exogenous enzymes with pentosanase activity will improve the feeding value of rye, but the response is not sufficient enough to use diets with rye as the only, or majority, cereal grain. Enzyme supplementation to rye-based diets is not always effective at removing the issue of sticky droppings, leaving a litter management challenge (Campbell and Bedford, 1992).

Carbohydrases

In animal nutrition carbohydrases can be broadly categorized into those that target either non-starch polysaccharides (NSP) or starch (Barletta, 2010). The main reason for inclusion of a NSP-degrading enzyme (NSPase) is to degrade the NSP and reduce the associated anti-nutritive effects. The two main NSPases commonly used in animal feed are xylanases and β-glucanases. Xylanases break down arabinoxylans and β-glucanases

breakdown β-glucans. Traditionally NSPases were used in diets containing 'viscous grains,' and the use in corn-based diets was less common because the benefits of inclusion are not well established as they are in wheat or barley- based diets. Due to the lower concentration of NSP in corn, and that they are more insoluble compared to those found in wheat, viscosity is not the main concern for corn (Masey O'Neill *et al*., 2014b; Rose *et al*., 2010). The use of carbohydrases provides an opportunity to improve feed utilization by monogastric animals as well as allow for more flexibility in the inclusion of alternative or low quality feed ingredients in formulated rations.

As discussed, soluble NSP can increase the viscosity of the digesta contents (Choct and Annison, 1992). It is not necessary to completely degrade all of the NSP present, but addition of NSPase can break up large aggregates of interacting NSP resulting in a reduction in digesta viscosity (Bedford, 1995). A reduction in viscosity could allow endogenous enzymes better access to nutrients in the lumen, as well as improved contact for mucosal surface enzymes. The addition of NSPases also allows NSP to be broken down in a more anterior portion of the small intestine, which can result in more efficient starch utilization by reducing microbial starch digestion (Classen, 1996; Hesselman and Aman, 1986). This moves the site of digestion of starch and protein to a more anterior portion of the small intestine, which allows the bird more opportunity to absorb the nutrients, and leaves a smaller fraction of undigested nutrients available to the microflora (Bedford, 2000; van der Klis *et al*., 1993; Hesselman and Aman, 1986). Reductions in undigested nutrients in the terminal ileum can influence the ileal digestible energy by altering the amount of fermentable substrate available to cecal microflora (Gehring *et al*., 2013). While NSPase

supplementation has been demonstrated to reduce digesta viscosity, this does not always correlate with differences in growth performance. Some authors have reported significant reductions in digesta viscosity with NSPase supplementation, correlating with weight gain and improved feed conversion efficiency (Wu *et al*., 2004; Choct *et al*., 1999; Choct *et al*., 1996; Almirall *et al*., 1995; Bedford and Classen, 1992). However, other studies have not always demonstrated this correlation (Woyengo *et al*., 2008; Leeson *et al*., 2000; Crouch *et al*., 1997; Choct and Annison, 1992).

In poultry diets NSPases are typically supplemented throughout the whole production cycle. However, most of the earlier research focused on the starter period when the viscous grains pose the most challenge to the immature gut of the birds. As a bird ages and the digestive tract develops, the bird is able to better tolerate NSP (Peterson *et al*., 1999; Bedford 1995; Chesson, 1993). Increased digestive capacity in older birds (greater than 2 weeks of age) due to gut maturity may also result in a reduced response to NSPase supplementation (Campbell and Bedford, 1992). However, recently there has started to be more investigation into supplementing in the later stages of production (Cowieson and Masey O'Neill, 2013). Birds eat the greatest quantity of feed towards the end of production, which could result in more potential savings if energy digestion and feed efficiency can be improved by exogenous enzyme inclusion during this time.

One of the main objectives for including exogenous carbohydrase enzymes is to improve energy utilization of a diet. The goal is to quantify the effects of the enzyme when it is included and assign it matrix values for feed formulation. Carbohydrates constitute the main energy source in poultry diets, but fats, oils, or greases are much more concentrated

energy sources. The concentrated energy sources are also typically much more expensive ingredients per kilogram than cereal grains. The most common method for including carbohydrases has been to assign an apparent metabolizable energy value to the enzyme and reduce the energy of the diet by reducing fat. However, unlike phytase, to which the response can be easily quantified, the response to carbohydrases are not easily determined. While the cost of fat is a contributing factor, there are many "extra-caloric" effects of fat inclusion in a diet that must be considered before fat is removed only of the basis of energy level of the diet. Fat is also an important source of essential fatty acids, the absorption of fat-soluble vitamins, palatability of the diet, pellet quality, density of the diet which can effect feed consumption and feed efficiency, and impacts passage rate of the digesta contents (Cowieson, 2010; Baiao and Lara, 2005). The presence of fat (lipids), rather than carbohydrates, in the duodenum stimulates cholecystokinin (CCK) secretion, which stimulates pancreatic enzyme secretion as well as regulates the flow of bile (Sherwood *et al*., 2005). Cowieson (2010) suggests that rather than only account for an energy value for the carbohydrase, that it may be more beneficial to evaluate enzyme's effect on energy metabolism through digestibility of specific nutrients such as starch, protein, and fat and the contributions they make to digestible energy.

Influencing intestinal microflora

Another current focus is to better understand the mechanisms in which enzymes can affect the microbial population in the gut, which is still largely unknown. This topic is increasingly more important to understand and manage gut microfloral populations as

consumer pressure for antibiotic free animal production increases. It was previously thought the shifts in microbial population were reactive to 'left-over' nutrients following digestion (Choct *et al*., 1996; Campbell and Bedford, 1992), however there is a hypothesis that the size and composition of the population can play a role in the extent of digestion the host can complete, which greatly influences growth rate and efficiency (Bedford and Cowieson, 2012). It has been known for decades that microbiota was part of the challenge associated with viscous grains, but the focus was always on the impaired digestion and microbes were secondary (Choct *et al*., 1996). It was demonstrated that the use of antibiotics could reduce the anti-nutritive effect with some viscous grains (Campbell *et al*., 1983; Misir and Marquardt, 1978). NSPase are able to alter microbial populations, but not in a precise or predictable way. If there is no longer an option to use antibiotics to help control the intestinal microflora of the bird, there is a need to better understand the mechanism in order to tailor these new-age enzymes to "fill in the gap" of the antibiotics.

Recently, Bedford and Cowieson (2012) discussed the topic that NSPases may not be as effective as they used to be resulting in less response by the poultry. The authors proposed that this could be a combination of factors such as advancements in genetics, improved understanding of nutrition, husbandry and biosecurity practices, as well as changes in the quality of the feed ingredients themselves. Modern broiler chickens have a high passage rate of digesta that may not allow for enough gut retention time for an enzyme to work optimally (Cowieson and Masey O'Neill, 2013). It may be more beneficial to slow digesta passage by increasing dietary fat or with larger feed particles to allow more time for the enzyme to function (Amerah *et al*., 2008; Engberg et *al*., 2004; Croom *et al*., 1999; Duke, 1982).

Summary

There is a large body of research evaluating enzymes as feed additives to poultry feeds. With all of the different products available on the market today, it is important to remember that products are developed and activities evaluated in controlled *in vitro* conditions which may be very different from the environment of the host animal (Igbasan *et al*., 2000). Available substrate, rate of gut passage, changes in pH, digesta viscosity, and endogenous enzymatic activity can all impact the efficacy of the enzyme (Ikegami *et al*., 1990) and an evaluation must be ultimately determined with *in vivo* bioassays.

In the research conducted for this dissertation, a novel mono-component endo-β-1,4-xylanase was utilized. This enzyme was engineered to be thermostable so it could withstand conditioning and pelleting processes during feed manufacturing. This novel xylanase was evaluated for optimal inclusion levels in broiler chicken diets, thermotolerance during feed manufacturing, and performance responses in broiler chickens in an attempt to quantify the energetic value of the product. The effect of bird age and presence of dietary phytase were also evaluated.

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MANUSCRIPT I. The evaluation of increasing concentrations of a novel, heat-stable xylanase in mash wheat-based broiler starter diets.

ABSTRACT: A study was conducted to evaluate the effect of increasing concentrations of a novel, heat-stable xylanase (Xyl) on digesta viscosity and nitrogen-corrected apparent metabolizable energy (AME_n) in broiler chicken diets fed from hatch until 20 days. A wheat (60%)-soybean meal (20%)-corn DDGS (10%)-based basal diet was fed in mash form and was supplemented with either no enzyme supplementation (NC), increasing levels of Xyl (1, 2, 4, 8, or 16 mg xylanase/kg finished feed (ppm)), or a commercially available carbohydrase (PC) at the manufacturer's recommended level. Birds were housed in 35 battery cages with 8 birds/cage and consumed feed and water *ad libitum* throughout the trial. At 20 days, 15 birds/treatment were euthanized and ileal digesta was collected for digesta viscosity analysis. Excreta samples were also collected at 20 days for AME_n analysis. Digesta viscosity was reduced as the level of Xyl increased ($p<0.05$). Digesta viscosity in birds fed Xyl at a level equal to or greater than 2 ppm was equal to birds fed PC. There was a linear increase in AME_n with increasing Xyl concentration ($p<0.005$), with an uplift of 140 kcal/kg with 16 ppm Xyl compared to the birds fed no Xyl. The addition of this novel, heat-stable xylanase decreased digesta viscosity and increased AME_n for starting broilers when included in mash wheat-based diets.

(*Keywords*: xylanase, enzyme, AME_n, viscosity, broilers)

Description of Problem

Grains, such as wheat and barley, contain high concentrations of soluble non-starch polysaccharides (NSP) that impair nutrient digestibility and negatively affect bird performance (Choct *et al*., 1996; Choct and Annison, 1992). As the use of grains such as wheat have increased in poultry diets as alternative ingredients to traditional corn. Due to this shift, there is a renewed interest in exogenous carbohydrases that has resulted in a large increase in new enzyme products that can be included in animal feed to improve performance.

Arabinoxylans are the main NSP in wheat, thus xylananse activity is the favored carbohydrase for wheat-based diets. Consumption of diets containing high concentrations of soluble NSP can increase digesta viscosity, thought to be the main cause of reduction in nutrient digestion. The inclusion of exogenous carbohydrase to these diets can reduce digesta viscosity (Campbell and Bedford, 1992) and result in increased apparent metabolizable energy (AME) of a diet (Nian *et al*., 2011).

In this study a novel exogenous xylanase was evaluated in mash diets. As with any novel product, proper inclusion level (dosage) must be evaluated.

Materials and Methods

Bird husbandry

Male Ross broiler chicks (280) were randomly distributed into groups of 8 chicks, weighed individually and assigned to 35 Alternative Design® battery cages[1]. Birds were randomly assigned to 1 of 7 dietary treatments and consumed feed and water *ad libitum.* Each cage was equipped with two adjustable-height nipple drinkers and one feed trough. Birds were provided with 23 hours of light and 1 hour of dark per day. Temperatures were provided at 32^0C for the first 48 hours after birds were placed. Temperature was then decreased 0.5^0C per day for an additional 5 days, after which it was decreased an additional 2.5^0C per week until 21^0C was reached.

Dietary treatments

One basal diet, formulated based on recommendations of the breeder (Ross, 2009), was mixed at the North Carolina State University Feed Mill. To create the dietary treatments, 7 aliquots were taken from the basal diet and supplemented with exogenous enzymes to create the dietary treatments. The 7 dietary treatments were allocated across 35 cages, resulting in 5 replicate cages per treatment. One treatment received no enzyme supplementation (NC). Five of the treatments were supplemented with increasing concentrations 1, 2, 4, 8, or 16 mg xylanase/kg finished feed (ppm)[2] of a novel xylanase (BioResource International, Inc., Durham, NC). The xylanase was included in a raw, dry

[1] Alternative Design Manufacturing & Supply, Siloam Springs, AR, USA.
[2] One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50 ^{0}C in 50mM trisodium citrate buffer at pH 6.0.

form and did not contain any carrier or filler. The seventh treatment (PC) was supplemented with a commercially available liquid carbohydrase enzyme (Rovabio® Excel, Adisseo, France)[3], included according to manufacturers' recommendations; this treatment was included to provide a comparison of a known product. An indigestible reference (Diatomaceous Earth)[4] was included in all treatments for analysis of nitrogen-corrected apparent metabolizable energy (AME_n).

Live performance

Individual birds and feeder weights were recorded at 7, 14, and 20 days of age to obtain bird body weights (BW), body weight gain (BWG), average feed intake (FI), calculated using feed disappearance, and feed conversion ratio (FCR). Cages were checked twice daily for mortality and morbidity, which was less than 3%. FCR was calculated as pen FI divided by pen BWG plus the weight of mortality that occurred during the period of interest.

Digesta viscosity and intestinal samples

At three weeks of age three birds per pen (15 birds/treatment) were euthanized by cervical dislocation and sampled to assess ileal digesta viscosity as well as intestinal and pancreas weight. The pancreas was removed from each bird and weighed. The small intestine was removed from each bird and was cut into segments: duodenum (duodenal loop),

[3] Rovabio® Excel enzyme cocktail contains main enzyme activities of endo-1,4-β-xylanase , endo-1,3(4)-β-glucanase, and endo-1,4-β-glucanase
[4] Celite®, World Minerals, Inc., Santa Barbara, CA

jejunum (duodenal loop to Meckel's diverticulum), and ileum (Meckel's diverticulum to ileal-cecal junction). Ileal contents were collected for viscosity evaluation and then all three segments of the small intestine were then flushed with 0.9% saline solution to remove any remaining contents and a longitudinal cut was made along the length of the segments so they could lay flat for more accurate measurements. Segment length and weight were recorded.

Viscosity measurements were taken on supernatant extracted from fresh ileal digesta contents. Ileal digesta contents manually expressed into 50 mL conical tubes and stored on ice for measurements of digesta viscosity. Ileal contents from each individual bird were mixed, sub-sampled, and centrifuged at 5.9 RCF[5] for 5 minutes to separate the supernatant from the solid digesta contents. The supernatant was extracted and placed in a clean 2 mL tube. Viscosity, in centipoise (cP), was measured on a 500 μL aliquot of the supernatant using a LVDV-II+ Brookfield digital viscometer (Brookfield Engineering Laboratories, Inc., Stoughton, Massachusetts, USA) equipped with a CP-40 cone spindle at shear rates of 22.5 sec^{-1} and 45 sec^{-1}. These two speed settings were chosen for these measurements because the readings were more stable and consistent then when using a higher or lower speed.

Apparent Metabolizable Energy

At three weeks of age fresh excreta samples were collected once/day for 3 days from a pan beneath each cage and pooled by cage for analysis and calculation of nitrogen-

[5] RCF=relative centrifugal force

corrected apparent metabolizable energy (AME_n)[6]. While collecting excreta, care was taken to avoid samples contaminated with feed particles or feathers. Samples from each cage were stored at -20^{0}C until analysis. Homogenized samples were removed from freezer and dried at 55^{0}C in a forced air oven (Blue M, Thermal Product Solutions) and ground using a small electric grinder. Gross energy was measured on feed and excreta samples using an adiabatic bomb calorimeter (IKA® calorimeter C 5000, IKA Works, Inc.). Nitrogen of both feed and excreta were measured by combustion analysis (LECO Corporation, St. Joseph, MI). Both feed and excreta were analyzed for Celite® recovery using the acid-insoluble ash procedure based on Vogtmann *et al*. (1975).

Feed analysis

Feed samples were analyzed by BioResource International, Inc. (Durham, NC, USA) to determine xylanase activity. Proximate analysis of feed completed by North Carolina Department of Agriculture (Raleigh, NC, USA).

Statistical analysis

Statistical analysis of the data was completed using Proc GLM of SAS (9.2) (SAS Institute, Cary, NC). Linear regression (Proc REG) was also used to analyze live performance, digesta viscosity, and AME_n; the PC treatment (commercial carbohydrase) was not included in linear regression analysis. Digesta viscosity was also analyzed using non-

[6] $AME_n=GE_{feed}-((GE_{excreta}*AiA_{feed})/AiA_{excreta})-(8.22*N_{retained})$; $N_{Retained}=N_{feed}-((N_{excreta}*AiA_{feed})/AiA_{excreta})$

linear regression, or segmented regression (Proc NLMIXED). Effects were considered significant at $p<0.05$.

Animal ethics

All husbandry practices and euthanasia were performed with full consideration of animal welfare. All bird handling procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University.

Results

Live performance

Results of the live performance can be found in Tables 2-5. No treatment response was observed on BW at 7 or 14 days. At 20 days, birds receiving 1ppm were heavier compared to both un-supplemented birds and other treatments supplemented with xylanase. Although there were no differences in BWG during the first (0-7 days) or third (14-20 days) week, during the second week (7-14 days) birds receiving xylanase at 8 or 16 ppm or the commercial carbohydrase (PC) had significantly greater BWG compared to un-supplemented treatments. Similarly to BW, during the third week (14-20 days) birds receiving 1ppm consumed more feed compared to both un-supplemented birds and other treatments supplemented with xylanase. No differences were observed in FCR throughout the trials and no linear effects were observed on BW, BWG, FI or FCR throughout the trial.

AME_n

A linear increase in AME_n was observed with increasing concentration of xylanase inclusion (Table 6). At the highest concentration (16 ppm) xylanase inclusion provided about 140 kcal/kg uplift compared to un-supplemented.

Digesta viscosity and intestinal samples

Xylanase inclusion resulted in reduction in digesta viscosity (Table 6). Linear reduction in viscosity was observed with increasing inclusion of xylanase. No treatment effect was observed on pancreas weight as a percentage of body weight (Table 7). Actual weight of the pancreas was also analyzed, using bird body weight as a covariate, however no difference was observed. We also evaluated weight of the small intestine segments both as a percentage of body weight, as well as actual weight, using bird body weight as a covariate. No treatment effect was observed on weight or length of duodenum, jejunum, or ileum.

Discussion

Although improvements in live performance were not observed in this study with the enzyme supplementation, it was evident that both the xylanase and commercial carbohydrase (PC) were active through responses measured in AME_n and digesta viscosity. There was no enzyme effect observed on BW during the first or second week, however at the end of the third week (20 days) birds receiving 1 ppm were heavier compared to both un-supplemented birds and other treatments supplemented with xylanase. This is likely due to a higher FI by this treatment during the third week (14-20 days). While a statistical effect was observed, a

biological explanation cannot be offered since these diets all originated from the same basal diet and were nutritionally similar. In this study, no treatment effect was observed on FCR. It is possible there was too much variation within treatments to measure statistical differences. This was the first *in vivo* evaluation of this novel enzyme being supplemented to birds. To include five concentration levels of xylanase, as well as a positive and negative control, we were limited to only five replications per treatment.

Due to this being the first *in vivo* evaluation of this xylanase, the priority was to evaluate if there was a linear response to increasing xylanase concentration. Although no linear response was observed in live performance, a linear increase in AME_n was observed as xylanase concentration increased. Therefore, the addition of the xylanase improved energy digestibility of the diet, although not enough to translate to differences live performance. At the highest xylanase concentration level in this study (16 ppm) an uplift in AME_n of about 140 kcal/kg was observed. However, the AME_n response did not seem to plateau at the higher concentration levels, indicating that the ideal inclusion level in mash diets may be higher than 16 ppm.

Similarly to the response in AME_n, there was a linear reduction in viscosity as xylanase inclusion level increased in concentration. However, the response appeared to plateau, for this reason segmented regression analysis, also referred to as broken-line analysis, was utilized to provide a more accurate depiction of the response. Using this non-linear regression analysis, it was estimated at an inclusion level of about 2 ppm the reduction in viscosity started to plateau and was similar to the response observed with inclusion of the commercial carbohydrase (PC). Reduction in digesta viscosity can improve nutrient

digestion (Bedford, 2000), which could explain the uplift in AME_n. While reduction in digesta viscosity and an improvement in AME_n can result in improved performance (Wu *et al*., 2004; Choct *et al*., 1996; Almirall *et al*., 1995; Bedford and Classen, 1992), sometimes there is no correlated improvement in performance (Woyengo *et al*., 2008; Leeson *et al*., 2000; Crouch *et al*., 1997).

It has been observed in previous studies that both the intestinal tract and digestive organs such as the pancreas can increase in size to adapt to diets high in indigestible polysaccharides, an effect that can be reversed when those diets are supplemented with carbohydrases (Gao *et al*., 2008; Almirall *et al*., 1995; Ikegami, 1990). However, in the current study, no differences were observed in the size or weight of the intestine or pancreas. Therefore the diets were digestible enough that the pancreas and digestive tract did not undergo hypertrophy, which can be costly for the animal. Although xylanase inclusion beneficially impacted viscosity and energy metabolism, there are indications that the diet overall was digestible enough to provide birds with adequate nutrients for optimal growth.

Conclusion and Applications

1. Supplementation of the novel xylanase in a wheat-based diet provided an uplift in AME_n of about 140 kcal/kg.
2. Supplementation of the novel xylanase reduced digesta viscosity comparably to the commercial carbohydrase at concentrations of greater than 2 ppm.
3. Optimal xylanase inclusion level may be higher than was provided in this study (16 ppm) to elicit measurable improvements in performance.

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Table 1: Composition and nutrient content of wheat-based starter phase diet fed to male broiler chickens from placement to 21 days of age.

Dietary phase	**Starter**
Days of age	**0-21**
Ingredients	***% of Total diet***
Wheat	59.40
Soybean meal	20.20
Corn DDGS	10.00
Poultry fat	2.24
Limestone (Calcium carbonate)	1.875
Monocalcium dicalcium phosphate (21% P)	1.70
L-lysine	0.71
D,L-methionine	0.50
L-threonine	0.275
Trace mineral premix[1]	0.20
Vitamin premix[2]	0.20
Choline chloride 60%	0.20
Sodium selenite premix[3]	0.10
Sodium chloride	0.25
Sodium bicarbonate	0.20
Celite ®	2.00
Nutrient content	
ME poultry, kcal/kg	2,900
Crude protein, % (calculated)	21.00
Crude protein, % (analyzed)	20.80
Crude fat, % (calculated)	4.26
Crude fat, % (analyzed)	4.40
Available phosphorus, % (calculated)	0.50
Total phosphorus, % (calculated)	0.74
Total phosphorus, % (analyzed)	0.83
Calcium, % (calculated)	1.11
Calcium, % (analyzed)	1.41
Sodium, % (calculated)	0.19
Total lysine, %	1.43
Total Met + Cys, %	1.08
Threonine, %	0.94
Choline, mg/kg	2,532

[1] Each kilogram of mineral premix (0.1% inclusion) supplied the following per kg of complete feed: 60 mg Zn as $ZnSO_4H_2O$; 60 mg of Mn as $MnSO_4H_2O$; 40 mg Fe as $FeSO_4H2O$; 5 mg Cu as $CuSO_4$; 1.25 mg I as $Ca(IO_3)_2$; 1 mg Co as $CoSO_4$.

[2] Each kilogram of vitamin premix (0.1% inclusion) supplied the following per kg of complete feed: 13,200 IU vitamin A; 4,000 IU cholecalciferol; 66 IU alpha-tocopherol; 110 mg niacin; 22 mg pantothenic acid; 13.2 mg riboflavin; 8 mg pyridoxine; 4 mg menadione; 2.2 mg folic acid; 4 mg thiamin; 0.253 mg biotin; 0.04 mg vitamin B_{12}; 100 mg ethoxyquin.

[3] $NaSeO_3$ premix provided 0.3 mg Se/kg of complete feed.

Table 2: Effect of dietary supplementation of a novel exogenous xylanase in increasing concentrations to broiler chickens in wheat-based diets on body weight (BW) from placement until 20 days of age.[1]

	Days of age			
	Placement	**7**	**14**	**20**
Treatment[3]	*grams body weight/bird*			
NC	40.0	134	345	653 [b]
1 ppm	40.2	134	369	704 [a]
2 ppm	39.5	132	349	654 [b]
4 ppm	39.7	133	350	660 [b]
8 ppm	40.0	137	364	681 [ab]
16 ppm	39.6	134	366	675 [ab]
PC	40.1	136	361	667 [b]
Source of variation	*P values*			
Treatment	0.77	0.84	0.13	0.03
Regression	0.41	0.87	0.30	0.97
SEM (25)[2]	0.34	2.6	6.8	10.8

[1]Values are means of 5 replicate pens of *ca.* 8 birds per pen.
[2]SEM (25)=Standard error of the mean with 25 degrees of freedom.
[3]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50 ^{0}C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 3: Effect of dietary supplementation of a novel exogenous xylanase in increasing concentrations to broiler chickens in wheat-based diets on body weight gain (BWG) from placement until 20 days of age.[1]

	Days of age				
	0-7	**7-14**	**14-20**	**0-14**	**0-20**
Treatment[3]	---------- *grams body weight gain/bird* ----------				
NC	94	210 [c]	308	306	613 [b]
1 ppm	94	232 [a]	334	323	663 [a]
2 ppm	93	217 [bc]	304	310	615 [b]
4 ppm	94	215 [bc]	309	311	620 [b]
8 ppm	97	226 [ab]	317	323	641 [ab]
16 ppm	94	231 [a]	310	326	636 [ab]
PC	98	225 [ab]	305	321	627 [b]
Source of variation	---------- *P values* ----------				
Treatment	0.85	0.03	0.13	0.38	0.04
Regression	0.86	0.14	0.46	0.20	0.95
SEM (25)[2]	2.8	4.8	7.7	7.6	10.8

[1]Values are means of 5 replicate pens of *ca.* 8 birds per pen.
[2]SEM (25)=Standard error of the mean with 25 degrees of freedom.
[3]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50 ^{0}C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 4: Effect of dietary supplementation of a novel exogenous xylanase in increasing concentrations to broiler chickens in wheat-based diets on feed intake (FI) from placement until 20 days of age.[1]

	Days of age				
	0-7	**7-14**	**14-20**	**0-14**	**0-20**
Treatment[3]	---------- *grams feed intake/bird* ----------				
NC	134	368	495 [b]	506	999 [bc]
1 ppm	132	371	535 [a]	511	1074 [a]
2 ppm	128	358	485 [b]	485	971 [c]
4 ppm	134	369	489 [b]	510	999 [bc]
8 ppm	139	379	503 [b]	518	1022 [b]
16 ppm	129	376	493 [b]	508	1003 [bc]
PC	133	378	497 [b]	512	1009 [bc]
Source of variation	---------- *P values* ----------				
Treatment	0.55	0.54	0.008	0.53	0.002
Regression	0.80	0.27	0.26	0.63	0.45
SEM (25)[2]	4.1	7.8	8.6	11.2	14.0

[1]Values are means of 5 replicate pens of *ca.* 8 birds per pen.
[2]SEM (25)=Standard error of the mean with 25 degrees of freedom.
[3]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50 ^{0}C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 5: Effect of dietary supplementation of a novel exogenous xylanase in increasing concentrations to broiler chickens in wheat-based diets on feed conversion ratio (FCR) from placement until 20 days of age.[1]

	Days of age				
	0-7	**7-14**	**14-20**	**0-14**	**0-20**
Treatment[3]	--------- *grams feed intake:grams body weight gain*----------				
NC	1.429	1.751	1.610	1.641	1.626
1 ppm	1.445	1.643	1.578	1.557	1.560
2 ppm	1.377	1.654	1.597	1.572	1.584
4 ppm	1.429	1.718	1.577	1.616	1.600
8 ppm	1.432	1.676	1.584	1.605	1.593
16 ppm	1.362	1.630	1.598	1.547	1.571
PC	1.372	1.683	1.624	1.592	1.607
Source of variation	---------- *P values* ----------				
Treatment	0.68	0.31	0.83	0.42	0.57
Regression	0.49	0.33	0.95	0.50	0.70
SEM (25)[2]	0.042	0.038	0.026	0.033	0.025

[1]Values are means of 5 replicate pens of *ca.* 8 birds per pen.
[2]SEM (25)=Standard error of the mean with 25 degrees of freedom.
[3]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50 ^{0}C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 6: Effect of dietary supplementation of a novel exogenous xylanase in increasing concentrations to broiler chickens in wheat-based diets on nitrogen-corrected apparent metabolizable energy (AME_n) and ileal digesta viscosity at 20 days of age.

	20 Days of age	
	AME_n[1]	**AvgVisc[2]**
Treatment[5]	kcal/kg	cP
NC	2776 [c]	13.2 [a]
1 ppm	2814 [bc]	9.89 [b]
2 ppm	2853 [abc]	7.91 [bc]
4 ppm	2874 [ab]	8.08 [bc]
8 ppm	2903 [ab]	5.95 [c]
16 ppm	2918 [a]	6.76 [c]
PC	2848 [abc]	6.79 [c]
Source of variation	---------- *P values* ----------	
Treatment	0.06	<0.0001
Regression	0.005	0.0002
SEM	32[3]	0.995[4]

[1]Values are means of 5 replicate pens.
[2]Values are means of 15 replicate birds per treatment.
[3]SEM (28)=Standard error of the mean with 28 degrees of freedom.
[4]SEM (95)=Standard error of the mean with 95 degrees of freedom.
[5]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50 ^{0}C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 7: Effect of dietary supplementation of a novel exogenous xylanase in increasing concentrations to broiler chickens in wheat-based diets on intestinal weight and length at 20 days of age.[1]

	Percentage of body weight				Length[2]			Weight[2]			
	---------- *(%)* ----------				-------- *(cm)* --------			---------- *(g)* ----------			
Treatment[4]	D	J	I	Panc	D	J	I	D	J	I	Panc
NC	1.15	1.95	1.52	0.36	22.2	52.6	52.1	7.78	13.20	10.32	2.39
1 ppm	1.01	1.86	1.43	0.36	21.0	53.5	53.3	6.95	12.79	9.85	2.50
2 ppm	1.08	1.82	1.37	0.38	22.2	51.6	50.0	7.30	12.35	9.30	2.55
4 ppm	1.03	1.87	1.38	0.37	21.2	53.4	53.7	6.98	12.78	9.44	2.54
8 ppm	1.09	1.88	1.44	0.38	21.3	52.3	52.6	7.37	12.77	9.80	2.56
16 ppm	1.03	1.84	1.47	0.36	21.0	51.1	52.2	6.91	12.43	9.95	2.39
PC	1.03	1.88	1.41	0.37	21.5	52.5	51.7	7.01	12.77	9.62	2.50
	---------- *P values* ----------										
Treatment	0.18	0.87	0.40	0.68	0.37	0.87	0.55	0.27	0.89	0.45	0.67
SEM (95)	0.040	0.065	0.051	0.014	0.048	1.28	1.28	0.28	0.450	0.346	0.089

[1]Values are means of 15 replicate birds per treatment. D=duodenum; J=jejunum; I=ileum; Panc=pancreas

[2]Weight and length were analyzed using bird bodyweight as a covariate.

[3]SEM (95)=Standard error of the mean with 95 degrees of freedom.

[4]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50 ^{0}C in 50mM trisodium citrate buffer at pH 6.0.

[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

MANUSCRIPT II. The evaluation of a novel, heat-stable xylanase in mash and pelleted wheat-based broiler starter diets.

ABSTRACT: Two trials (T1 and T2) were conducted to evaluate the effect of a novel, heat-stable xylanase (Xyl) on the growth performance of broiler chicks from hatch until 3 weeks of age when included in wheat-based rations. T1 was designed as 3 x 2 factorial, with 3 energy levels (2770, 2920, and 3070 kcal/kg ME), and 2 levels of Xyl (no Xyl or 20 mg xylanase/kg finished feed (ppm)). Diets in T1 were pelleted at 85^0C and then crumbled. T2 was designed as a 5 x 2 factorial with 5 levels of Xyl inclusion (0, 5, 10, 20, 40 ppm) and 2 feed forms (mash & crumble); all treatments in T2 were formulated to have 2770 kcal/kg ME. The xylanase was added to the diets in a dry form during feed manufacturing prior to the pelleting process. Birds were housed in 36 (T1) or 60 (T2) battery cages with 6 birds/cage and consumed feed and water *ad libitum* throughout the trial. Bird and feeder weights were recorded at 3 weeks of age and used to calculate average pen body weights (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR). At the conclusion of the trial, fresh excreta samples were collected for nitrogen-corrected apparent metabolizable energy (AME_n) analysis. Data were analyzed using JMP 10 and responses were considered significant at $p \leq 0.05$. In T1, chicks fed diets containing 20 ppm Xyl had an improved FCR and an uplift in AME_n at 3 weeks of age when compared to the un-supplemented treatments. BW, BWG, and FI were affected by the energy level, with the lower energy treatments consuming more feed resulting in higher BW and BWG. In T2, BW, BWG, and AME_n linearly increased and FCR was linearly improved with increasing Xyl concentration in the crumbled diets at 3 weeks of age. The addition of this heat-stable

xylanase to both mash and pelleted wheat-based diets was able to improve the 3 week growth performance of broiler chicks.

(*Keywords*: xylanase, enzyme, heat-stable, broilers, growth performance)

Description of Problem

Most broiler chicken diets in the United States are pelleted. When feed undergoes the conditioning and pelleting process ingredients are exposed to high temperatures, moisture levels and pressure. Exposure to these conditions can be detrimental to some feed additives, such as enzymes, which can lose stability and subsequently activity before being presented to the animal in the finished feed (Demirjian *et al*., 2001; Campbell and Bedford, 1992). In the past decade advances have been made in developing thermostable feed additives, including enzymes, either through genetic manipulation or applying protective coatings on the enzyme (Gilbert and Cooney, 2010; Turner *et al*., 2007). All new products must be evaluated for thermostability to ensure viability of the product post-pellet when it reaches the animal in the feed.

The feeding of soluble non-starch polysaccharides (NSP) found in high concentrations in cereal grains such as wheat, barley and rye, can result in increased viscosity of the digesta contents, inhibiting nutrient digestibility (Choct and Annison, 1992). It was previously demonstrated that this novel xylanase reduced digesta viscosity and improved apparent metabolizable energy (AME_n) in broilers chicks fed wheat-based diets, both in mash (Manuscript I) and pelleted diets (Biggs *et al*., 2012—unpublished data).

In the current study a heat-stable xylanase is supplemented in diets formulated to various dietary energy levels to evaluate the bird's response. This xylanase has previously been evaluated in mash and pelleted diets, but not in the same diets in the same study. In the current study the birds are also offered mash and pelleted diets of the same composition with increasing levels of xylanase inclusion.

Materials and Methods

Bird husbandry

Male Ross broiler chicks were randomly distributed into groups of 6 chicks, weighed individually and assigned to 36 Alternative Design (Trial 1) or 60 Petersime (Trial 2) battery cages. Birds were randomly assigned to 1 of 6 (Trial 1) or 1 of 10 (Trial 2) dietary treatments and consumed feed and water *ad libitum.* Each Alternative Design cage was equipped with two adjustable-height nipple drinkers and one food trough. Petersime cages were each equipped with one water trough and one food trough. Birds were provided with 23 hours of light and 1 hour of dark per day. Temperatures were provided at 32^0C for the first 48 hours after birds were placed. Temperature was then decreased 0.5^0C per day for an additional 5 days, after which it was decreased another 2.5^0C per week until 21^0C was reached.

Dietary treatments

Trial 1. One basal diet, formulated based on recommendations of the breeder (Ross, 2009), was mixed at the North Carolina State University Feed Mill. The basal was split in half and half received the dry, raw form of the heat stable xylanase at 20 mg xylanase/kg

finished feed (ppm)[7] (BioResource International, Inc., Durham, NC), the remaining half of the basal received no enzyme supplementation. Each of these two aliquots was further split into thirds and energy was adjusted using cornstarch or sand to achieve a high, medium and low ME diet. Cornstrach was used to adjust the dietary energy level so that fat would not confound results by affecting feed efficiency independently of the xylanase. Sand was used to replace cornstarch to avoid changes in energy dilution in the diet. All diets were then pelleted at 85^0C. In total 6 dietary treatments were utilized in this study, set up as a 3 x 2 factorial with 3 energy levels: high (H), Medium (M), and Low (L); and 2 levels of xylanase inclusion: No xylanase (0) or 20 ppm (X).

Trial 2. One basal diet, formulated based on recommendations of the breeder (Ross, 2009), was mixed at the North Carolina State University Feed Mill. The basal was split in half and half received the dry form of the heat stable xylanase at 40 ppm (BioResource International, Inc., Durham, NC), the remaining half of the basal received no enzyme supplementation. Each of these two aliquots was divided in half, half was left in mash form, and the remaining half was pelleted at 85^0C and subsequently crumbled. For both the mash form (M) and crumble form (C), the 2 xylanase concentrations (0 or 40 ppm) were blended to create 5 levels of xylanase concentration (0, 5, 10, 20, 40 ppm). In total 10 dietary treatments were utilized in this study, set up as a 2 x 5 factorial with 2 feed forms (mash and crumble) and 5 xylanase concentrations (0, 5, 10, 20, 40 ppm).

[7] One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50 ^{0}C in 50mM trisodium citrate buffer at pH 6.0.

An indigestible reference (Diatomaceous Earth)[8] was included in all treatments for analysis of nitrogen-corrected apparent metabolizable energy (AME_n)

Live performance

Individual birds and feeder weights were recorded 7, 14, and 19 (Trial 1) or 21 (Trial 2) days of age to obtain bird body weights (BW), body weight gain (BWG), average feed intake (FI), calculated using feed disappearance, and feed conversion ratio (FCR). Cages were checked twice daily for mortality and morbidity. FCR was calculated as pen FI divided by pen BWG plus the weight of mortality that occurred during the period of interest.

Apparent Metabolizable Energy

At three weeks of age fresh excreta samples were collected from a pan beneath each cage for analysis and calculation of nitrogen-corrected apparent metabolizable energy (AME_n)[9]. While collecting excreta, care was taken to avoid samples with abnormal appearances or that were contaminated with feed or feathers. Samples from each pen were stored at -20^{0}C until analysis. Samples were removed from freezer and dried at 55^{0}C in a forced air oven (Blue M, Thermal Product Solutions). Gross energy was measured on feed and excreta samples using an adiabatic bomb calorimeter (IKA® calorimeter C 5000, IKA Works, Inc.). Nitrogen of both feed and excreta were measured by combustion analysis

[8] Celite®, World Minerals, Inc., Santa Barbara, CA

[9] $AME_n = GE_{feed} - ((GE_{excreta} * AiA_{feed})/AiA_{excreta}) - (8.22 * N_{retained})$
$N_{Retained} = N_{feed} - ((N_{excreta} * AiA_{feed})/AiA_{excreta})$

(LECO Corporation, St. Joseph, MI). Both feed and excreta were analyzed for Celite® recovery using the acid-insoluble ash procedure based on Vogtmann *et al*. (1975).

Feed analysis

Feed samples were analyzed by BioResource International, Inc. (Durham, NC, USA) to determine xylanase activity. Proximate analysis of feed completed by Carolina Analytical Services, LLC (Bear Creek, NC, USA).

Statistical analysis

Statistical analysis of the data was completed using JMP 10 (SAS Institute, Cary, NC). Effects were considered significant at $p<0.05$.

Animal ethics

All husbandry practices and euthanasia were performed with full consideration of animal welfare. All bird handling procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University.

Results

Trial 1. The live performance results for Trial 1 are presented in Tables 2-5. A main effect of Energy was observed on BW and BWG, in which the birds fed the high ME diets were lighter compared to the birds fed the medium or low ME diets. However, no Xylanase inclusion main effect or Energy*Xylanase interaction was observed. Similarly to BW and

BWG, the birds fed the high ME diets had lower feed intake compared to the birds fed the medium and low ME diets. During the first week (0-7 days) those receiving xylanase supplementation consumed less feed compared to those fed the un-supplemented diets. An Energy*Xylanase interaction was observed during the first two weeks (0-14 days); birds receiving xylanase had consumed less feed compared to un-supplemented birds, but only at the low ME. Xylanase supplementation improved FCR during the first week, as well as cumulatively (0-19 days). During the second week (7-14 days and 0-14 days) there was an Energy*Xylanase interaction, in which xylanase supplementation improved FCR only at low ME. In the third week, both energy and xylanase main effects, but no interaction were observed. Birds on the high ME diets had improved FCR compared to low or medium ME diets. In the second and third weeks xylanase supplementation also improved FCR compared to un-supplemented diets.

At the conclusion of the trial, excreta samples were collected to analyze AME_n (Table 6). Although no main effect of energy was observed for AME_n, xylanase inclusion provided an uplift in AME_n of about 170 kcal/kg. An Energy*Xylanase interaction was also observed for AME_n; at both the medium and high ME diets, there was an AME_n uplift, but not at the low energy diets.

Trial 2. The live performance results for Trial 2 are presented in Tables 7-10. In Trial 2, greater BW, BWG, and FI were observed for crumble diets compared to mash diets. During the third week (14-21 days) and cumulatively (0-21 days) there was a linear increase in BWG as xylanase concentration increased, but only for birds fed the crumble diets. Feeder weights were not recorded at 14 days, thus FI and FCR are only available for the first

week and the cumulative 21 days period. FCR (0-21 days) improved linearly as xylanase concentration increased, however this was only in the crumble diets, not in mash diets.

A linear increase in AME_n was measured with increasing xylanase concentration. As with BWG and FCR, this response was observed in crumble diets but not mash diets (Table 11).

Discussion

Based on measured responses in live performance and AME_n, it was evident that the xylanase was active in both mash and pelleted diets. In Trial 1 there were three dietary energy levels. Birds on the high ME diets consumed less feed compared to the medium and low ME diets. The reduced FI of the birds consuming high ME diets resulted in lower BW and BWG. Overall the birds fed the low ME had poorer FCR; birds fed low ME diets had to consume a greater amount of feed to gain the amount of BW as the medium ME treatment. The birds fed the medium ME diets had the best FCR, indicating this energy level was closest to optimal for the bird. Throughout the study, FCR was improved with xylanase supplementation; therefore, there was improved nutrient digestibility with xylanase. This was supported by the measured improvement in AME_n with xylanase supplementation. Xylanase is often included in poultry diets to increase AME_n, in some cases the energy level of the diet is reduced and a xylanase is included in a diet given a matrix value for energy, or AME_n uplift. We observed further improvement in FCR when xylanase was supplemented at low ME diets compared to un-supplemented low ME diets. This improvement with the xylanase may not be observed at higher dietary energy levels; if the bird is already able to

metabolize adequate energy from the diet, the potential improvements gained from enzyme supplementation may be smaller and thus less noticeable (Adeola and Cowieson, 2011; Coweison, 2010). However, an unexpected response was the improved FCR in the low energy diets with xylanase supplementation did not correlate to interaction results of AME_n values. Higher AME_n was observed with xylanase supplementation, but at the medium and high level, not at the low ME, where improvements in FCR were observed.

In Trial 2 diets were formulated based on the low ME energy level from Trial 1, where the improvement with xylanase supplementation was observed most clearly. In Trial 2 xylanase concentrations were included above and below the inclusion level in Trial 1. The purpose of this was to compare diets in mash form and crumble form side-by-side. This was to demonstrate enzyme efficacy following the pelleting process. It was evident that birds fed diets in crumble form had greater BWG and FI over those consuming mash diets of the same composition. This result was not surprising since if has long been understood that offering feed in pelleted form versus mash form allows the bird to expend less energy and spend less time feeding to consume the same amount of nutrients (Jensen *et al*., 1962). There is also less segregation in pelleted diets resulting in more uniform consumption and performance of birds. In crumble diets there was a linear improvement in BWG and FCR demonstrating that the xylanase was still efficacious and increasing levels resulted in increased improvements. As in Trial 1, in Trial 2 improvements in performance due to xylanase were supported by correlated improvements in AME_n.

Conclusion and Applications

1. Supplementation of this novel xylanase improved feed efficiency in broiler chicks during the starter period (0-21days).
2. Supplementation of this novel xylanase provided 120-170 kcal/kg uplift in AME_n in wheat-based broiler diets.
3. Bird response to xylanase was more consistent and measureable in pelleted (crumbled) diets compared to mash diets.

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Table 8: Composition and nutrient content of wheat-based diets fed to male broiler chickens from placement until three weeks of age.

Trial	**---------- 1 ----------**			**---- 2 ----**
Dietary treatment	**Low ME**	**Medium ME**	**High ME**	**Low ME**
Ingredients	***----------% of Total diet----------***			
Wheat	49.8	49.8	49.8	57.5
Soybean meal	23.4	23.4	23.4	23.4
Corn DDGS	5.5	5.5	5.5	10.0
Poultry fat	6.2	6.2	6.2	2.0
Monocalcium dicalcium phosphate (21% P)	1.5	1.5	1.5	1.5
Limestone (Calcium carbonate)	1.7	1.7	1.7	1.6
L-lysine	0.5	0.5	0.5	0.5
D,L-methionine	0.4	0.4	0.4	0.4
L-threonine	0.4	0.4	0.4	0.4
Trace mineral premix[1]	0.1	0.1	0.1	0.1
Vitamin premix[2]	0.1	0.1	0.1	0.1
Choline chloride 60%	0.3	0.3	0.3	0.3
Sodium selenite premix[3]	0.1	0.1	0.1	0.1
Sodium chloride	0.2	0.2	0.2	0.2
Sodium bicarbonate	0.2	0.2	0.2	0.2
Celite ®	2.0	2.0	2.0	2.0
Sand	8.8	4.0	--	--
Cornstarch	--	4.0	8.0	--
Nutrient content				
ME poultry, Kcal/kg	2,770	2,920	3,070	2,770
Crude protein, % (calculated)	20.03	20.03	20.03	22.15
Crude protein, % (analyzed)	21.23	20.73	21.11	22.45
Crude fat, % (calculated)	7.67	7.67	7.67	4.11
Crude fat, % (analyzed)	7.59	7.57	7.62	3.78
Available phosphorus, % (calculated)	0.43	0.43	0.43	0.47
Total phosphorus, % (analyzed)	0.75	0.71	0.75	0.71
Calcium, % (calculated)	0.94	0.94	0.94	0.95
Calcium, % (analyzed)	1.11	1.12	1.16	0.99
Sodium, % (analyzed)	0.20	0.20	0.20	0.20
Total lysine, %	1.27	1.27	1.27	1.33
Total Met + Cys, %	0.90	0.90	0.90	0.97
Threonine, %	0.98	0.98	0.98	1.02
Choline, mg/kg	1,161	1,161	1,161	1,258

[1] Each kilogram of mineral premix (0.1% inclusion) supplied the following per kg of complete feed: 60 mg Zn as $ZnSO_4H_2O$; 60 mg of Mn as $MnSO_4H_2O$; 40 mg Fe as $FeSO_4H2O$; 5 mg Cu as $CuSO_4$; 1.25 mg I as $Ca(IO_3)_2$; 1 mg Co as $CoSO_4$.

[2] Each kilogram of vitamin premix (0.1% inclusion) supplied the following per kg of complete feed: 13,200 IU vitamin A; 4,000 IU cholecalciferol; 66 IU alpha-tocopherol; 110 mg niacin; 22 mg pantothenic acid; 13.2 mg riboflavin; 8 mg pyridoxine; 4 mg menadione; 2.2 mg folic acid; 4 mg thiamin; 0.253 mg biotin; 0.04 mg vitamin B_{12}; 100 mg ethoxyquin.

[3] NaSeO3 premix provided 0.3 mg Se/kg of complete feed.

Table 9: Effect of dietary supplementation of a novel exogenous xylanase to broiler chickens in wheat-based diets with three dietary energy levels on body weight (BW) from placement until 19 days of age (Trial 1).[1]

		Age (days)		
Effect		**7**	**14**	**19**
Energy		---------- *grams body weight/bird* ----------		
2770		156 [a]	443 [a]	733 [a]
2920		150 [ab]	450 [a]	748 [a]
3070		145 [b]	406 [b]	687 [b]
SEM (2)		2.4	8.3	9.3
Xylanase[3]				
0 ppm		148	429	714
20 ppm		152	437	731
SEM (1)		1.9	6.9	7.7
Energy	**Xylanase[3]**			
2770	0 ppm	154	442	725
2770	20 ppm	158	444	741
2920	0 ppm	149	444	742
2920	20 ppm	151	455	754
3070	0 ppm	142	402	675
3070	20 ppm	148	411	699
SEM (28)[2]		3.3	11.7	13.2
Source of variation		---------- *P values* ----------		
Energy		0.009	0.003	0.0004
Xylanase		0.14	0.46	0.13
Energy*Xylanase		0.83	0.92	0.92

[1]Values are means of 6 replicate cages of *ca.* 6 birds per cage.
[2]SEM (28)=Standard error of the mean with 28 degrees of freedom.
[3]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50^0C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 10: Effect of dietary supplementation of a novel exogenous xylanase to broiler chickens in wheat-based diets with three dietary energy levels on body weight gain (BWG) from placement until 19 days of age (Trial 1).[1]

		Age (days)				
Effect		**0-7**	**7-14**	**14-19**	**0-14**	**0-19**
Energy		*grams body weight gain/bird*				
2770		112 [a]	293 [a]	293 [a]	398 [a]	689 [a]
2920		107 [ab]	296 [a]	299 [a]	403 [a]	704 [a]
3070		101 [b]	262 [b]	281 [b]	361 [b]	643 [b]
SEM (2)		2.4	7.3	3.4	8.8	9.4
Xylanase[3]						
0 ppm		105	284	285 [b]	384	670
20 ppm		109	284	297 [a]	390	687
SEM (1)		1.9	5.8	2.7	7.2	7.7
Energy	**Xylanase[3]**					
2770	0 ppm	110	300	282	399	681
2770	20 ppm	115	287	303	397	697
2920	0 ppm	106	291	298	397	698
2920	20 ppm	107	300	300	409	710
3070	0 ppm	98	260	274	357	631
3070	20 ppm	104	264	287	364	655
SEM (28)[2]		3.3	9.7	4.6	12.4	13.2
Source of variation		*P values*				
Energy		0.008	0.004	0.003	0.004	0.0004
Xylanase		0.13	0.98	0.004	0.58	0.13
Energy*Xylanase		0.70	0.56	0.18	0.86	0.92

[1]Values are means of 6 replicate cages of *ca.* 6 birds per cage.
[2]SEM (28)=Standard error of the mean with 28 degrees of freedom.
[3]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50^0C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 11: Effect of dietary supplementation of a novel exogenous xylanase to broiler chickens in wheat-based diets with three dietary energy levels on feed intake (FI) from placement until 19 days of age (Trial 1).[1]

		Age (days)				
Effect		**0-7**	**7-14**	**14-19**	**0-14**	**0-19**
Energy		*grams feed intake/bird*				
2770		195 [a]	474 [a]	463 [a]	668 [a]	1123 [a]
2920		184 [ab]	470 [a]	452 [a]	657 [a]	1115 [a]
3070		177 [b]	433 [b]	423 [b]	611 [b]	1033 [b]
SEM (2)		3.6	9.3	7.4	15.1	11.8
Xylanase[3]						
0 ppm		190 [a]	458	443	648	1,092
20 ppm		181 [b]	460	449	642	1,096
SEM (1)		2.9	7.5	6.0	12.2	9.5
Energy	**Xylanase[3]**					
2770	0 ppm	206	492 [a]	448	698 [a]	1145
2770	20 ppm	185	456 [ab]	477	638 [bc]	1120
2920	0 ppm	184	462 [ab]	458	647 [abc]	1106
2920	20 ppm	183	478 [a]	447	667 [ab]	1124
3070	0 ppm	181	419 [b]	424	601 [c]	1025
3070	20 ppm	174	446 [ab]	423	621 [bc]	1043
SEM (28)[2]		5.1	12.5	10.4	21.2	16.0
Source of variation		*P values*				
Energy		0.005	0.008	0.002	0.0002	<0.0001
Xylanase		0.03	0.84	0.49	0.55	0.76
Energy*Xylanase		0.12	0.05	0.15	0.004	0.33

[1]Values are means of 6 replicate cages of *ca.* 6 birds per cage.
[2]SEM (28)=Standard error of the mean with 28 degrees of freedom.
[4]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50^0C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 12: Effect of dietary supplementation of a novel exogenous xylanase to broiler chickens in wheat-based diets with three dietary energy levels on feed conversion ratio (FCR) from placement until 19 days of age (Trial 1).[1]

		Age (days)				
Effect		**0-7**	**7-14**	**14-19**	**0-14**	**0-19**
Energy		----- *grams feed intake: grams body weigh gain* -----				
2770		1.704	1.628	1.624 [b]	1.662	1.629 [a]
2920		1.725	1.570	1.530 [b]	1.613	1.575 [b]
3070		1.770	1.618	1.507 [a]	1.657	1.591 [ab]
SEM (2)		0.035	0.028	0.018	0.027	0.014
Xylanase[3]						
0 ppm		1.795 [a]	1.636	1.579 [a]	1.688 [a]	1.630 [a]
20 ppm		1.671 [b]	1.574	1.531 [b]	1.600 [b]	1.567 [b]
SEM (1)		0.029	0.023	0.014	0.022	0.011
Energy	**Xylanase[3]**					
2770	0 ppm	1.783	1.725 [a]	1.645	1.767 [a]	1.687
2770	20 ppm	1.624	1.531 [b]	1.604	1.556 [b]	1.572
2920	0 ppm	1.742	1.576 [ab]	1.545	1.621 [ab]	1.584
2920	20 ppm	1.708	1.564 [ab]	1.520	1.605 [ab]	1.567
3070	0 ppm	1.86	1.608 [ab]	1.545	1.676 [ab]	1.619
3070	20 ppm	1.68	1.628 [ab]	1.469	1.639 [ab]	1.563
SEM (28)[2]		0.050	0.040	0.024	0.039	0.020
Source of variation		---------- *P values* ----------				
Energy		0.43	0.33	0.0002	0.40	0.03
Xylanase		0.01	0.07	0.03	0.01	0.001
Energy*Xylanase		0.29	0.03	0.56	0.03	0.06

[1]Values are means of 6 replicate cages of *ca.* 6 birds per cage.
[2]SEM (28)=Standard error of the mean with 28 degrees of freedom.
[4]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50^0C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 13: Effect of dietary supplementation of a novel exogenous xylanase to broiler chickens in wheat-based diets with three dietary energy levels on nitrogen-corrected apparent metabolizable energy (AME_n) at 19 days of age (Trial 1).[1]

		Age (days)
Effect		**19**
Energy		*kcal ME/kg*
2770		3191
2920		3226
3070		3274
SEM (2)		27
Xylanase[3]		
0 ppm		3147 [b]
20 ppm		3320 [a]
SEM (1)		22
Energy	**Xylanase[3]**	
2770	0 ppm	3159 [b]
2770	20 ppm	3224 [ab]
2920	0 ppm	3109 [b]
2920	20 ppm	3363 [a]
3070	0 ppm	3175 [b]
3070	20 ppm	3374 [a]
SEM (28)[2]		37
Source of variation		*P values*
Energy		0.10
Xylanase		<0.0001
Energy*Xylanase		0.05

[1]Values are means of 6 replicate cages.
[2]SEM (28)=Standard error of the mean with 28 degrees of freedom.
[3]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50^0C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 14: Effect of dietary supplementation of a novel exogenous xylanase in increasing concentrations to broiler chickens in both mash and crumbled wheat-based diets on body weight (BW) from placement until 21 days of age (Trial 2).[1]

			Age (days)		
Effect		**Placement**	**7**	**14**	**21**
Feed form		-------- *grams body weight/bird* --------			
Mash		44.4	144 [b]	425 [b]	864 [b]
Crumble		44.3	186 [a]	517 [a]	983 [a]
SEM (1)		0.02	2.1	4.9	11.1
Xylanase[3]					
0 ppm		44.4	163	464	928 [ab]
5 ppm		44.5	163	461	874 [b]
10 ppm		44.4	164	471	934 [ab]
20 ppm		44.4	171	478	934 [ab]
40 ppm		43.9	165	482	950 [a]
SEM(4)		0.29	3.3	7.8	17.8
Feed form	**Xylanase[3]**				
Mash	0 ppm	44.4	142	418	874
Mash	5 ppm	44.9	145	412	823
Mash	10 ppm	44.1	142	424	875
Mash	20 ppm	44.1	150	437	871
Mash	40 ppm	44.1	144	436	880
Crumble	0 ppm	44.4	185	511	983
Crumble	5 ppm	44.1	180	511	925
Crumble	10 ppm	44.7	186	518	994
Crumble	20 ppm	44.7	192	519	996
Crumble	40 ppm	43.7	187	528	1019
SEM(43)[2]		0.40	4.6	10.7	25.0
Source of variation		---------- *P values* ----------			
Feed form		0.93	<0.0001	<0.0001	<0.0001
Xylanase		0.62	0.39	0.30	0.04
Feed form*Xylanase		0.32	0.84	0.95	0.96
$Regression_{MASH}$		0.31	0.59	0.09	0.15
$Regression_{CRUMBLE}$		0.53	0.30	0.13	0.12

[1]Values are means of 6 replicate cages of *ca.* 6 birds per cage.
[2]SEM (43)=Standard error of the mean with 28 degrees of freedom.
[3]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50^0C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 15: Effect of dietary supplementation of a novel exogenous xylanase in increasing concentrations to broiler chickens in both mash and crumbled wheat-based diets on body weight gain (BWG) from placement until 21 days of age (Trial 2).[1]

		Age (days)				
Effect		**0-7**	**7-14**	**14-21**	**0-14**	**0-21**
Feed form		*grams body weight gain/bird*				
Mash		98	274 [b]	445 [b]	375 [b]	820 [b]
Crumble		141	323 [a]	480 [a]	473 [a]	939 [a]
SEM (1)		1.8	5.7	5.1	5.5	11.1
Xylanase[3]						
0 ppm		118	301	462 [ab]	418	884 [ab]
5 ppm		116	283	436 [b]	417	829 [b]
10 ppm		119	299	462 [ab]	417	890 [ab]
20 ppm		124	303	462 [ab]	434	890 [ab]
40 ppm		122	308	489 [a]	434	906 [a]
SEM(4)		2.9	9.2	8.0	8.6	17.8
Feed form	**Xylanase[3]**					
Mash	0 ppm	95	274	456 [bc]	369	829
Mash	5 ppm	97	269	427 [c]	368	778
Mash	10 ppm	96	265	452 [bc]	361	832
Mash	20 ppm	100	284	440 [bc]	394	828
Mash	40 ppm	98	280	450 [bc]	384	836
Crumble	0 ppm	141	327	468 [bc]	466	938
Crumble	5 ppm	135	296	446 [bc]	466	881
Crumble	10 ppm	141	333	473 [abc]	474	949
Crumble	20 ppm	147	322	483 [ab]	474	952
Crumble	40 ppm	143	336	528 [a]	483	976
SEM(43)[2]		4.1	12.9	11.2	12.0	25.0
Source of variation		*P values*				
Feed form		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Xylanase		0.40	0.37	0.002	0.37	0.04
Feed form*Xylanase		0.79	0.53	0.05	0.76	0.96
$Regression_{MASH}$		0.22	0.13	0.41	0.18	0.14
$Regression_{CRUMBLE}$		0.22	0.58	0.04	0.39	0.0002

[1]Values are means of 6 replicate cages of *ca.* 6 birds per cage.
[2]SEM (43)=Standard error of the mean with 28 degrees of freedom.
[3]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50^0C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference (P≤0.05).

Table 16: Effect of dietary supplementation of a novel exogenous xylanase in increasing concentrations to broiler chickens in both mash and crumbled wheat-based diets on feed intake (FI) from placement until 21 days of age (Trial 2).[1]

		Age (days)	
Effect		**0-7**	**0-21**
Feed form		----- *grams feed intake/bird* -----	
Mash		251	1400 [b]
Crumble		235	1524 [a]
SEM (1)		5.4	21.9
Xylanase[3]			
0 ppm		239	1480
5 ppm		240	1421
10 ppm		238	1444
20 ppm		253	1488
40 ppm		248	1478
SEM(4)		8.7	34.0
Feed form	**Xylanase[3]**		
Mash	0 ppm	236	1417
Mash	5 ppm	252	1362
Mash	10 ppm	243	1403
Mash	20 ppm	267	1415
Mash	40 ppm	239	1403
Crumble	0 ppm	241	1543
Crumble	5 ppm	228	1481
Crumble	10 ppm	232	1485
Crumble	20 ppm	240	1560
Crumble	40 ppm	239	1553
SEM(43)[2]		11.9	48.0
Source of variation		---------- *P values* ----------	
Feed form		0.06	0.0002
Xylanase		0.65	0.61
Feed form*Xylanase		0.65	0.96
$Regression_{MASH}$		0.48	0.44
$Regression_{CRUMBLE}$		0.97	0.84

[1]Values are means of 6 replicate cages of *ca.* 6 birds per cage.
[2]SEM (43)=Standard error of the mean with 28 degrees of freedom.
[3]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50^0C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 17: Effect of dietary supplementation of a novel exogenous xylanase in increasing concentrations to broiler chickens in both mash and crumbled wheat-based diets on feed conversion ratio (FCR) from placement until 21 days of age (Trial 2).[1]

		Age (days)	
Effect		**0-7**	**0-21**
Feed form		*grams feed intake:grams body weight gain*	
Mash		2.653 [b]	1.730 [b]
Crumble		1.670 [a]	1.609 [a]
SEM (1)		0.077	0.022
Xylanase[3]			
0 ppm		2.134	1.677
5 ppm		2.171	1.709
10 ppm		2.199	1.647
20 ppm		2.162	1.696
40 ppm		2.143	1.619
SEM(4)		0.124	0.036
Feed form	**Xylanase[3]**		
Mash	0 ppm	2.550	1.714
Mash	5 ppm	2.643	1.767
Mash	10 ppm	2.765	1.732
Mash	20 ppm	2.685	1.746
Mash	40 ppm	2.622	1.693
Crumble	0 ppm	1.717	1.640
Crumble	5 ppm	1.699	1.652
Crumble	10 ppm	1.633	1.562
Crumble	20 ppm	1.639	1.646
Crumble	40 ppm	1.664	1.546
SEM(43)[2]		0.173	0.050
Source of variation		---------- *P values* ----------	
Feed form		<0.0001	0.0004
Xylanase		1.00	0.36
Feed form*Xylanase		0.93	0.88
$Regression_{MASH}$		0.89	0.57
$Regression_{CRUMBLE}$		0.35	0.02

[1]Values are means of 6 replicate cages of *ca.* 6 birds per cage.
[2]SEM (43)=Standard error of the mean with 28 degrees of freedom.
[3]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50^0C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 18: Effect of dietary supplementation of a novel exogenous xylanase in increasing concentrations to broiler chickens in both mash and crumbled wheat-based diets on nitrogen-corrected apparent metabolizable energy (AME_n) at 21 days of age (Trial 2).[1]

		Age (days)
Effect		**21**
Feed form		*kcal/kg*
Mash		2955 [a]
Crumble		2899 [b]
SEM (1)		11
Xylanase[3]		
0 ppm		2887 [bc]
5 ppm		2880 [c]
10 ppm		2963 [a]
20 ppm		2959 [ab]
40 ppm		2947 [abc]
SEM(4)		18
Feed form	**Xylanase[3]**	
Mash	0 ppm	2928 [abc]
Mash	5 ppm	2930 [abc]
Mash	10 ppm	3019 [a]
Mash	20 ppm	2975 [a]
Mash	40 ppm	2925 [abc]
Crumble	0 ppm	2846 [bc]
Crumble	5 ppm	2829 [c]
Crumble	10 ppm	2907 [abc]
Crumble	20 ppm	2944 [abc]
Crumble	40 ppm	2969 [ab]
SEM(43)		25
Source of variation		*P values*
Feed form		0.001
Xylanase		0.002
Feed form*Xylanase		0.04
$Regression_{MASH}$		0.17
$Regression_{CRUMBLE}$		0.004

[1]Values are means of 6 replicate cages.
[2]SEM (43)=Standard error of the mean with 28 degrees of freedom.
[3]One ppm xylanase inclusion provided 667 XU/kg finished feed. One xylanase unit of activity (XU) is the amount of xylanase needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50^0C in 50mM trisodium citrate buffer at pH 6.0.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

MANUSCRIPT III. The evaluation of a heat-stable xylanase, alone and in combination with a commercial phytase on broiler performance from day-of-hatch until 42 days of age.

ABSTRACT: A study was conducted to evaluate the efficacy of an exogenous, heat-stable xylanase in broiler chicken diets when supplemented in combination with a commercial exogenous phytase. The study was designed as a 2 x 2 x 2 factorial with the factors being: energy level, xylanase inclusion, and phytase inclusion. Eight wheat-based diets (60%) containing DDGS (10%) were fed to male broiler chicks from hatch until 41 days. Birds were housed in 96 litter floor pens with 16 birds per pen in a curtain-sided house. Bird and feeder weights were collected at 13, 27, and 41days to obtain BW, BW gain (BWG), feed intake (FI), and feed conversion ratio (FCR). Fresh excreta samples were also collected prior to each weigh-day to evaluate nitrogen-corrected apparent metabolizable energy (AME_n). Data were analyzed using JMP 10 and effects were considered significant at $p<0.05$. Higher BW and BWG were observed with the addition of xylanase and phytase when added individually compared to diets containing no enzymes. When xylanase and phytase were added in combination, both BW and BWG were higher than the controls or xylanase alone, but were not different than the treatments receiving only phytase. FCR was improved with the inclusion of xylanase and phytase alone compared to the control; further improvement in FCR was observed when xylanase and phytase were included in combination. In agreement to the observed response in FCR, uplifts in AME_n were observed with the inclusion of both the xylanase and phytase individually, and further improvement was observed when the two enzymes were included in combination. This exogenous xylanase improved broiler

performance from hatch until 41days when included in wheat-based diets alone or in combination with a commercial phytase.

(*Keywords*: xylanase, enzyme, broilers, heat-stable, phytase)

Description of Problem

Over the past several years there has been increased interest in finding alternative ingredients to include in poultry diets to replace corn. However, there are anti-nutritional factors associated with the use of some ingredients, such as wheat, barley, and dried distillers grains (DDGS). These anti-nutritional factors, such as high concentrations of non-starch polysaccharides (NSP), cause an increase in the viscosity of the digesta contents, often resulting in reduced nutrient digestibility and performance (Bedford and Morgan, 1996; Choct and Annison, 1992). To ameliorate the negative effects associated with NSP, dietary NSP-degrading enzymes, or carbohydrases, can be supplemented to poultry diets (Santos *et al*., 2004; Choct *et al*., 1999).

When incorporating a new feed additive into a diet formulation, there is always a concern of possible interactions with the additives already included in that formulation. Most commercial poultry operations in the United States include a dietary phytase in their feeding program, and in recent years it there has been interest in evaluating the interaction of a dietary xylanase when included in combination with a dietary phytase (Selle *et al*., 2003).

Both phytase (Singh *et al*., 2003; Ravindran *et al*., 2001) and xylanase (Cowieson and Masey O'Neill, 2013; Nian *et al*., 2011) have been demonstrated to improve bird

performance when included in poultry diets. However, previous research conducted with the inclusion of both carbohydrases and phytases included in combination has resulted in conflicting outcomes. Some have reported observing additive effects in performance when both enzymes are included together (Pourreza and Classen, 2001; Zyla *et al*., 1999). In other cases, there have been reports of measureable improvements only from each enzyme included alone (Gehring *et al*., 2013b; Woyengo *et al*., 2008).

This current study was designed to evaluate the effect of an exogenous heat-stable xylanase on bird performance when added in combination with a commercially available, heat-stable exogenous phytase. The study was designed as a 2 x 2 x 2 factorial with the factors: energy level, xylanase inclusion, and phytase inclusion. This provided an opportunity to evaluate the effect of each enzyme alone and any additive effects from including both enzymes on live performance and nitrogen-corrected apparent metabolizable energy (AME_n).

Materials and Methods

Bird husbandry

Ross 708 chicks were sexed on day of hatch at the North Carolina State University Chicken Education Unit. Male chicks (1,536) were randomly distributed into groups of 16 birds, weighed individually, and assigned to pens. All 96 litter-floor pens were contained in one curtain-sided house, with 16 pens allocated to each of six blocks based on location within the house. Each (2.23 m^2) pen was set up with one Plasson® bell drinker and one tube feeder. Prior to the start of this trial, any caked litter present was removed from each pen and pens

were top-dressed with fresh pine-shaving litter. Light was provided for 23 hours per day for the first 7 days, 21 hours of light per day from 7 to 21 days, after which natural day length (approximately 13 hours/day) was used until 42 days. Temperatures were provided at 35^0C for the first 48 hours after birds were placed. Temperature was then decreased to 32^0C for an additional 5 days, after which it was decreased another 2.5^0C per week until ambient temperature of approximately 25^0C was reached.

Dietary treatments

All diets were formulated based on nutrition recommendations of the breeder (Ross, 2009). This experiment was designed to as a 2 x 2 x 2 factorial, resulting in a total of eight wheat-based dietary treatments. The three factors included: 1) energy (recommended energy or 200 kcal/kg ME reduction), xylanase inclusion (no enzyme or 20,000 XU/kg of feed[10]), and phytase inclusion (no enzyme or 500 FTU/kg feed[11]). The positive control (PC) energy level were based on breeder-recommended energy levels, but were slightly lower and were increased in smaller increments between dietary phases than recommendations: 2,975 kcal/kg (starter), 3,005 kcal/kg (grower), and 3,035 kcal/kg (finisher). Diets were formulated to be wheat-based with inclusion of DDGS in order to provide adequate substrate for the xylanase enzyme and elicit a measureable response. In addition, diets containing exogenous phytase were formulated with a standard reduction (0.1%) in calcium (Ca) and available phosphorus

[10] One xylanase unit of activity (XU) is the amount of Xylamax™ needed for the release of 1 nanomol of reducing sugars from 0.5% beechwood xylan per second at 50 ^{0}C in 50mM trisodium citrate buffer at pH 6.0.
[11] One phytase unit of activity (FTU) is the amount of phytase needed to liberate 1 micromol of inorganic phosphate in one minute under the conditions of the assay at 37 ^{0}C and pH 5.5.

(aP). In reduced Ca and P diets, sand was included as filler to reduce variation in nutrient density between dietary treatments. Both the xylanase (Xylamax ^{TM}, BioResource International, Durham, NC) and phytase (Axtra® PHY 2500 TPT, Danisco Animal Nutrition, Dupont, St. Louis, MO) used in the study were dry, heat-stable products that could be included in the feed prior to pelleting. Additional fat added to diets to increase energy level was applied post-pellet to reduce pellet quality difference due to fat inclusion.

To manufacture the feed one basal diet was formulated to the reduced level of energy, Ca, and available phosphorus (aP). The basal was then split into 4 aliquots and remixed with additional calcium carbonate as a calcium source and monocalcium dicalcium phosphate as a source of phosphorus, xylanase, and/or phytase according to the treatment. These four diets were then pelleted at 85^0C. The four pelleted diets were each split in half and sent back into the mixer, half was sent though the mixer with no additions (NC diets), the other half received post-pellet liquid application (PPLA) of poultry fat to reach the energy level of the PC. Complete feeds were fed in crumble (starter) or pelleted (short pellet for grower, pellet for finisher) form.

An indigestible reference (Diatomaceous Earth)[12] was included in all treatments for analysis of nitrogen-corrected apparent metabolizable energy (AME_n).

Live performance

Individual bird weights and feeder weigh-backs were recorded at 13, 27, and 41 days of age in order to obtain bird body weights (BW), body weight gain (BWG), average feed

[12] Celite®, World Minerals, Inc., Santa Barbara, CA

intake (FI), calculated using feed disappearance, and feed conversion ratio (FCR). Pens were checked twice daily for mortality and morbidity. FCR was calculated as pen FI divided by pen BWG plus the weight of mortality that occurred during the period of interest. Birds were fed based on kilograms of feed per bird for each dietary phase; therefore, feed was removed after mortality was found to adjust the amount of feed allocation.

Apparent Metabolizable Energy

Fresh excreta samples were collected from each pen at 12 and 40 days for analysis and calculation of nitrogen-corrected apparent metabolizable energy (AME_n)[13]. For each day of excreta collection, feeders were shaken and each pen was lined with brown paper to provide separation between litter and fresh excreta. Birds were allowed an hour after the pens were lined to acclimate lining in the pens and return to normal activity. While collecting excreta, care was taken to avoid samples contaminated with feed, feathers, or litter. Samples were collected and pooled over a two-day period (for a six hour period per day) for each collection, homogenized, and stored at -20^{0}C until further analysis. Samples were removed from freezer, thawed, and dried at 55^{0}C in a forced air oven (Blue M, Thermal Product Solutions). Dried excreta were hand-ground through a 1 mm^2 screen to ensure that samples did not contain litter or feathers before they were ground with an electric coffee grinder to reduce the size of any larger particles. Gross energy was measured on feed and excreta samples using an adiabatic bomb calorimeter (1341 Plain Jacket Calorimeter, Parr

[13] $AME_n=GE_{feed}-((GE_{excreta}*AiA_{feed})/AiA_{excreta})-(8.22*N_{retained})$
$N_{Retained}=N_{feed}-((N_{excreta}*AiA_{feed})/AiA_{excreta})$

Instrument Company, Moline, IL, USA). Nitrogen of both feed and excreta were measured by combustion analysis (LECO Corporation, St. Joseph, MI). Both feed and excreta were analyzed for Celite® recovery using the acid-insoluble ash procedure based on Vogtmann *et al*. (1975).

Tibia Ash

At 14, 28, and 42 days of age one bird per pen (12 birds per treatment) was randomly selected, euthanized by cervical dislocation and both legs removed. Meat was manually removed using a scalpel from the right tibia of each bird. Clean bones were wrapped in cheesecloth and soaked in ethyl ether to extract fat from the bones. Bones were soaked in ethyl ether for approximately 9 days total; ethyl ether was changed every 3 days (approximately 3 times) until it was clear. Following fat extraction, bones were placed in a fume hood for 48 hours to allow time for evaporation of any residual ethyl ether. Bones were then dried in a forced air oven (Blue M, Thermal Product Solutions) at 105^0C for 48 hours. Dried bones were weighed and ashed in a furnace at 600^0C for 14 hours.

Feed analysis

Feed samples were analyzed by BioResource International, Inc. (Durham, NC, USA) to determine xylanase activity and by Dupont (St. Louis, MO, USA) to determine phytase activity. Proximate and phytic acid analyses of diets were conducted by Carolina Analytical Services (Bear Creek, NC, USA).

Statistical analysis

Statistical analysis of the data was completed using JMP 10 ® (SAS Institute, Cary, NC). Data were analyzed as a 2 x 2 x 2 factorial with the main effects of energy level, xylanase inclusion, and phytase inclusion. Effects were considered significant at $p<0.05$.

Animal Ethics

All husbandry practices and euthanasia were performed with full consideration of animal welfare. All bird handling procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University.

Results

Live performance

Overall mortality for the trial was at a reasonable and acceptable level (2.4%) and was not related to dietary treatment. The results for average bird BW are in Table 2. No main effect of energy on BW was observed at 13, 27, or 41 days of age. However, higher BW were observed with xylanase or phytase inclusion at all three ages. At 13d there was a Xylanase*Phytase interaction in which xylanase inclusion increased BW over the un-supplemented diets, and phytase inclusion resulted in higher BW than the un-supplemented diets and the xylanase alone, but was not different than when both enzymes were included together. The results for BWG by period and cumulatively can be found in Table 3. Throughout the study, the birds supplemented with phytase had greater BWG compared to un-supplemented diets. During the starter and grower phases the birds supplemented with

xylanase also had greater BWG than un-supplemented birds. However, in the finisher period (27-41days) there was no xylanase effect, but there was an energy effect, with greater BWG with higher dietary energy. Cumulatively, 0-27 days and 0-41 days, both xylanase and phytase supplemented treatments yielded greater BWG than un-supplemented treatments, but there was no energy effect on BWG.

The results for average bird FI can be found in Table 4. Although, no energy or xylanase effect was observed in the starter, grower or finisher periods, a cumulative (0-41 days) effect for both energy and xylanase inclusion was observed. Cumulatively (0-41 days) there was also as Energy*Xylanase interaction, in which xylanase inclusion reduced FI in the higher energy diets (PC), but not in reduced energy diets (NC). In the starter period (0-13 days) the birds receiving phytase had a lower FI compared to un-supplemented diets, but this effect did not persist past 13 days.

The results for FCR are presented in Table 5. Main effects on FCR were observed throughout the study for xylanase and phytase supplementation, with the inclusion of each of the enzymes improving FCR. During the grower and finisher periods, there was also a main effect for dietary energy level, with reduced-energy diets (NC) having poorer FCR compared to PC diets. Interactions were observed with each other the individual enzymes with energy. The improvements in FCR were greater for each of the enzymes when included in PC diets compared to NC diets.

Bone Ash

No main effects or interactions were observed on tibia ash percentage at 14, 28, or 42 days of age (Table 10).

AME_n

Results for AME_n analysis are presented in Table 11. At both 12 and 40 days, higher AME_n values were observed in PC diets compared to NC diets. Higher AME_n was also observed when both enzymes were supplemented individually. At 12 days an Energy*Phytase interaction was observed with a greater improvement in the PC diets compared to NC diets. At 40 days an Energy*Xylanase interaction was observed with a greater uplift in AME_n in PC diets (173 kcal/kg) compared to NC diets (81 kcal/kg) due to xylanase inclusion. At 12 and 40 days both enzymes improved AME_n when included alone, however a further improvement was observed when the two enzymes were included in combination (p=0.001 and 0.08, respectively).

Discussion

In the current study, it was evident that both the xylanase and phytase were active in the feed through main effects observed for both enzymes in live performance as well as AME_n. Main effects for both xylanase and the phytase supplementation were observed for BW at 13, 27, and 41 days of age, with higher BW observed in treatments receiving enzyme. This agrees with previous research observing higher BW and BWG with supplementation of phytase or xylanase to wheat-based diets (Wu *et al*., 2004; Zyla *et al*., 1999). Higher BW

were observed when xylanase and phytase were added in combination compared to the no-enzyme controls. Treatments in which birds received xylanase alone, body weights were intermediary between the no-enzyme controls and the treatments receiving phytase and the xylanase-phytase combination. Similar to the response in BW, higher BWG were observed with the supplementation of xylanase and phytase individually throughout the trial. Higher BWG were also observed when xylanase and phytase were added in combination compared to the no-enzyme controls in the starter period. Similar to the response in BW, treatments in which birds received xylanase alone, body weights were intermediary between the no-enzyme controls and the treatments receiving phytase and the phytase-xylanase combination. However, this effect did not persist for the remainder of the trial. In the finishing period (27-41 days), an energy effect was observed on BWG; however, a xylanase effect was not during this period. There was no treatment effect on feed intake except during the starter period (0-13 days), in which birds receiving phytase supplementation consumed greater quantity of feed compared to un-supplemented diets.

As expected, the birds consuming reduced energy diets (NC) demonstrated poorer FCR compared to PC. This effect was not observed in the starter period (0-13 days); however during this period an Energy*Xylanase interaction was observed in which FCR was improved by xylanase supplementation in PC diets, but not in the reduced energy diets (NC). The same Energy*Xylanase interaction was observed in the finisher period (27-41 days), but not in the grower period (13-27 days). Interestingly, during the grower period, an Energy*Phytase interaction was observed with a greater improvement in FCR in the PC diets

(8 pts ± 1 pt) compared to in the NC diets (5 pts, ± 1 pt). While each of the enzymes improved FCR when included individually, no Xylanase*Phytase interaction was observed.

Some researchers have reported observing performance improvements when xylanase and phytase are supplemented individually, but no further improvement when the two are added in combination (Gehring *et al*., 2013a; Woyengo *et al*., 2008) while others observed further improvement when xylanase and phytase were included in combination (Wu *et al*., 2004; Pourreza and Classen, 2001). Cowieson (2010) predicted with the inclusion of a phytase enzyme that a xylanase would provide little improvement in feeding value of a diet over a phytase alone. The author attributed this to the phytase improving the digestibility of the diet, reducing the concentration of undigested amino acids and energy that could benefit from a xylanase. This may explain why BW and BWG for the starter period statistically showed a Xylanase*Phytase interaction, but the treatment receiving only phytase were not different that those receiving the xylanase-phytase combination.

In the current study tibia ash was analyzed at the end of each dietary phase as an indication of bone mineralization. Bone ash can be used as an indicator of phosphorus status of a bird (Ravindran *et al*., 2001), with higher percent ash indicating greater quantities of available mineral. This analysis is often utilized when evaluating phytase inclusion; phytase can liberate more Ca and P from a diet, thus increasing minerals available for deposition into the bones. No treatment effect was observed on tibia ash percentage at 14, 28, or 42 days of age. Other researchers have also observed no effect of phytase on bone ash (Wu *et al*., 2004; Ravindran *et al*., 2001). Wu *et al*. (2004) suggested since no enzyme response was observed on bone ash, that the observed performance response to phytase supplementation was not

related to P, but likely due to the release of other nutrients. Observing no treatment differences in tibia ash percentage indicates that the phytase, included in the reduced Ca and available P diets, was able to work properly and utilize nutrients in reduced Ca and P diets. The efficacy of the enzyme was further supported by the growth performance improvements observed with phytase supplementation.

Higher AME_n values were observed for PC diets compared to NC diets at both 12 and 40 days. This difference can be attributed to the energy level (fat inclusion) to the diets. The supplementation of either xylanase or phytase individually resulted in an uplift in AME_n compared to un-supplemented diets. At the end of the starter period (12 days) an Energy*Phytase interaction was observed with a greater uplift in the higher energy diets (PC) compared to the lower energy diets (NC). This interaction was not observed at 40 days. This could have been related to the age of the bird, which could explain why the interaction was not observed with the older birds. During the first two weeks post hatch there is rapid bone mineralization occurring to develop a strong skeletal structure for the fast-growing bird (Williams *et al*., 2000). The uplift in AME_n due to xylanase inclusion was similar in the PC and NC diets at 12 days (117 kcal/kg), however, at 40 days an uplift in AME_n was observed when xylanase was supplemented in the PC diets, but not the NC diets. This could be due to the fat inclusion in the diet, which could have increased gut retention time. Perhaps this had a greater impact in the finisher diet when there was more substrate present; with greater gut retention time more time is allowed for enzymatic action (Singh *et al*., 2012; Selle *et al*., 2010).

While both the xylanase and phytase provided an uplift in AME_n when supplemented individually, further improvement was observed when the two enzymes were included in combination. Therefore, there is added benefit to included both xylanase and phytase in combination throughout the production phase. Improvements in AME are not always sufficient enough to result in measureable improvements in live performance (Bao *et al*., 2013). This may explain why further improvement was measured in AME_n when enzymes were included in combination compared to either enzyme supplemented alone, but a FCR response was not observed for Xylanase*Phytase interaction.

While there was not full additivity, an improvement was observed in performance and AME_n from both enzymes when included individually and in combination. It is suggested that improvements in nutrient digestibility from xylanase supplementation will be less when added in combination with a phytase because there will be a smaller undigested fraction (Selle *et al*., 2010; Cowieson and Bedford, 2009). Selle *et al*. (2010) rules out the possibility of full additivity and suggest about a 20% reduction to the energy matrix when added in combination.

Conclusion and Applications

1. Xylanase or phytase inclusion wheat-based broiler diets can improve BWG (0-41 days).
2. Xylanase or phytase inclusion in wheat-based broiler diets can improve FCR in reduced energy diets, however greater improvements can be observed in diets not reduced in energy.

3. Xylanase or phytase inclusion can provide an uplift in AME_n in wheat-based broiler diets, however greater uplifts in AME_n are possible when the two enzymes are included in combination.

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Table 19: Composition and nutrient content of dietary feeding phases of positive control (PC)[5] diets fed to male broiler chickens from placement to 42 days of age.

Dietary phase	**Starter**	**Grower**	**Finisher**
Days of age	**~0-14**	**~14-28**	**~28-42**
Ingredients	---------- *% of Total diet* ----------		
Wheat	56.63	60.77	65.19
Soybean meal	21.15	17.31	12.98
Corn DDGS	9.62	9.62	9.62
Poultry fat	5.77 (2.00)	5.77 (2.00)	5.75 (1.98)
Celite ®	1.92	1.92	1.92
Limestone (Calcium carbonate)[4]	1.60 (1.55)	1.50 (1.40)	1.45 (1.40)
Monocalcium dicalcium phosphate (21% P)[4]	1.60 (1.10)	1.48 (1.00)	1.30 (0.08)
L-lysine HCl	0.05	0.46	0.48
D,L-methionine	0.39	0.29	0.31
L-threonine	0.34	0.19	0.29
Sodium chloride	0.24	0.24	0.24
Sodium bicarbonate	0.14	0.14	0.14
Trace mineral premix[1]	0.10	0.10	0.10
Vitamin premix[2]	0.10	0.10	0.10
Sodium selenite premix[3]	0.05	0.05	0.05
Choline chloride (60%)	---	0.19	0.19
Nutrient content			
ME poultry, kcal/kg	2,977 (2,779)	3,005 (2,806)	3,035 (2,838)
Crude protein, % (calculated)	21.23	19.58	18.07
Crude protein, % (analyzed)	20.35	18.29	16.80
Crude fat, % (calculated)	7.71 (4.10)	7.75 (4.14)	7.77 (4.16)
Crude fat, % (analyzed)	7.40 (4.13)	7.44 (4.11)	7.70 (4.01)
Available phosphorus, % (calculated)	0.47 (0.35)	0.46 (0.35)	0.42 (0.31)
Total phosphorus, % (analyzed)	0.70 (0.61)	0.63 (0.55)	0.58 (0.49)
Calcium, % (calculated)	0.93 (0.85)	0.91 (0.80)	0.85 (0.76)
Calcium, % (analyzed)	0.94 (0.86)	0.91 (0.81)	0.91 (0.78)
Sodium, % (calculated)	0.20	0.20	0.20
Sodium, % (analyzed)	0.19	0.19	0.18
Total lysine, %	1.28	1.16	1.06
Total Met + Cys, %	0.95	0.83	0.80
Threonine, %	0.96	0.75	0.77
Choline, mg/kg	1,378	2,303	2,215

[1] Each kilogram of mineral premix (0.1% inclusion) supplied the following per kg of complete feed: 60 mg Zn as $ZnSO_4H_2O$; 60 mg of Mn as $MnSO_4H_2O$; 40 mg Fe as $FeSO_4H2O$; 5 mg Cu as $CuSO_4$; 1.25 mg I as $Ca(IO_3)_2$; 1 mg Co as $CoSO_4$.

[2] Each kilogram of vitamin premix (0.1% inclusion) supplied the following per kg of complete feed: 13,200 IU vitamin A; 4,000 IU cholecalciferol; 66 IU alpha-tocopherol; 110 mg niacin; 22 mg pantothenic acid; 13.2 mg riboflavin; 8 mg pyridoxine; 4 mg menadione; 2.2 mg folic acid; 4 mg thiamin; 0.253 mg biotin; 0.04 mg vitamin B_{12}; 100 mg ethoxyquin.

[3] NaSeO3 premix provided 0.3 mg Se/kg of complete feed.

[4]Sand was added as a filler when reduction in calcium carbonate or monocalcium dicalcium phosphate were made to reduced dilution effect of nutrients.

[5]Nutrient reductions for negative control (NC) diets are shown in parentheses when appropriate.

Table 20: Effect of dietary supplementation of exogenous xylanase and phytase, alone and in combination, to wheat-based broiler chicken diets on body weight (BW) from placement until 41 days of age.[1]

Age (days)		Placement	13	27	41
Effect		*(grams body weight/bird)*			
Energy					
NC		42	435	1610	3245
PC		42	433	1614	3267
Xylanase (X)[2]					
0		42	430 [b]	1598 [b]	3238 [b]
X		42	437 [a]	1626 [a]	3275 [a]
Phytase (Phy)[3]					
0		42	423 [b]	1572 [b]	3203 [b]
Phy		42	444 [a]	1652 [a]	3310 [a]
SEM (1)		0.2	1.5	7	9
Energy	**Xylanase (X)[2]**				
NC	0	42	432	1599	3230
NC	X	42	437	1620	3261
PC	0	42	428	1597	3246
PC	X	41	438	1632	3289
Energy	**Phytase (Phy)[3]**				
NC	0	42	426	1576	3200
NC	Phy	42	443	1643	3291
PC	0	42	421	1567	3206
PC	Phy	42	445	1661	3329
Xylanase (X)[2]	**Phytase (Phy)[3]**				
0	0	42	417 [c]	1551	3186
X	0	41	429 [b]	1592	3220
0	Phy	42	443 [a]	1644	3290
X	Phy	42	446 [a]	1660	3330
SEM (1)		0.28	2.1	9	13
Source of variation		*P values*			
Energy		0.56	0.46	0.63	0.10
Xylanase		0.17	0.001	0.004	0.01
Phytase		0.29	<0.0001	<0.0001	<0.0001
Energy*Xylanase		0.05	0.37	0.47	0.66
Energy*Phytase		0.19	0.11	0.15	0.23
Xylanase*Phytase		0.98	0.03	0.19	0.81
Energy*Xylanase*Phytase[4]		0.38	0.61	0.55	0.93

[1]Values are means of 12 replicate pens of *ca.* 16 birds per pen.
[2]Xylanase (X) was supplemented at 20,000 XU/kg feed.
[3]Phytase (Phy) was supplemented at 500 FTU/ kg feed.
[4]Three-way interactions were not statically significant, LSMeans not presented in table.
[a,b]Means within a column with no common superscript are significantly difference (P≤0.05).

Table 21: Effect of dietary supplementation of exogenous xylanase and phytase, alone and in combination, to wheat-based broiler chicken diets on average body weight gain (BWG) from placement until 41 days of age.[1]

Age (days)		0-13	13-27	27-41	0-27	0-41
Effect		*(grams body weight gain/bird)*				
Energy						
NC		393	1175	1619 [b]	1568	3203
PC		391	1183	1648 [a]	1572	3225
Xylanase (X)[2]						
0		388 [b]	1169 [b]	1627	1556 [b]	3195 [b]
X		396 [a]	1188 [a]	1640	1584 [a]	3233 [a]
Phytase (Phy)[3]						
0		382 [b]	1149 [b]	1618 [b]	1530 [b]	3161 [b]
Phy		402 [a]	1208 [a]	1649 [a]	1610 [a]	3268 [a]
SEM (1)		1.4	6	7	7	9
Energy	**Xylanase (X)[2]**					
NC	0	390	1168	1615	1557	3188
NC	X	396	1182	1624	1578	3219
PC	0	386	1171	1640	1554	3203
PC	X	396	1194	1656	1590	3248
Energy	**Phytase (Phy)[3]**					
NC	0	385 [b]	1151	1604	1534	3158
NC	Phy	401 [a]	1199	1635	1601	3249
PC	0	379 [b]	1147	1633	1525	3164
PC	Phy	403 [a]	1218	1663	1619	3287
Xylanase (X)[2]	**Phytase (Phy)[3]**					
0	0	376 [c]	1135	1620	1509	3144
X	0	389 [b]	1163	1617	1550	3179
0	Phy	401 [a]	1204	1635	1602	3247
X	Phy	404 [a]	1213	1664	1618	3288
SEM (1)		0.28	2	8	10	9
Source of variation		*P values*				
Energy		0.27	0.36	0.005	0.64	0.09
Xylanase		<0.0001	0.03	0.19	0.003	0.005
Phytase		<0.0001	<0.0001	0.002	<0.0001	<0.0001
Energy*Xylanase		0.32	0.61	0.72	0.43	0.63
Energy*Phytase		0.04	0.15	0.96	0.15	0.23
Xylanase*Phytase		0.01	0.29	0.10	0.21	0.81
Energy*Xylanase*Phytase[4]		0.46	0.51	0.47	0.57	0.93

[1]Values are means of 12 replicate pens of *ca.* 16 birds per pen.
[2]Xylanase (X) was supplemented at 20,000 XU/kg feed.
[3]Phytase (Phy) was supplemented at 500 FTU/ kg feed.
[4]Three-way interactions were not statically significant, LSMeans not presented in table.
[a,b]Means within a column with no common superscript are significantly difference (P≤0.05).

Table 22: Effect of dietary supplementation of exogenous xylanase and phytase, alone and in combination, to wheat-based broiler chicken diets on average feed intake (FI) from placement until 41 days of age.[1]

Age (days)		0-13	13-27	27-41	0-27	0-41
Effect		*(grams feed intake/bird)*				
Energy						
NC		568	2011	3332	2580	5924
PC		565	1976	3296	2541	5826
Xylanase (X)[2]						
0		567	2006	3355 [a]	2574	5929 [a]
X		566	1980	3274 [b]	2546	5821 [b]
Phytase (Phy)[3]						
0		559 [b]	1989	3311	2550	5873
Phy		573 [a]	1997	3317	2570	5876
SEM (1)		2.4	13	23	14	29
Energy	**Xylanase (X)[2]**					
NC	0	564 [ab]	2026	3341	2592	5934 [a]
NC	X	571 [a]	1996	3323	2567	5915 [a]
PC	0	569 [ab]	1987	3368	2556	5924 [a]
PC	X	561 [b]	1965	3225	2525	5728 [b]
Energy	**Phytase (Phy)[3]**					
NC	0	562	1995	3324	2559	5908
NC	Phy	557	2027	3340	2600	5941
PC	0	557	1984	3299	2540	5839
PC	Phy	573	1968	3294	2541	5812
Xylanase (X)[2]	**Phytase (Phy)[3]**					
0	0	557	1994	3351	2553	5905
X	0	562	1984	3272	2546	5842
0	Phy	576	2019	3358	2595	5953
X	Phy	570	1976	3276	2546	5800
SEM (1)		0.28	3.4	18	32	19
Source of variation		*P values*				
Energy		0.39	0.06	0.27	0.05	0.02
Xylanase		0.83	0.15	0.01	0.15	0.01
Phytase		0.0001	0.65	0.87	0.28	0.94
Energy*Xylanase		0.03	0.83	0.06	0.90	0.03
Energy*Phytase		0.49	0.19	0.74	0.29	0.46
Xylanase*Phytase		0.09	0.38	0.98	0.29	0.27
Energy*Xylanase*Phytase[4]		0.75	0.88	0.83	0.92	0.85

[1]Values are means of 12 replicate pens of *ca.* 16 birds per pen.
[2]Xylanase (X) was supplemented at 20,000 XU/kg feed.
[3]Phytase (Phy) was supplemented at 500 FTU/ kg feed.
[4]Three-way interactions were not statically significant, LSMeans not presented in table.
[a,b]Means within a column with no common superscript are significantly difference (P≤0.05).

Table 23: Effect of dietary supplementation of exogenous xylanase and phytase, alone and in combination, to wheat-based broiler chicken diets on feed conversion ratio adjusted for mortality (FCR) from placement until 41 days of age.[1]

Age (days)		0-13	13-27	27-41	0-27	0-41
Effect		-------- *(grams feed intake: grams body weight gain)* --------				
Energy						
NC		1.443	1.676 [a]	2.011 [a]	1.611 [a]	1.799 [a]
PC		1.440	1.640 [b]	1.967 [b]	1.584 [b]	1.764 [b]
Xylanase (X)[2]						
0		1.459 [a]	1.683 [a]	2.011 [a]	1.621 [a]	1.805 [a]
X		1.424 [b]	1.633 [b]	1.967 [b]	1.574 [b]	1.759 [b]
Phytase (Phy)[3]						
0		1.463 [a]	1.690 [a]	2.015 [a]	1.627 [a]	1.812 [a]
Phy		1.420 [b]	1.626 [b]	1.963 [b]	1.568 [b]	1.752 [b]
SEM (1)		0.0045	0.0062	0.007	0.0048	0.0048
Energy	**Xylanase (X)[2]**					
NC	0	1.448 [ab]	1.697	2.018 [a]	1.628	1.812 [a]
NC	X	1.437 [b]	1.656	2.003 [a]	1.594	1.787 [b]
PC	0	1.470 [a]	1.670	2.004 [a]	1.615	1.797 [ab]
PC	X	1.411 [c]	1.610	1.929 [b]	1.554	1.732 [c]
Energy	**Phytase (Phy)[3]**					
NC	0	1.46	1.699 [a]	2.030	1.632 [a]	1.821 [a]
NC	Phy	1.425	1.654 [b]	1.992	1.589 [b]	1.777 [b]
PC	0	1.465	1.682 [ab]	1.999	1.621 [a]	1.802 [ab]
PC	Phy	1.415	1.598 [c]	1.934	1.548 [c]	1.726 [c]
Xylanase (X)[2]	**Phytase (Phy)[3]**					
0	0	1.483	1.716	2.033	1.652	1.836
X	0	1.442	1.664	1.996	1.602	1.788
0	Phy	1.436	1.650	1.989	1.591	1.773
X	Phy	1.405	1.601	1.937	1.546	1.730
SEM (1)		0.2800	0.0064	0.0088	0.0098	0.0068
Source of variation		---------- *P values* ----------				
Energy		0.71	<0.0001	<0.0001	0.0002	<0.0001
Xylanase		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Phytase		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Energy*Xylanase		0.0004	0.28	0.004	0.05	0.01
Energy*Phytase		0.23	0.03	0.17	0.03	0.02
Xylanase*Phytase		0.45	0.89	0.45	0.70	0.77
Energy*Xylanase*Phytase[4]		0.83	0.30	0.06	0.33	0.30

[1]Values are means of 12 replicate pens of *ca.* 16 birds per pen.
[2]Xylanase (X) was supplemented at 20,000 XU/kg feed.
[3]Phytase (Phy) was supplemented at 500 FTU/ kg feed.
[4]Three-way interactions were not statically significant, LSMeans not presented in table.
[a,b]Means within a column with no common superscript are significantly difference (P≤0.05).

Table 24: Effect of dietary supplementation of exogenous xylanase and phytase, alone and in combination, to wheat-based broiler chicken diets on tibia bone ash percentage at 14, 28, and 42 days of age.[1]

Age (days)		14	28	42
Effect		--------- *(% tibia ash)* ---------		
Energy				
NC		42.54	42.72	42.53
PC		42.04	43.05	42.32
Xylanase (X)[2]				
0		42.40	42.85	42.5
X		42.17	42.91	42.35
Phytase (Phy)[3]				
0		42.18	43.04	42.71 [a]
Phy		42.40	42.73	42.14 [b]
SEM (1)		0.208	0.201	0.189
Energy	**Xylanase (X)[2]**			
NC	0	42.47	42.64	42.57
NC	X	42.61	42.80	42.49
PC	0	42.34	43.07	42.43
PC	X	41.74	43.03	42.21
Energy	**Phytase (Phy)[3]**			
NC	0	42.48	43.13	42.85
NC	Phy	42.61	42.31	42.21
PC	0	41.87	42.94	42.56
PC	Phy	42.19	43.15	42.07
Xylanase (X)[2]	**Phytase (Phy)[3]**			
0	0	42.00 [ab]	43.00	42.96
X	0	42.36 [ab]	43.08	42.45
0	Phy	42.81 [a]	42.71	42.04
X	Phy	41.98 [b]	42.75	42.24
SEM (1)		0.295	0.210	0.285
Source of variation		---------- *P values* ----------		
Energy		0.08	0.25	0.43
Xylanase		0.42	0.83	0.56
Phytase		0.46	0.28	0.04
Energy*Xylanase		0.21	0.73	0.80
Energy*Phytase		0.77	0.07	0.77
Xylanase*Phytase		0.04	0.94	0.19
Energy*Xylanase*Phytase[4]		0.71	0.29	0.07

[1]Values are means of 12 replicate birds per treatment.
[2]Xylanase (X) was supplemented at 20,000 XU/kg feed.
[3]Phytase (Phy) was supplemented at 500 FTU/ kg feed.
[4]Three-way interactions were not statically significant, LSMeans not presented in table.
[a,b]Means within a column with no common superscript are significantly difference (P≤0.05).

Table 25: Effect of dietary supplementation of exogenous xylanase and phytase, alone and in combination, to wheat-based broiler chicken diets on nitrogen-corrected apparent metabolizable energy (AME_n) at 12 and 40 days of age.[1]

Age (days)		12	40
Effect		--------- *(kcal/kg)* ---------	
Energy			
NC		3077 [b]	2975 [b]
PC		3193 [a]	3151 [a]
Xylanase (X)[2]			
0		3076 [b]	3002 [b]
X		3193 [a]	3124 [a]
Phytase (Phy)[3]			
0		3062 [b]	2945 [b]
Phy		3207 [a]	3181 [a]
SEM (1)		±8	±14
Energy	**Xylanase (X)[2]**		
NC	0	3018	2939 [c]
NC	X	3135	3020 [b]
PC	0	3134	3064 [b]
PC	X	3252	3237 [a]
Energy	**Phytase (Phy)[3]**		
NC	0	3041 [c]	2868
NC	Phy	3112 [b]	3083
PC	0	3083 [bc]	3022
PC	Phy	3303 [a]	3279
Xylanase (X)[2]	**Phytase (Phy)[3]**		
0	0	3023 [c]	2904 [d]
X	0	3100 [b]	2986 [c]
0	Phy	3130 [b]	3100 [b]
X	Phy	3286 [a]	3262 [a]
SEM (1)		0.295	±12
Source of variation		--------- *P values* ---------	
Energy		<0.0001	<0.0001
Xylanase		<0.0001	<0.0001
Phytase		<0.0001	<0.0001
Energy*Xylanase		0.94	0.02
Energy*Phytase		<0.0001	0.21
Xylanase*Phytase		0.001	0.08
Energy*Xylanase*Phytase[4]		0.20	0.40

[1]Values are means of 12 replicate pens per treatment.
[2]Xylanase (X) was supplemented at 20,000 XU/kg feed.
[3]Phytase (Phy) was supplemented at 500 FTU/ kg feed.
[4]Three-way interactions were not statically significant, LSMeans not presented in table.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

MANUSCRIPT IV. The evaluation of a heat-stable xylanase in corn-soybean meal-based diets on broiler performance at various dietary phases from day-of-hatch until 42 days of age.

ABSTRACT: The efficacy of a heat-stable xylanase (Xyl) was evaluated in broiler diets when supplemented during different dietary phases. Corn-soy diets were fed to Ross 344 x 708 male chicks from hatch to 42 days of age. There were two formulated energy levels, PC (3,000 kcal/kg in starter; breeder recommendations) and NC (200 kcal/kg ME reduction) achieved by removing fat from the formulation. The eight dietary treatments were: 1) PC, 2) PC + Xyl, 3) NC, 4) NC + Xyl, 5) Xyl in starter phase (0-14 days), 6) Xyl in starter and grower phases (0-28 days), 7) Xyl in grower and finisher phases (14-42 days), and 8) Xyl in finisher phase only (28-42 days). For treatments 5 to 8, during phases receiving Xyl supplementation (20,000 XU/kg of feed; Xylamax^{TM}, BioResource International, Inc.), enzyme was included in NC energy level and PC diets were fed during remaining phases. Birds were housed in 96 litter-floor pens with each treatment replicated 12 times with 12 birds per 2.23m^2 pen in a curtain-sided house. Bird and feeder weights were collected at 14, 28, and 42 days to obtain BW gain (BWG), feed intake (FI), and feed conversion ratio (FCR). Data were analyzed using JMP 10 with treatment means separated by LSMeans; treatment effects were considered significant at $p<0.05$. Xyl addition during the first 14 days increased BWG and improved FCR when compared to un-supplemented controls at the PC energy level. However, for the remainder of the study no treatment effects were observed on BWG. Cumulative FCR (0-42 days) was significantly improved in the PC diet over the NC diet (1.71 vs 1.75 ± 0.006; $p<0.0001$), however the treatments receiving Xyl in the starter

(1.71) or finisher (1.71) phases only, had similar FCR to those receiving PC throughout the study. In this study, the greatest advantage in performance was observed during the starter phase when Xyl was supplemented in PC energy level diets. Based on results, it may be possible to reduce dietary fat and include Xyl in the finisher phase without negatively impacting overall performance. This regimen may also provide significant cost savings in dietary energy.

(Keywords: xylanase, enzyme, broilers, heat-stable, energy)

Description of Problem

Some cereal grains and co-products have high concentrations of non-starch polysaccharides (NSPs) that can impair nutrient digestion. The antinutritive effects caused by the NSPs in the diet, such as increased digesta viscosity, can be reduced with the addition of exogenous carbohydrases, such as xylanases and β-glucanases (Bedford, 1996; Petterson and Aman, 1989). Although carbohydrases are typically supplemented to diets containing "viscous" grains, there is increasing interest in the potential of these enzymes supplemented into corn-based diets (Masey O'Neill *et al*., 2012; Barletta, 2010; Zanella *et al*., 1999).

Corn is considered a highly digestible and consistent feed ingredient (Cowieson, 2005; Odetallah *et al*., 2003); however, corn can be variable in quality and digestibility like other grains (Cowieson, 2005; Belyea *et al*., 2004; Leeson *et al*., 1993). Corn does contain arabinoxylans (Smits and Annison, 1996) and may benefit from xylanase supplementation. The concentration of NSP is lower and more insoluble in corn compared to wheat and digesta

viscosity is not the main concern for corn (Masey O'Neill *et al*., 2014; Rose *et al*., 2010). The mechanism of action by which xylanases improve bird performance in corn-based diets is unclear, but may be due to overall improvement in feed utilization (Singh *et al*., 2012). Masey O'Neill *et al*. (2014) suggests that even though xylanase may have different mechanisms of action in corn compared to wheat-based diets, enzyme use in both can improve bird performance. Improvements in bird performance have been observed in corn-based diets with the addition of xylanase (Masey O'Neill *et al*., 2014; Masey O'Neill *et al*., 2012; Liu *et al*., 2011; Yu *et al*., 2007).

Improved nutrient utilization can lead to improvements in live performance such as increases in body weight gain as well as improvements in feed conversion ratio. Supplementation of exogenous xylanase can also improve the apparent metabolizable energy (AME) of poultry diets. Carbohydrates contribute the largest proportion of energy in poultry diets while energy in the form of fats or oils is a more concentrated source of energy. The cost of energy from fats and oils contribute greatly to the already high cost of feed. With an uplift in AME due to enzyme addition, smaller quantities of fat or oil can be used to achieve the same ME of a diet, potentially reducing overall feed costs. The starch in cereal grains provides over half of the AME in a poultry diet, so even relatively small improvements in starch digestibly can have a sizeable effect of AME of a diet (Cowieson, 2005). NSP present in the diet can act as anti-nutritional factors, reducing nutrient digestibility. By supplementing carbohydrases some of these negative effects can be mitigated, improving energy digestibility through improved access for native enzymes or increased nutrient

absorption. Improvements in AME through improvements in starch digestibility could allow for reductions in the dietary energy contributed by fats and oils.

In practice, most carbohydrases are included in poultry diets throughout the entire production period. There has been little research on identification of the most advantageous time to include exogenous carbohydrases. Good nutrient digestion in the starter period is very important for optimal growth performance and health of the bird. Digestion is impaired to the greatest degree by NSP in the starter period due to immaturity of the digestive tract (Peterson *et al*., 1999; Campbell and Bedford, 1992); however, this is more of a challenge with viscous grains. By about three weeks of age broilers have mature digestive tracts, including endogenous enzyme secretion and microfloral population, and are better able to tolerate anti-nutritional factors (Campbell and Bedford, 1992). The finisher period is when birds are consuming the greatest quantity of feed and reducing energy in the diet could have the greatest economic impact.

The objective of this study was to determine in which periods of broiler chicken production the inclusion of an exogenous xylanase would be most beneficial.

Materials and Methods

Bird husbandry

Ross 708 chicks were sexed on day of hatch at the North Carolina State University Chicken Education Unit. Male chicks (1,152) were randomly distributed into groups of 12 birds, weighed individually, and assigned to pens. All 96 litter-floor pens were contained in one curtain-sided house, with 16 pens allocated to each of six blocks based on location within

the house. Each pen (2.23 m^2) was set up with one Plasson® bell drinker and one tube feeder. Prior to the start of this trial, all litter was removed from pens and replaced with fresh pine-shaving litter. For the first 7 days 23 hours of light per day was provided and decreased to 21 hours of light 7 to 21 days, after which natural day length (approximately 10 hours/day) was used until 42 days. Temperatures were provided at 35^0C for the first 48 hours after birds were placed. Temperature was then decreased to 32^0C for an additional 5 days, after which it was decreased another 2.5^0C per week until ambient temperature of approximately 18^0C was reached.

Dietary treatments

All diets were formulated based on recommendations of the breeder (Ross, 2009). There were 8 dietary treatments allocated across 96 pens, resulting in 12 replicate pens per treatment. There were four diet formulations fed during each of the three feed phases (starter, grower, finisher). One basal industry-type diet including corn, soybean meal, wheat, and dried distillers grains (DDGS) was manufactured at the North Carolina State University Feed Mill. Half of the basal diet was pelleted (Bliss Industries, Ponca City, OK) with a target temperature of 85^0C and cooled. Cooled pellets were split in half and remixed with one half receiving no additions (NC) and the second half receiving additional poultry fat to increase the energy of the diet about 200 kcal ME/kg feed (PC). A thermo-stable xylanase (Xylamax®, BioResource International, Inc., Durham, NC) was added to the second half of

the basal at 20,000 XU/kg[14] of finished feed and was subsequently pelleted with a target temperature of 85^0C. Feed containing xylanase was then split as before and half received no additions (NC-X) and half received post pellet liquid application of poultry fat (PC-X). Each of the 8 dietary treatments received a different combination of the four diets throughout the production cycle: 1) PC fed throughout trial (PC); 2) PC-X fed throughout trial (PC-X); 3) NC fed throughout trial (NC); 4) NC-X fed throughout trial (NC-X); 5) NC-X fed in starter phase (X-S); 6) NC-X fed starter and grower phases (X-SG); 7) NC-X fed in grower and finisher phases (X-GF); 8) NC-X fed in finisher phase only (X-F). For treatments 5 to 8, during phases receiving Xyl supplementation, enzyme was included in NC energy level & PC diets were fed during remaining phases. An indigestible reference (Diatomaceous Earth)[15] was included in all treatments for analysis of nitrogen-corrected apparent metabolizable energy (AME_n).

Live performance

Individual bird weights and feeder weigh-backs were recorded at 14, 28, and 42 days of age in order to obtain bird body weights (BW), body weight gain (BWG), average feed intake (FI), calculated using feed disappearance, and feed conversion ratio (FCR). Pens were checked twice daily for mortality and morbidity. FCR was calculated as pen FI divided by pen BWG plus the weight of mortality that occurred during the period of interest. Birds were

[14] XU is defined as amount of Xylamax™ needed for the release of 1 nanomol of reducing sugars per second from 0.5% beechwood xylan at 50^0C in 50 mM trisodium citrate buffer at pH 6.0
[15] Celite®, World Minerals, Inc., Santa Barbara, CA

fed based on kilograms of feed per bird for each dietary phase; therefore, feed was removed after mortality was found to adjust the amount of feed allocation.

Apparent Metabolizable Energy

At 14 and 42 days fresh excreta samples were collected from each pen for analysis and calculation of nitrogen-corrected apparent metabolizable energy (AME_n)[16]. For each day of excreta collection, feeders were shaken and each pen was lined with brown paper to provide separation between litter and fresh excreta. Birds were allowed an hour after the pens were lined to acclimate lining in the pens and return to normal activity. While collecting excreta over a 12 hour time period, care was taken to avoid samples with abnormal appearances or that were contaminated with feed, feathers, or litter. Samples from each pen were stored at -20^{0}C until analysis. Samples were removed from freezer and dried at 55^{0}C in a forced air oven (Blue M, Thermal Product Solutions). Dried excreta were hand-ground through a 1 mm^2 screen to ensure that samples did not contain litter or feathers before they were ground with an electric coffee grinder to reduce the size of any larger particles. Gross energy was measured on feed and excreta samples using an adiabatic bomb calorimeter (IKA® calorimeter C 5000, IKA Works, Inc.). Nitrogen of both feed and excreta were measured by combustion analysis (LECO Corporation, St. Joseph, MI). Both feed and excreta were analyzed for Celite® recovery using the acid-insoluble ash procedure based on Vogtmann *et al*. (1975).

[16] $AME_n=GE_{feed}-((GE_{excreta}*AiA_{feed})/AiA_{excreta})-(8.22*N_{retained})$
$N_{Retained}=N_{feed}-((N_{excreta}*AiA_{feed})/AiA_{excreta})$

Processing and cutup

After body weights were measured at 42 days of age, two birds of average weight per pen were selected for processing. Feed was removed from pens 16 hours prior to processing. Immediately before processing, a fasted live weight was recorded and each bird was hung on a shackle on an automatic processing line. Birds were electrically stunned (approximately 15 mA DC for 10 seconds), manually cut and bled out for approximately 120 seconds, scalded in a single pass countercurrent scalder at approximately 60^0C for 120 seconds, then picked in a commercial 2-bank picker for 30 seconds. Following removal from the shackles, shanks and necks were removed before carcasses were manually eviscerated. A raw carcass weight was then recorded to calculate dressing percent, before placing the carcass in an ice-bath for 3 hours to pre-chill. Carcasses were then transferred to chill tanks and placed in walk-in coolers for 16 hours. Carcasses were removed from chillers and hung on a rack to drip for at least 5 minutes. Chilled carcass weight was recorded prior to carcass being placed on a cone and commercial cuts were manually removed. Weights were recorded for wings, fillets, tenders, thighs, drums, and the frame with the skin.

Feed analysis

Feed samples were analyzed by BioResource International, Inc. (Durham, NC, USA) to determine xylanase activity. Proximate analysis of feed completed by Carolina Analytical Services (Bear Creek, North Carolina).

Statistical analysis

Statistical analysis of the data was completed using JMP 10 (SAS Institute, Cary, NC). Effects were considered significant at $p<0.05$.

Animal Ethics

All husbandry practices and euthanasia were performed with full consideration of animal welfare. All bird handling procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University.

Results

Overall mortality was low in this study (1.91%) and bird performance was similar to projected values (Ross, 2009). Performance results are presented in Tables 2 to 5. During the first 14 days of this trial, while there were eight treatment assignments, there were only four different diets provided to birds. There were no significant differences between treatments receiving the same diets during this period; therefore it may be concluded that the like treatment diets were nutritionally similar. This trial was designed to have a 2 x 2 factorial to serve as controls to use as comparison. During the starter period (0-14 days) performance was evaluated as a 2 x 2 factorial using the four treatment groups (PC, PC-X, NC, NC-X) (Table 6). During this period we observed an Energy * Xylanase interaction in mean BW and FCR. The birds supplemented with xylanase had greater BWG, but only in the higher energy (PC) diets. With birds fed the reduced energy diets supplemented with xylanase (NC-X), BWG was similar to the birds on the un-supplemented reduced energy diets (NC). A

similar pattern was observed for FCR; xylanase supplementation improved FCR for birds fed the higher energy diets, but not for those fed the lower energy diets.

No treatment differences were observed on BW or BWG during the grower (14-28 days) or finisher (28-42 days) periods or on a cumulative basis (0-42 days). By the completion of the trial the FCR was similar for PC and PCX, both of which were improved over NC and NCX. The treatments receiving xylanase only in the starter (X-S) or the finisher phase (X-F) had similar FCR to the PC and PCX. Those receiving xylanase in the starter and grower (X-SG) or grower and finisher (X-GF) had similar FCR to the NC and NCX treatments.

The results for AME_n are presented in Tables 7 and 8. At 14 days an energy main effect was observed, with higher AME_n for the higher energy diet (PC) compared to reduced energy diet (NC). The main effect for xylanase inclusion was significant ($p=0.05$), with a higher AME_n for xylanase inclusion over un-supplemented control. There was no interaction effect observed at 14 days for AME_n. At 42 days the AME_n was higher for birds receiving the higher energy diets in the finisher period. However, if the treatments receiving the same diets throughout the whole study (NC, NCX, PC, PCX) are evaluated as a 2 x 2 factorial, there was an uplift in AME_n due to both the higher energy level (fat inclusion) as well as the xylanase inclusion. No Energy*Xylanase interaction was observed. However, at both 14 and 42 days there was an Energy*Xylanase interaction for retained nitrogen. At both ages, retained nitrogen was increased when xylanase was included at the higher energy level, but not for the reduced energy diets.

At 42 days two birds per pen of average size for the pen were selected and processed. Data collected for carcass parts yield were analyzed as percentage of each part of the chilled carcass weight (Table 9). Carcass parts yield data were also analyzed using the weight of each part, with chilled carcass weight as a covariate; this analysis demonstrated the same result so data were not presented in this paper. No treatment differences were observed in processing or cut-up, except for the drums. Birds receiving xylanase only in the starter phase (X-S) had the lightest drums, while birds receiving xylanase in the starter and grower phase (X-SG) had the heaviest drums, with all other treatments intermediary. While a statistical effect was observed, a biological significance cannot be offered.

Discussion

Based on improvements observed in live performance, AME_n uplift, and retained N, it was evident that the xylanase enzyme used in the study was efficacious. In the starter period, birds receiving reduced energy diets (NC) had a greater BWG than birds receiving the PC energy level. No difference was observed in FCR between the two energy levels, so the difference in BWG can be attributed to the greater FI by these birds resulting in higher BWG. During the starter period an improvement in bird performance was observed with the addition of xylanase, but only in the PC energy level diets, not in the reduced energy diets. This agrees with observations from a previous study (Barasch, 2015, Manuscript III) in which a greater effect was observed when xylanase was supplemented to wheat-based diets not reduced in energy. Masey O'Neill *et al*. (2014) also observed improved FCR in the starter period (0-14 days) with xylanase addition to corn-soybean meal based diets. The

energy of the diets used by Masey O'Neill *et al*. (2014) were not reduced to include the xylanase, but was of a similar energy value to the one used in the PC diets in the current study. In this study no treatment effect was observed on BW or BWG during the grower (14-28 days) or finisher (28-42 days) periods or on a cumulative basis (0-42 days). By 42 days, no differences were observed with the addition of xylanase to either the NC or PC diets. However, the PC diet did result in improved FCR compared to the reduced energy diets, which is an expected response when reducing the fat content of a diet (Baiao and Lara, 2005).

Part of the objective of the current study was to evaluate if xylanase could replace fat in a diet at any phase of production without having a negative effect. Fat is an expensive ingredient in poultry diets and if it can be reduced without hampering performance it could have economical benefits. In this study fat was removed from the diets to reduce the energy level of the negative control diets. Carbohydrases are included to 'release' extra energy from a diet, so fat is often removed to demonstrate improvement in energy utilization when the birds are supplemented with an exogenous carbohydrase. It is understood that the addition of enzymes are not a direct replacement for fat because they can not improve pellet quality, increase essential fatty acids or fat soluble vitamins, or effect gastric emptying in the way that dietary fat can (Cowieson *et al*., 2010; Selle *et al*., 2010). Perhaps the improvements observed when the xylanase was included in the PC level diets were due to 'extra-caloric' effects. Slowing gut passage by dietary fat may improve the efficacy of the xylanase by increasing gut retention time. Birds have a relatively fast gut passage rate, which may not allow enough time for enzymes to hydrolyze to the fullest extent (Singh *et al*., 2012).

The treatments receiving xylanase only in the starter (X-S) or the finisher phase (X-F) had similar FCR to the PC and PCX treatments. Therefore, fat may be reduced and xylanase supplemented in either the starter or the finisher. While any improvement in performance is beneficial, there may be a greater economic benefit by removing fat in the finisher period. During the finisher phase of production is when birds are consuming the greatest quantities of feed and the feed is formulated at its highest energy level. Removing fat in the starter period may not result in as significant cost savings. Based on the results of this study and others, it may be more beneficial to feed at a higher energy level (recommended by breeder) during the starter period to get the bird off to a good start (Cowieson and Masey O'Neill, 2013) and then replace fat with a xylanase during the finisher period.

Apparent metabolizable energy (AME) is a common parameter used in poultry to estimate how much of a diet the bird is able to utilize as energy. When supplementing diets with exogenous enzymes, a quantifiable response of the bird to the enzyme is desired. This allows for nutrient reductions to be made in formulations that can be attributed to the enzyme. Carbohydrases are included to improve energy utilization in a diet through improved starch, protein, and fat digestion, and are often associated with an AME value. In the current study a main effect of energy was observed for AME_n at both 14 and 42 days. As expected, lower AME_n values were observed with the reduced energy diets (NC) compared to PC. This can be attributed to the energy level (fat inclusion) in the diet provided. This effect was evident at 42 days when the statistical significance of the AME_n values correlated with whether the treatment was fed a NC or PC energy level diet in the finisher period. At 14 and 42 days we observed an uplift in AME_n (about 40 and 60 kcal/kg, respectively) with the

addition of xylanase. Additionally, at both 14 and 42 days there was an Energy*Xylanase interaction for retained nitrogen. At both ages, there was an increase in retained nitrogen when xylanase was supplemented to PC diets, but not in NC diets. This may be due to improved protein utilization with xylanase supplementation. Perhaps at the reduced energy diets birds consumed more feed, which resulted in greater total protein consumption. If the PC birds consumed less total feed, the inclusion of the xylanase could have improved nutrition utilization, including improved protein digestibility.

Most AME uplift values from xylanase supplementation in corn-soy diets are approximately 40-60 kcal/kg. In this study we reduced energy in the negative control about 200 kcal ME/kg compared to the PC. This energy reduction was selected based on previous work done with this enzyme, which was conducted in wheat-based diets. In wheat-based diets AME_n uplifts of 100-120 kcal/kg were observed when the xylanase was included in a reduced energy level diet. Not knowing what uplift to expect in a corn-soy diet, a large gap between the PC and NC was desired in order to enhance the ability to measure a response. Measured AME_n uplifts in this study of 40-60 kcal/kg were observed when xylanase was supplemented, which agrees with previous research with carbohydrase supplementation in corn-soybean meal diets (Kiarie *et al*., 2014; Cowieson and Ravindran, 2008; Zanella *et al*., 1999; Marquardt *et al*., 1994).

A reduction in AME_n not only can depress body weight gain and increase feed conversion ratio, but has also been demonstrated to decrease carcass yields in broilers at market age (Singh *et al*., 2012). However, carcass yield differences were not observed in this study. This could be a result of very similar body weights at 42 days between treatments.

The only difference in carcass characteristics was found with the drums, which were larger for X-SG compared to X-S. While a statistical effect was observed, a biological significance cannot be offered.

In the current study performance responses were observed when xylanase was supplemented in the starter period. Masey O'Neill *et al*. (2012) suggested that in corn-soy diets there may be a delay in response to exogenous xylanase because it is thought to impact the microfloral population, promoting a more beneficial caecal microflora. This delay may be until about 21d of age, around the time when the digestive tract is becoming fully mature. Masey O'Neill *et al*. (2012) observed improvements in FCR when xylanase was supplemented to corn-soybean meal diets, but did not observed this response until 35 and 42 days of age. This response was observed in both the PC and reduced energy diets when xylanase was included. However, these authors only reduced the dietary energy about 100 kcal ME/kg, whereas in the present study energy was reduced 200 kcal ME/kg.

In order to have the 2 x 2 factorial of energy level and xylanase inclusion fed throughout the study as control treatments, we were limited to the number of additional treatment groups without sacrificing number of replications per treatment. Further investigation is necessary to better evaluate the optimal period and method of inclusion for this xylanase.

Conclusions and Applications:

1. Xylanase can improve bird performance (0-14 days) when included in corn-soy diets not reduced in dietary energy.

2. Xylanase can be supplemented in reduced energy diets in the finisher phase (28-42 days) without negatively impacting bird performance.
3. Xylanase addition to corn-soybean meal diets provided an uplift in AME_n (40-60 kcal/kg).

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Table 26: Composition and nutrient content of dietary feeding phases of negative control diets fed to male broiler chickens from placement to 42 days of age.

Dietary phase	**Starter**	**Grower**	**Finisher**
Days of age	**~0-14**	**~14-28**	**~28-42**
Ingredients	***----------% of Total diet----------***		
Corn	36.6	39.1	40.6
Soybean meal	28	23	18
Wheat	20	20	20
Corn DDGS	7.5	10.0	12.5
Poultry fat	4.98 (0.98)	5.40 (1.40)	6.48 (2.48)
Celite ®	2	2	2
Limestone (Calcium carbonate)	1.20	1.05	1.05
Dicalcium phosphate (18.5% P)	2.1	1.9	1.7
L-lysine HCl	0.400	0.375	0.375
D,L-methionine	0.300	0.250	0.275
L-threonine	0.125	0.125	0.125
Sodium chloride	0.30	0.30	0.30
Sodium bicarbonate	0.15	0.15	0.15
Trace mineral premix[1]	0.10	0.10	0.10
Vitamin premix[2]	0.10	0.10	0.10
Sodium selenite premix[3]	0.05	0.05	0.05
Choline chloride 60%	0.10	0.10	0.10
Nutrient content			
ME poultry, kcal/kg	2,809	2,869	2,948
Crude protein, % (calculated)	22.34	20.69	19.14
Crude protein, % (analyzed)	20.50	19.50	18.50
Crude fat, % (calculated)	6.91 (3.31)	7.56 (3.96)	8.80 (5.20)
Crude fat, % (analyzed)	6.00 (3.00)	5.48 (3.46)	8.79 (5.06)
Available phosphorus, % (calculated)	0.48	0.45	0.41
Total phosphorus, % (analyzed)	0.80	0.73	0.68
Calcium, % (calculated)	1.02	0.91	0.86
Calcium, % (analyzed)	1.12	1.00	0.96
Sodium, % (analyzed)	0.20	0.20	0.21
Total lysine, %	1.38	1.24	1.11
Total Met + Cys, %	0.94	0.85	0.83
Threonine, %	0.86	0.78	0.80
Choline, mg/kg	1,965	1,923	1,874

[1] Each kilogram of mineral premix (0.1% inclusion) supplied the following per kg of complete feed: 60 mg Zn as $ZnSO_4H_2O$; 60 mg of Mn as $MnSO_4H_2O$; 40 mg Fe as $FeSO_4H2O$; 5 mg Cu as $CuSO_4$; 1.25 mg I as $Ca(IO_3)_2$; 1 mg Co as $CoSO_4$.

[2] Each kilogram of vitamin premix (0.1% inclusion) supplied the following per kg of complete feed: 13,200 IU vitamin A; 4,000 IU cholecalciferol; 66 IU alpha-tocopherol; 110 mg niacin; 22 mg pantothenic acid; 13.2 mg riboflavin; 8 mg pyridoxine; 4 mg menadione; 2.2 mg folic acid; 4 mg thiamin; 0.253 mg biotin; 0.04 mg vitamin B_{12}; 100 mg ethoxyquin.

[3] NaSeO3 premix provided 0.3 mg Se/kg of complete feed.

[4]Nutrient reductions for negative control (NC) diets are shown in parentheses when appropriate.

Table 27: Effect of dietary supplementation of exogenous xylanase to broiler chickens during various feeding phases on body weight (BW) from placement until 42 days of age.[1]

	Days of age			
	Placement	**14**	**28**	**42**
Treatment	---------- *(grams body weight/bird)* ----------			
PC	46.3	465 [ab]	1710	3181
PCX	46.0	484 [a]	1708	3194
NC	46.5	482 [a]	1681	3174
NCX	46.3	472 [ab]	1702	3164
X-S[3]	46.3	467 [ab]	1705	3188
X-SG[3]	46.5	475 [ab]	1715	3213
X-GF[3]	46.5	457 [b]	1676	3141
X-F[3]	46.1	468 [ab]	1709	3179
Source of variation	---------- *P values* ----------			
Treatment	0.94	0.003	0.40	0.78
SEM (83)[2]	0.34	5	14	28

[1]Values are means of 12 replicate pens of *ca.* 12 birds per pen.
[2]SEM (83)=Standard error of the mean with 83 degrees of freedom.
[3]Xylanase (X) was supplemented at 20,000 XU in specified dietary phases (starter (S), grower (G), or finisher (F)), on top of reduced energy diets.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 28: Effect of dietary supplementation of exogenous xylanase to broiler chickens during various feeding phases on body weight gain (BWG) from placement until 42 days of age.[1]

	Days of age				
	0-14	**14-28**	**28-42**	**0-28**	**0-42**
Treatment	*---------- (grams body weight gain/bird) ----------*				
PC	418 [ab]	1244	1453	1664	3134
PCX	438 [a]	1234	1475	1662	3148
NC	435 [a]	1198	1492	1634	3128
NCX	426 [ab]	1229	1468	1656	3117
X-S[3]	425 [ab]	1233	1453	1659	3142
X-SG[3]	428 [ab]	1240	1502	1669	3167
X-GF[3]	411 [b]	1220	1462	1629	3094
X-F[3]	423 [ab]	1240	1467	1663	3133
Source of variation	*---------- P values ----------*				
Treatment	0.002	0.17	0.46	0.39	0.77
SEM (83)[2]	5	12	18	15	28

[1]Values are means of 12 replicate pens of *ca.* 12 birds per pen.
[2]SEM (83)=Standard error of the mean with 83 degrees of freedom.
[3]Xylanase (X) was supplemented at 20,000 XU in specified dietary phases (starter (S), grower (G), or finisher (F)), on top of reduced energy diets.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 29: Effect of dietary supplementation of exogenous xylanase to broiler chickens during various feeding phases on feed intake (FI) from placement until 42 days of age.[1]

	Days of age				
	0-14	**14-28**	**28-42**	**0-28**	**0-42**
Treatment	---------- *(grams feed intake/bird)* ----------				
PC	642 [ab]	1938 [ab]	2950	2580 [ab]	5530
PCX	633 [ab]	1901 [b]	3049	2534 [b]	5583
NC	655 [ab]	1977 [ab]	3080	2624 [ab]	5705
NCX	659 [ab]	1970 [ab]	3021	2629 [ab]	5649
X-S[3]	627 [ab]	1943 [ab]	2977	2579 [ab]	5556
X-SG[3]	655 [a]	2015 [a]	3020	2673 [a]	5673
X-GF[3]	623 [b]	1901 [b]	3073	2533 [b]	5630
X-F[3]	640 [ab]	1937 [ab]	2961	2577 [ab]	5529
Source of variation	---------- *P values* ----------				
Treatment	0.01	0.01	0.14	0.01	0.18
SEM (83)[2]	8	23	39	28	55

[1]Values are means of 12 replicate pens of *ca.* 12 birds per pen.
[2]SEM (83)=Standard error of the mean with 83 degrees of freedom.
[3]Xylanase (X) was supplemented at 20,000 XU in specified dietary phases (starter (S), grower (G), or finisher (F)), on top of reduced energy diets.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 30: Effect of dietary supplementation of exogenous xylanase to broiler chickens during various feeding phases on feed conversion ratio adjusted for mortality weight (FCR) from placement until 42 days of age.[1]

	Days of age				
	0-14	**14-28**	**28-42**	**0-28**	**0-42**
Treatment	*------ (grams feed intake:grams body weight gain) ------*				
PC	1.508 [ab]	1.559 [b]	1.896 [b]	1.545 [ab]	1.703 [c]
PCX	1.442 [b]	1.553 [b]	1.939 [ab]	1.523 [b]	1.701 [c]
NC	1.490 [ab]	1.637 [a]	1.922 [ab]	1.594 [a]	1.744 [ab]
NCX	1.538 [a]	1.610 [ab]	1.953 [ab]	1.591 [a]	1.755 [a]
X-S[3]	1.503 [ab]	1.571 [b]	1.902 [b]	1.553 [ab]	1.709 [bc]
X-SG[3]	1.531 [a]	1.606 [ab]	1.902 [b]	1.587 [a]	1.732 [abc]
X-GF[3]	1.523 [a]	1.570 [b]	1.964 [a]	1.562 [ab]	1.746 [ab]
X-F[3]	1.516 [a]	1.561 [b]	1.908 [ab]	1.549 [ab]	1.711 [bc]
Source of variation	---------- *P values* ----------				
Treatment	0.001	0.0003	0.002	0.0002	<0.0001
SEM (83)[2]	0.015	0.014	0.014	0.012	0.009

[1]Values are means of 12 replicate pens of *ca.* 12 birds per pen.
[2]SEM (83)=Standard error of the mean with 83 degrees of freedom.
[3]Xylanase (X) was supplemented at 20,000 XU in specified dietary phases (starter (S), grower (G), or finisher (F)), on top of reduced energy diets.
[a-c]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 31: Effect of dietary energy level and dietary supplementation of exogenous xylanase to broiler chickens on performance during the starter phase (0-14 days).[1]

Effect		14 days BW (g/bird)	0-14 days BWG (g/bird)	0-14 days FI (g/bird)	0-14 days FCR (g:g)
Energy					
NC		477	431	654	1.514 [a]
PC		474	428	637	1.475 [b]
Xylanase (X)[3]					
0		473	427	645	1.499
X		478	432	646	1.490
SEM (1)		3.4	3.3	6.2	0.0113
Energy	**Xylanase**				
NC	0	482	435 [ab]	648	1.490 [ab]
NC	X	472	426 [ab]	659	1.538 [a]
PC	0	465	418 [b]	642	1.508 [a]
PC	X	484	438 [a]	633	1.442 [b]
SEM (39)[2]		4.8	4.7	8.8	0.0160
Source of variation		---------- *P values* ----------			
Energy		0.57	0.59	0.08	0.02
Xylanase		0.30	0.27	0.93	0.57
Energy*Xylanase		0.01	0.01	0.25	0.001

[1]Values are means of 12 replicate pens of *ca.* 12 birds per pen.
[2]SEM (39)=Standard error of the mean with 39 degrees of freedom.
[3]Xylanase (X) was supplemented at 20,000 XU/kg finished feed.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 32: Effect of dietary supplementation of exogenous xylanase to broiler chickens during various feeding phases on nitrogen-corrected apparent metabolizable energy (AME_n) at 42 days of age.[1]

Treatment	42 days AME_n (kcal/kg)
PC	3277 [a]
PCX	3349 [a]
NC	3005 [b]
NCX	3058 [b]
X-S[3]	3295 [a]
X-SG[3]	3298 [a]
X-GF[3]	3039 [b]
X-F[3]	3063 [b]
Source of variation	***P values***
Treatment	<0.0001
SEM (83)[2]	19.5

[1]Values are means of 12 replicate pens of *ca.* 12 birds per pen.
[2]SEM (83)=Standard error of the mean with 83 degrees of freedom.
[3]Xylanase (X) was supplemented at 20,000 XU in specified dietary phases (starter (S), grower (G), or finisher (F)), on top of reduced energy diets.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 33: Effect of dietary energy level and dietary supplementation of exogenous xylanase to broiler chickens on nitrogen-corrected apparent metabolizable energy (AME_n) and retained nitrogen (retained N) at 14 and 42 days of age.[1]

Days of age		---------- 14 ----------		---------- 42 ----------	
Effect		**AME_n (kcal/kg)**	**Retained N (%)**	**AME_n (kcal/kg)**	**Retained N (%)**
Energy					
NC		2913 [b]	2.35 [b]	3031 [b]	1.84 [b]
PC		3132 [a]	2.40 [a]	3313 [a]	1.91 [a]
Xylanase (X)[3]					
0		3004 [b]	2.29 [b]	3141 [b]	1.74 [b]
X		3041 [a]	2.46 [a]	3203 [a]	2.00 [a]
SEM (1)		13.8	0.018	14.8	0.019
Energy	**Xylanase**				
NC	0	2884	2.33 [bc]	3005	1.82 [b]
NC	X	2942	2.36 [b]	3058	1.85 [b]
PC	0	3124	2.24 [c]	3276	1.67 [c]
PC	X	3141	2.56 [a]	3349	2.14 [a]
SEM (39)[2]		21	0.028	20.7	0.026
Source of variation		---------- *P values* ----------			
Energy		<0.0001	0.04	<0.0001	0.01
Xylanase		0.05	<0.0001	0.004	<0.0001
Energy*Xylanase		0.29	<0.0001	0.63	<0.0001

[1]Values are means of 12 replicate pens of *ca.* 12 birds per pen.
[2]SEM (39)=Standard error of the mean with 39 degrees of freedom.
[3]Xylanase (X) was supplemented at 20,000 XU/kg finished feed.
[a-c]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 34: Effect of dietary supplementation of exogenous xylanase to broiler chickens during various feeding phases on carcass parts yield as a percent of the chilled carcass at 42 days of age.[1]

Treatment	DP%[4]	Fillets	Tenders	Wings	Drums	Thighs	Frame & Skin
	% of Chilled WOG[5]						
PC	73.8	24.82	4.52	9.96	11.85 [ab]	16.66	31.89
PCX	74.1	24.55	4.68	10.10	11.64 [ab]	16.78	31.84
NC	74.2	25.1	4.73	9.89	11.65 [ab]	16.76	31.49
NCX	74.5	24.47	4.62	10.05	11.94 [ab]	17.18	31.39
X-S[3]	74.3	24.92	4.60	9.98	11.62 [b]	16.59	31.93
X-SG[3]	74.3	24.14	4.79	10.10	12.17 [a]	16.90	31.64
X-GF[3]	74.7	24.53	4.66	10.20	11.92 [ab]	16.94	31.50
X-F[3]	74.6	24.99	4.54	10.07	11.92 [ab]	17.08	31.15
Source of variation	*P-values*						
Treatment	0.15	0.61	0.70	0.52	0.02	0.57	0.66
SEM (177)[2]	0.25	0.365	0.113	0.103	0.123	0.224	0.325

[1]Values are means of 24 replicate birds per treatment.
[2]SEM (177)=Standard error of the mean with 177 degrees of freedom.
[3]Xylanase (X) was supplemented at 20,000 XU in specified dietary phases (starter (S), grower (G), or finisher (F)), on top of reduced energy diets.
[4]DP%=Dressing Percent (or WOG yield)—Raw WOG as a percentage of fasted live weight. Raw WOG defined as carcass weight following evisceration and removal of neck and shanks, but prior to chilling.
[5]Chilled WOG= carcass weight after evisceration, removal of neck and shanks, chilling over night, and drip-dried for 5 minutes.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

SUMMARY

The overall objective of this research was to evaluate a novel xylanase for its potential as a feed additive in broiler chicken diets. Through a series of trials, we evaluated optimal inclusion levels, efficacy through the pelleting process, inclusion with other enzymes, and supplementation into both wheat and corn based diets. Efficacy of the enzyme was evaluated based on measurable responses of the birds for growth performance, digestion viscosity, and nitrogen-corrected apparent metabolizable energy (AME_n).

Xylanases are most commonly included in wheat-based diets due to the relatively high concentration of arabinoxylans, thus the majority of this research was conducted utilizing wheat-based diets. Measuring reductions in digesta viscosity was a helpful parameter in evaluating efficacy *in vivo,* but is not of practical interest since it does not always directly correlate to improvements in growth performance. After establishing that the novel xylanase reduced viscosity of the digesta contents, this parameter was no longer measured. The main evaluation parameters for measuring response to xylanase supplementation were growth performance and AME_n, since these parameters are of more interest to the industry. Live performance is of the greatest interest to production. Feed cost account for a large proportion of production cost, and improvements in feed efficiency are a priority.

One of the main points of interest regarding this enzyme was its heat-stability, since more broiler chicken feed in the United States is pelleted. The xylanase evaluated in this research is inherently heat-stable in its raw form, thus it does not require a protective coating to survive the pelleting process. Based on this research, this xylanase is heat-stable enough

to sufficiently survive the pelleteing process at temperatures up to 85^0C and remain active through the time feed is consumed by the bird. Through evaluation of inclusion level, it was estimated that the optimal inclusion level in a pelleted diet should be approximately 20,000 XU/kg of feed.

AME_n was a parameter of interest because of the desire to quantify the expected contribution of the enzyme when included in a diet. Attributing an energy "value" to the enzyme potentially allows for nutrient reductions in the feed formulation, reducing feed costs. Dietary energy is typically reduced when xylanases are included to improve the AME_n of a lower energy diet. Based on the research conducted, this xylanase resulting in uplifts in AME_n of approximately 120-170 kcal/kg in wheat diets and 40-60 kcal/kg in corn diets. These values are comparable to AME_n values reported by other researchers.

In this research growth performance improvements were observed in low energy diets when diets with xylanase supplementation. This effect was observed when dietary energy level was adjusted using cornstarch, an easily digested energy source, however it is recognized that this is not a practical choice for industry diets. In subsequent studies, dietary energy levels were reduced through the removal of fat, which is typically the method used to industry. Interestingly, it was observed that the response to enzyme inclusion was more pronounced in diets not reduced in energy. The non-reduced energy diets were based on recommendations by the breeder or slightly lower, but did include higher percentage of fat compared to the reduced energy diets. It was determined that the level of fat inclusion itself may have provided 'extra-caloric' effects independent of the level of the dietary energy, such as slowing the transit time of digesta thought the gastrointestinal tract resulting in overall

improved nutrient digestion. More research may be required to further investigate the possibility of enhanced enzyme effect due to dietary fat inclusion.

Since dietary phytase is included almost ubiquitously in broiler diets, xylanase efficacy was evaluated when supplemented in combination with phytase. With the inclusion of both xylanase and phytase enzymes individually, improvements in growth performance were observed. When the two enzymes were supplemented in combination, no further improvements were observed in growth performance. However, additional benefit may be achieved when xylanase and phytase are supplemented in combination rather than individually. When supplemented in combination, there was further improvement in AME_n than when either enzyme was supplemented individually. Thus, when the xylanase and phytase were supplemented in combination, not only were both enzymes efficacious, but the combination may have allowed for further improvements in energy digestibility.

There has been little research on when dietary enzymes should be included in poultry diets. The starter period (0-3 weeks) was the focus of this research early on, however in the grow-out studies conducted, benefit was observed with xylanase supplementation until market age (42 days). As expected, this research supported that dietary enzymes provide the most benefit to performance during the starter period before the bird's digestive tract is fully developed. In wheat-based diets, improvements in performance were observed from placement to market age (42 days). In corn-based diets, clear improvements in performance were only observed in the starter period. However, in both wheat and corn-based diets uplifts in AME_n were observed at the conclusion of both the starter and finisher phases, demonstrating the xylanase was improving energy digestibility throughout the production

cycle. In the corn-based diets fat was replaced with xylanase in the finisher phase only and performance was comparable overall to the positive control treatments, indicating that xylanase is still improving energy digestibility in this late dietary phase. There is opportunity for further investigation of the optimal dietary phase to include xylanase, which may depend on what main ingredients constitute the diet.

The majority of this research was conducted in wheat-based diets due to higher concentrations of NSP present in wheat compared to corn. However, based on our observations there may be potential for inclusion of xylanase in corn-based diets. In agreement with research conducted by others, we conclude that the action of the xylanase may be different in wheat-based versus corn-based diets and may require different strategies when supplementing xylanase to poultry diets.

APPENDICES

Appendix A

Pilot study—Evaluation of an exogenous carbohydrase when fed to broiler chicks

Introduction

The following two trials were designed in order for us to create a model in which we would be able to evaluate the effects of a carbohydrase enzyme added to poultry feed. We selected a commercially available carbohydrase product (Rovabio® Excel, Adisseo, France)[17] and supplemented it into broiler chicken starter diets with high levels of non-starch polysaccharides (NSP). The enzyme product selected was an enzyme cocktail with the main activities of xylanase and β-glucanase; diets were formulated to include a combination of wheat and barley to provide adequate substrate for both of the main enzyme activities. Carbohydrase enzymes are often supplemented to poultry diets to improve overall energy utilization, thus in the current trials dietary energy levels of the diets were adjusted to evaluate bird response to enzyme supplementation. Utilizing a known product with expected results could provide the opportunity to compare various methods for evaluating bird response, which could be used as a model when a novel carbohydrase is utilized. A commercially available exogenous protease (Versazyme ®, BioResource International, Durham, NC, USA) was also utilized in the study to evaluate if there was any interference when two enzymes were supplemented in combination. The protease enzyme was selected since the effects should not be similar to the carbohydrase, which could mask the effect being evaluated.

[17] Rovabio® Excel enzyme cocktail contains main enzyme activities of endo-1,4-β-xylanase , endo-1,3(4)-β-glucanase, and endo-1,4-β-glucanase

Materials and Methods

Bird husbandry

Two trials were conducted with Ross 708 broiler chicks. Male chicks (288) were placed on day-of-hatch and raised in 36 Alternative Design battery cages, with 8 birds per cage until 21 days (Trial 1) or 28 days (Trial 2). Birds were randomly assigned to 1 of 6 dietary treatments and consumed feed and water *ad libitum*. Each cage was equipped with two adjustable-height nipple drinkers and one feed trough. Birds were provided with 23 hours of light and 1 hour of dark per day. Temperatures were provided at 32^0C for the first 48 hours after birds were placed. Temperature was then decreased 0.5^0C per day for an additional 5 days, after which it was decreased another 2.5^0C per week until 21^0C was reached.

Dietary treatments

All diets met NRC and breeder recommendations for all nutrients including protein and amino acids. In Trial 1 the treatments were: 1) high (3,025 kcal/ kg, recommended) energy level with no enzyme additions (PC); 2) reduced energy (2,880 kcal/kg) diet with no enzyme additions (NC); 3) NC with carbohydrase addition (CH); 4) NC with protease addition (Pro); 5) NC with both protease and carbohydrase addition (PrCH); and 6) NC with protease and a double dose of carbohydrase (Pr2C). In Trial 2 the treatments were: 1) high (2,980 kcal/kg) energy level with no enzyme additions (PC); 2) medium energy (2,830 kcal/kg) diet with no enzyme additions (NC1); 3) low energy (2,680 kcal/kg) diet with no enzyme additions (NC2); 4) NC2 with carbohydrase addition (CH); 5) NC2 with protease

addition (Pro); and 6) NC2 with both protease and carbohydrase addition (PrCH). Reduction in dietary energy level was achieved by removing the inclusion level of poultry fat and were based on suggested energy increase attributed to enzyme inclusion in wheat (85 kcal/kg ME uplift) and barley diets (140 kcal/kg ME uplift). An indigestible reference (Diatomaceous Earth)[18] was included in all treatments for analysis of nitrogen-corrected apparent metabolizable energy (AME_n).

Live performance

Individual bird weights and feeder weigh-backs were recorded weekly in order to obtain bird body weights (BW), body weight gain (BWG), average feed intake (FI), calculated using feed disappearance, and feed conversion ratio (FCR). Pens were checked twice daily for mortality and morbidity. FCR was calculated as pen FI divided by pen BWG plus the weight of mortality that occurred during the period of interest.

Digesta viscosity and intestinal samples

At the end of each study, 21 days (Trial 1) or 28 days (Trial 2), 48 birds per treatment were euthanized by cervical dislocation and body weights were recorded. The pancreas was removed from each bird and weighed. The small intestine was removed from each bird and was cut into segments: duodenum (duodenal loop), jejunum (duodenal loop to Meckel's diverticulum), and ileum (Meckel's diverticulum to ileal-cecal junction). Ileal digesta contents were collected and stored on ice for measurements of digesta viscosity. Viscosity

[18] Celite®, World Minerals, Inc., Santa Barbara, CA

measurements were taken on 36 birds/treatment (Trial 1) or 18 birds/treatment (Trial 2). All three segments of the small intestine were then flushed with 0.9% saline solution to remove any remaining contents and a longitudinal cut was made along the length of the segments so they could lay flat for more accurate measurements. Segment length and weight were recorded.

Viscosity measurements were taken on supernatant extracted from fresh ileal digesta contents. Ileal digesta contents manually expressed into 50 mL conical tubes and stored on ice for measurements of digesta viscosity. Ileal contents from each individual bird were mixed, sub-sampled, and centrifuged at 5.9 RCF[19] for 5 minutes to separate the supernatant from the solid digesta contents. The supernatant was extracted and placed in a clean 2 mL tube. Viscosity, in centipoise (cP), was measured on a 500 µL aliquot of the supernatant using a LVDV-II+ Brookfield digital viscometer (Brookfield Engineering Laboratories, Inc., Stoughton, Massachusetts, USA) equipped with a CP-40 cone spindle at shear rates of 22.5 sec^{-1} and 45 sec^{-1}. These two speed settings were chosen for these measurements because the readings were more stable and consistent then when using a higher or lower speed.

Energy digestibility

At the conclusion of each trial, fresh excreta samples were collected from pans beneath each cage for analysis of nitrogen-corrected apparent metabolizable energy (AME_n)[20]. Excreta, free of feathers and feed particles, was collected and frozen at -20^{0}C

[19] RCF=relative centrifugal force

[20] $AME_n=GE_{feed}-((GE_{excreta}*AiA_{feed})/AiA_{excreta})-(8.22*N_{retained})$
$N_{Retained}=N_{feed}-((N_{excreta}*AiA_{feed})/AiA_{excreta})$

until analysis. Samples were removed from freezer and dried at 55^0C in a forced air oven (Blue M, Thermal Product Solutions) for 48 hours. Dried excreta and feed samples were ground and analyzed for gross energy using an adiabatic bomb calorimeter (IKA® calorimeter C 5000, IKA Works, Inc.) and nitrogen by combustion analysis (LECO Corporation, St. Joseph, MI). Both feed and excreta were analyzed for Celite® recovery using the acid-insoluble ash procedure based on Vogtmann *et al*. (1975).

Statistical analysis

Data were analyzed using the GLM procedure of SAS 9.2 (SAS Institute, Cary, North Carolina, USA) with means separated using LS means ($p<0.05$).

Animal ethics

All husbandry practices and euthanasia were performed with full consideration of animal welfare. All bird handling procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University.

Results and Discussion

Live performance results for Trial 1 and 2 are presented in Tables 2-5. In Trial 1, no treatment effects observed on BW, BWG, FI or FCR. It was surprising that we did not see a performance response between the positive and negative controls. The negative control was formulated to 145 kcal ME/kg lower than the positive control. It is possible that the diet was not deficient enough in energy to observe a measurable difference of increased feed

consumption to compensate for a lower energy density. For Trial 2, a second negative control treatment was included to reduce the energy level an additional 150 kcal/kg to more clearly measure treatment differences when the carbohydrase was included. Another factor in evaluating feed consumption was as a result of the cage design; there was an immeasurable amount of feed spillage during Trial 1. The feed was not able to spill into feed troughs of other cages, but this wastage was also not accounted for in measurements of feed consumption. Although the volume of feed wastage appeared to be consistent between treatments, blocks, and cages, this could have resulted in an inaccurate calculation of the feed conversion ratio. For Trial 2, feed spillage was collected and accounted for. Additionally, in Trial 2 birds were raised an additional week (28 days) to observe if an additional week of growth provided additional clarity of a treatment effect.

In Trial 2, no treatment effect was observed on BW. However, at 7 and 14 days there was a trend of the treatments receiving carbohydrase having greater BW compared to the un-supplemented birds ($p=0.06$ and 0.08, respectively). A treatment effect was observed for BWG, but only for the first 14 days. Greater BWG (0-14 days) was observed for those treatments receiving carbohydrase supplementation compared to the control treatments and the treatment receiving only protease supplementation. All diets were formulated with adequate protein and amino acid balance, thus it was not expected that improvements in performance would be observed in these trials with protease supplementation alone. Greater FI was also observed for birds receiving carbohydrase supplementation (0-14 days). The greater consumption of feed could account for the higher BWG observed in the corresponding treatments. However, similar to the BWG response, treatment differences in

FI were not measurable during the third and fourth week of the trial. A difference in FCR was only observed during the first week of Trial 2, with an improvement observed in the treatments receiving carbohydrase. Overall, raising the birds to 27 days rather than 20 days did not provide clearer differences in bird performance due to enzyme supplementation.

Reports of carbohydrase supplementation effects on bird performance have been inconsistent. Some researchers have reported improvements in performance (Chesson, 2001; Choct *et al*., 1996; Ferket 1993), while others not observed improvements in live performance (Odatellah *et al*., 2002; Bedford, 2000). As with any feed additive, improving live performance with carbohydrase supplementation is dependent on many factors. These factors can include grain age and quality (Chesson, 2001), concentration and type of NSP (Odatellah *et al*., 2002), other antinutritional factors (Choct, 2006), dietary energy level (Chesson, 2001), and bird age (Gao *et al*., 2008; Choct, 2006; Chesson, 2001).

Another common parameter for evaluating efficacy of dietary carbohydrases is measuring the effect on the viscosity of the digesta contents. Birds are able to tolerate moderate increases in digesta viscosity without negative effects on their performance, but large increases in viscosity due to soluble NSP can reduce performance and impair nutrient digestion (Choct and Annison, 1992). Based on the viscosity measurements obtained in the current two trials, carbohydrase successfully reduced digesta viscosity compared to un-supplemented treatments (Table 6). Additionally, the carbohydrase was efficacious even when supplemented in combination with the exogenous protease. In Trial 1, the reduced viscosity was observed in those treatments receiving exogenous carbohydrase (CH, PrCH, Pr2C). There was not a significant difference in viscosity measurements between the

treatments receiving protease and carbohydrase (PrCH) and treatments with protease and a double dose of carbohydrase (Pr2C). For Trial 2, the carbohydrase and protease were only included together at only one concentration, based on manufacture's recommendation, since no additional improvements were observed with the higher concentration of carbohydrase in Trial 1. As observed in Trial 1, digesta viscosity was significantly lower in treatments receiving the carbohydrase in Trial 2, both alone and in combination with the protease, compared with the negative control diets. This further supports that the combination of the two enzymes did not negatively impact the efficacy of each other. The ability to clearly and consistently measure this response suggests digesta viscosity as a good parameter to measure for evaluation of carbohydrase activity. In Trial 1, viscosity measurements were taken on about 36 birds per treatment. Due to the clear statistical differences, despite some expected variability between birds, the number of birds sampled for digesta viscosity in Trial 2 was reduced to 18 birds per treatment. Statistical differences were still clear, thus 15 to 18 birds per treatment is the number of birds we will use for this parameter in the future.

AME_n is of interest as a method to quantify the expected response of a carbohydrase. This value provides us an estimation of the overall energy value an animal can metabolize from a feed, but is not specific to fat, starch or protein digestibility. Quantifying an expected response can allow possible reductions in feed formations, which would result in cost savings. High concentrations of NSP in a diet can result in decreased AME (Danicke, 2001), but supplementation of carbohydrases to those diets can improve AME (Nian *et al*., 2011).

Results for AME_n analysis for Trial 1 and 2 are presented in Table 6. In Trial 1 we expected to see a difference in AME_n between the positive and negative control due to the

difference in dietary energy level. This difference was not observed. We also did not observe a difference in AME_n with the addition of carbohydrase or protease alone. However, an uplift in AME_n was observed in Pr2C compared to the enzymes alone or the NC. Additionally, when the two enzymes were included in combination (PrCH) the response was intermediary between Pr2C and NC or when enzymes were supplemented individually. Therefore, the reduction in digesta viscosity from the carbohydrase may have allowed the protease to improve energy digestibility through improved protein digestibility. Unlike Trial 1, there was a difference in AME_n between PC and NC in Trial 2. As expected, the PC had highest AME_n. However, there was no difference between NC1 and NC2. While there was no AME_n uplift with the carbohydrase compared to un-supplemented controls, the diet with protease supplementation alone had the lowest AME_n. While there was no improvement in AME_n observed with the supplementation of the carbohydrase to reduced energy diets in Trial 2, there was also no negative effect of including the protease in combination with the carbohydrase. Although significant uplifts in AME_n due to carbohydrase inclusion were not observed in either Trial 1 or 2, this parameter is of interest and will still be considered as a method of evaluation in further study.

In previous research it has been demonstrated that the intestinal tract and digestive organs can enlarge to adapt to diets high in indigestible polysaccharides (Choct, 2001; Bedford, 1996; Almirall *et al*., 1995; Ikegami, 1990). The pancreas can adapt in size and enzymatic production in response to dietary changes (Bedford, 1996; Brannon, 1990), this can result in hypertrophy as the pancreas adapts to increased enzymatic demand. In Trial 1, no treatment effect was observed for pancreas weight when it was analyzed as a percentage

of body weight or when the pancreas weight was analyzed using bird body weight as covariate (Table 7). It is possible that the diet in Trial 1 was not indigestible enough. Although we used new-harvest wheat, perhaps it was a good quality. Since the carbohydrase product supplemented in these trials included both xylanase and β-glucanase activities, the diets included both wheat and barley. Perhaps the percentage of barley in diet was not high enough to cause digestive challenges. For Trial 2, the proportion of the barley in the diet was increased and the wheat decreased to provide more substrate for the β-glucanase.

In Trial 2 the NC 1 and NC2 treatments had increased pancreas size compared to the PC. Therefore the birds in the reduced energy diets were adapting to the more indigestible diet. The birds supplemented with the combination of carbohydrase and protease had pancreases reduced to the same size as the PC; those supplemented with only the carbohydrase had pancreases intermediary between the PC and NC treatments. Almirall *et al*. (1995) observed increased pancreas weights as a proportion of body weight in chick fed barley diets, and a subsequent decrease in pancreas weight with the addition of β-glucanase. Based on observations in Trial 2, pancreas size may be a parameter to include in the evaluation of a carbohydrase.

In addition to pancreas weight, the small intestine was also evaluated for changes in length and weight due to treatment. Some researchers have reported reductions in relative weights of the small intestine when carbohydrase enzymes were supplemented to either wheat-based (Gao *et al.,* 2008) or barley-based (Hesselman and Aman, 1986) diets. In Trial 1, no differences were observed in relative weights of duodenum or jejunum, however there was a reduction in relative weight of the ileum in Pr2C compared to the un-supplemented

controls (PC and NC), with other treatments intermediary. These data were also analyzed using the weight of the intestine and its corresponding bird as a covariate. This analysis yielded the same results.

In Trial 2, no treatment effect was observed on duodenum or jejunum weight as a percentage of bird body weight. However, the ileum weight as a percentage of bird body weight was reduced with the supplementation of carbohydrase when compared to all other treatments. The data produced the same result when the weight of the ileum was analyzed using bird body weight as a covariate. Perhaps the ileum is more affected than the other segments of the small intestine. Chyme (digesta) collects in the ileum and has a lower moisture content due to water re-absorption. This could increase the bulk of the chyme in the lumen, resulting in the increased intestinal length to accommodate increased bulk. In Trial 1 there was a reduction in the length of the jejunum and ileum in Pr2C compared to the PC or NC, with other treatments receiving one or both enzymes intermediary. In Trial 2, the length of the jejunum, using the body weight as a covariate was reduced in those birds receiving carbohydrase and further reduced when the carbohydrase and protease were supplemented in combination. A measureable response to dietary carbohydrase was observed on the weight and length of the small intestine and these measurements could be utilized as a parameter for evaluating bird response to carbohydrase supplementation. However due to great variability between animals, this parameter is not as sensitive and requires great numbers of samples to be collected.

Conclusions and Applications

1. Digesta viscosity is a good parameter to evaluate efficacy of a carbohydrase enzyme in wheat and barley-based diets, but may not be an accurate prediction of live performance.
2. Improvements in AME_n may not be evident with carbohydrase supplementation; however, AME_n measurements should still be considered as a parameter for evaluation.
3. The protease used in this study can be used in combination with this commercial carbohydrase since there were no observations indicating interference of the protease activity on the carbohydrase activity.
4. Pancreas and intestinal weights may be utilized as indicators of carbohydrase response, but should not be relied upon as the sole evaluation parameter.

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Table 1: Composition and nutrient content of wheat and barley-based diets fed to male broiler chickens from placement to 3 or 4 weeks of age.

Ingredients	Trial 1	Trial 2
	% of Total diet	
Soybean meal	32.97	29.00
Wheat, soft	30.00	16.00
Barley	24.80	39.20
Poultry fat	6.50	8.00
Monocalcium dicalcium phosphate (21% P)	1.80	1.80
Calcium carbonate	1.75	1.75
DL-methionine	0.43	0.44
L-Lysine	0.33	0.38
Trace mineral premix[1]	0.20	0.20
Choline chloride 60%	0.20	0.20
Sodium chloride	0.25	0.25
Vitamin premix[2]	0.20	0.20
Sodium selenite premix[3]	0.10	0.10
Filler (sand)	0.10	2.11
Sodium bicarbonate	0.23	0.23
L-Threonine	0.15	0.15
Nutrient content		
ME Poultry, kcal/kg[4]	3025 (2880)	2980 (2830) (2680)
Crude protein, % (calculated)	23.26	21.58
Crude protein, % (analyzed)	19.17	19.29
Crude fat, %	7.70	9.27
Crude fat, % (analyzed)	8.47	7.85
Crude fiber, %	3.11	3.32
Crude fiber, % (analyzed)	4.87	5.90

[1] Each kilogram of mineral premix (0.1% inclusion) supplied the following per kg of complete feed: 60 mg Zn as $ZnSO_4H_2O$; 60 mg of Mn as $MnSO_4H_2O$; 40 mg Fe as $FeSO_4H2O$; 5 mg Cu as $CuSO_4$; 1.25 mg I as $Ca(IO_3)_2$; 1 mg Co as $CoSO_4$.

[2] Each kilogram of vitamin premix (0.1% inclusion) supplied the following per kg of complete feed: 13,200 IU vitamin A; 4,000 IU cholecalciferol; 66 IU alpha-tocopherol; 110 mg niacin; 22 mg pantothenic acid; 13.2 mg riboflavin; 8 mg pyridoxine; 4 mg menadione; 2.2 mg folic acid; 4 mg thiamin; 0.253 mg biotin; 0.04 mg vitamin B_{12}; 100 mg ethoxyquin.

[3] NaSeO3 premix provided 0.3 mg Se/kg of complete feed.

[4] Values in parentheses represent reductions in calculated dietary ME.

Table 2: Effect of dietary supplementation of a carbohydrase and protease in reduced energy wheat and barley based diets fed to broiler chickens on bird body weight from placement until 20 or 27 days of age.[1]

		Days of Age				
		Placement	**7**	**14**	**20***	**27**
T[3]	**Treatment**	---------- *Grams of body weight per bird* ----------				
	PC	43.5	163	447	832	-
	NC	43.3	164	447	837	-
	CH	43.7	168	453	843	-
1	Pro	43.8	163	444	823	-
	PrCH	43.6	169	459	855	-
	Pr2C	43.5	172	459	837	-
	SEM(27)[2]	0.21	2.6	5.9	12.1	-
	PC	42.0	170	458	906	1400
	NC 1	42.1	167	445	895	1398
	NC 2	42.0	164	433	863	1333
2	CH	41.8	181	474	901	1353
	Pro	42.0	165	450	895	1378
	PrCH	42.0	177	474	914	1378
	SEM(27)[2]	0.23	4.4	10.9	18.2	28.6
		---------- *P values* ----------				
	Trial 1	0.76	0.10	0.32	0.56	-
	Trial 2	0.95	0.06	0.08	0.48	0.54

[1]Values are means of 6 replicate pens of *ca.* 8 birds per pen.
[2]SEM (27)=Standard error of the mean with 27 degrees of freedom.
[3]T=Trial; signifies if results were from Trial 1 (T1) or Trial 2 (T2).
*Data were collected at 20d for birds in Trial 1 and 21d for birds in Trial 2.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 3: Effect of dietary supplementation of a carbohydrase and protease in reduced energy wheat and barley based diets fed to broiler chickens on bird body weight gain from placement until 20 or 27 days of age.[1]

		Days of Age						
		0-7	**7-14**	**14-20***	**21-27**	**0-14**	**0-20***	**0-27**
T[3]	**Treatment**	---------- *grams of body weight gain per bird* ----------						
	PC	119	282	384	-	401	785	-
	NC	121	280	390	-	401	790	-
	CH	124	285	390	-	409	799	-
1	Pro	120	281	379	-	400	779	-
	PrCH	124	290	395	-	414	810	-
	Pr2C	129	286	379	-	415	794	-
	SEM(27)[2]	2.6	4.7	8.2	-	6.2	12.1	-
	PC	126 [b]	267 [bc]	454	493	393 [b]	858	1357
	NC 1	122 [b]	272 [abc]	444	502	397 [ab]	847	1356
	NC 2	120 [b]	255 [c]	431	470	378 [b]	822	1291
2	CH	139 [a]	289 [ab]	426	453	429 [a]	859	1311
	Pro	121 [b]	266 [bc]	441	483	389 [b]	849	1336
	PrCH	131 [ab]	295 [a]	439	464	430 [a]	872	1336
	SEM(27)[2]	4.1	8.3	10.9	15.4	11.8	17.5	28.6
		---------- *P values* ----------						
	Trial 1	0.09	0.63	0.66	-	0.30	0.56	-
	Trial 2	0.02	0.02	0.54	0.23	0.02	0.49	0.54

[1]Values are means of 6 replicate pens of *ca.* 8 birds per pen.
[2]SEM (27)=Standard error of the mean with 27 degrees of freedom.
[3]T=Trial; signifies if results were from Trial 1 (T1) or Trial 2 (T2).
*Data were collected at 20d for birds in Trial 1 and 21d for birds in Trial 2.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 4: Effect of dietary supplementation of a carbohydrase and protease in reduced energy wheat and barley based diets fed to broiler chickens on feed intake from placement until 20 or 27 days of age.[1]

		Days of Age						
		0-7	**7-14**	**14-20***	**21-27**	**0-14**	**0-20***	**0-27**
T[3]	**Treatment**	*grams of feed intake per bird*						
	PC	131 [c]	396	623	-	527 [c]	1150	-
	NC	138 [b]	400	639	-	538 [bc]	1177	-
	CH	147 [a]	421	656	-	568 [a]	1224	-
1	Pro	140 [ab]	402	632	-	543 [bc]	1175	-
	PrCH	141 [ab]	411	620	-	551 [ab]	1171	-
	Pr2C	141 [ab]	412	637	-	553 [ab]	1190	-
	SEM(27)[2]	2.3	6.3	15.6	-	7.4	21.4	-
	PC	140	373 [bc]	701	796	513 [b]	1214	2009
	NC 1	140	375 [bc]	689	816	515 [b]	1203	2019
	NC 2	141	362 [c]	680	784	503 [b]	1183	1967
2	CH	151	396 [ab]	684	750	546 [a]	1230	1980
	Pro	141	372 [bc]	690	805	512 [b]	1203	2007
	PrCH	145	404 [a]	704	779	549 [a]	1253	2032
	SEM(27)[2]	3.3	8.6	11.0	18.4	10.4	18.3	29.6
		P values						
	Trial 1	0.002	0.11	0.65	-	0.01	0.30	-
	Trial 2	0.21	0.01	0.63	0.21	0.01	0.15	0.65

[1]Values are means of 6 replicate pens of *ca.* 8 birds per pen.
[2]SEM (27)=Standard error of the mean with 27 degrees of freedom.
[3]T=Trial; signifies if results were from Trial 1 (T1) or Trial 2 (T2).
*Data were collected at 20d for birds in Trial 1 and 21d for birds in Trial 2.
[a,b]Means within a column with no common superscript are significantly difference (P≤0.05).

Table 5: Effect of dietary supplementation of a carbohydrase and protease in reduced energy wheat and barley based diets fed to broiler chickens on feed conversion ratio adjusted for mortality from placement until 20 or 27 days of age.[1]

		Days of Age						
		0-7	**7-14**	**14-20***	**21-27**	**0-14**	**0-20***	**0-27**
T[3]	**Treatment**	---------- *grams of feed intake: grams of body weight gain* ----------						
	PC	1.100	1.406	1.623	-	1.315	1.466	-
	NC	1.136	1.435	1.641	-	1.342	1.487	-
	CH	1.190	1.475	1.684	-	1.388	1.511	-
1	Pro	1.177	1.437	1.673	-	1.360	1.511	-
	PrCH	1.132	1.419	1.570	-	1.332	1.446	-
	Pr2C	1.098	1.438	1.648	-	1.335	1.484	-
	SEM(27)[2]	0.028	0.028	0.048	-	0.025	0.030	-
	PC	1.122 [abc]	1.399	1.547	1.616	1.300	1.423	1.490
	NC 1	1.109 [bc]	1.381	1.553	1.625	1.287	1.417	1.491
	NC 2	1.180 [a]	1.420	1.581	1.675	1.334	1.450	1.530
2	CH	1.077 [c]	1.369	1.609	1.658	1.275	1.437	1.513
	Pro	1.171 [ab]	1.397	1.569	1.670	1.319	1.435	1.512
	PrCH	1.101 [c]	1.372	1.606	1.682	1.281	1.440	1.524
	SEM(27)[2]	0.022	0.022	0.027	0.025	0.020	0.018	0.017
		---------- *P values* ----------						
	Trial 1	0.13	0.65	0.63	-	0.41	0.63	-
	Trial 2	0.02	0.57	0.47	0.32	0.28	0.81	0.48

[1]Values are means of 6 replicate pens of *ca.* 8 birds per pen.
[2]SEM (27)=Standard error of the mean with 27 degrees of freedom.
[3]T=Trial; signifies if results were from Trial 1 (T1) or Trial 2 (T2).
*Data were collected at 20d for birds in Trial 1 and 21d for birds in Trial 2.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 6: Effect of dietary supplementation of a carbohydrase and protease in reduced energy wheat and barley based diets fed to broiler chickens on ileal digesta viscosity and nitrogen-corrected apparent metabolizable energy (AME_n) at 20 or 27 days of age.

T[2]	Treatment	Viscosity *(cP)*	AME_n[1] *(kcal/kg)*
1*	PC	10.18 [a]	2732 [ab]
	NC	8.50 [b]	2679 [b]
	CH	6.10 [c]	2676 [b]
	Pro	8.92 [b]	2658 [b]
	PrCH	6.20 [c]	2706 [ab]
	Pr2C	5.53 [c]	2811 [a]
	SEM	0.460[#]	26
2*	PC	9.60 [bc]	3108 [a]
	NC 1	14.00 [a]	2963 [b]
	NC 2	14.29 [a]	2984 [b]
	CH	5.89 [c]	2990 [b]
	Pro	9.94 [b]	2893 [c]
	PrCH	6.99 [bc]	2977 [b]
	SEM(93)	1.27[^]	23
		---------- *P values* ----------	
	Trial 1	<0.0001	0.0003
	Trial 2	<0.0001	<0.0001

[1]Values are means of 6 replicate pens; Standard error of the mean (SEM) with 27 degrees of freedom.
[#]Values are means of *ca.* 36 replicate birds per treatment; Standard error of the mean (SEM) with 183 degrees of freedom.
[^]Values are means of *ca.* 18 replicate birds per treatment; Standard error of the mean (SEM) with 93 degrees of freedom.
[2]T=Trial; signifies if results were from Trial 1 (T1) or Trial 2 (T2).
[*]Data were collected at 20d for birds in Trial 1 and 27d for birds in Trial 2.
[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Table 7: Effect of dietary supplementation of a carbohydrase and protease in reduced energy wheat and barley based diets fed to broiler chickens on intestinal weight and length at 20 or 27 days of age.[1]

		Percentage of body weight (%)				Length[2] (cm)			Weight[2] (g)			
T[4]	Treatment	D	J	I	Panc	D	J	I	D	J	I	Panc
1*	PC	1.13	2.01	1.55[a]	0.32	25.0	58.0[a]	60.2[a]	10.63	18.98	14.65[a]	3.05
	NC	1.10	1.87	1.57[a]	0.32	25.7	56.8[ab]	60.7[a]	10.41	17.71	14.80[a]	3.03
	CH	1.25	1.95	1.47[ab]	0.33	25.2	55.9[abc]	58.8[ab]	11.66	18.40	14.07 [ab]	3.08
	Pro	1.07	1.93	1.46[ab]	0.34	25.2	55.7[abc]	56.4[b]	10.04	18.28	13.73 [ab]	3.15
	PrCH	1.07	1.90	1.50[ab]	0.31	25.2	55.4[bc]	57.9[ab]	10.12	18.01	14.26 [ab]	2.93
	Pr2C	1.04	1.89	1.42[b]	0.32	24.5	53.7[c]	56.3[b]	9.86	17.87	13.40 [b]	3.01
	SEM (269)[3]	0.084	0.039	0.030	0.007	0.35	0.90	1.06	0.75	0.36	0.29	0.061
2*	PC	0.77	1.39	1.15[a]	0.25[b]	27.38	67.9[ab]	64.6	11.60	20.84	17.27 [a]	3.72[b]
	NC 1	0.74	1.37	1.20[a]	0.27[a]	27.20	68.4[ab]	66.2	11.11	20.60	17.96 [a]	4.09[a]
	NC 2	0.76	1.36	1.16[a]	0.27[a]	27.46	69.4[a]	67.2	11.10	20.20	17.10 [a]	3.99[ab]
	CH	0.75	1.33	1.06[b]	0.26[ab]	27.43	66.3[bc]	64.1	11.09	19.99	15.92 [b]	3.84[b]
	Pro	0.73	1.34	1.14[a]	0.27[a]	26.83	66.7[abc]	65.2	10.88	19.99	17.05 [a]	4.00[ab]
	PrCH	0.72	1.34	1.03[b]	0.25[b]	26.54	63.8[c]	63.1	10.86	20.23	15.52 [b]	3.74[b]
	SEM (241)[3]	0.020	0.030	0.026	0.006	0.42	1.10	1.20	0.27	0.45	0.38	0.08
		P values										
	Trial 1	0.58	0.13	0.004	0.11	0.24	0.02	0.01	0.58	0.16	0.02	0.21
	Trial 2	0.41	0.74	<0.0001	0.01	0.58	0.01	0.21	0.43	0.70	<0.0001	0.01

[1]Values are means of *ca.* 48 replicate birds per treatment. D=duodenum; J=jejunum; I=ileum; Panc=pancreas

[2]Weight and length were analyzed using bird bodyweight as a covariate.

[3]SEM (269)=Standard error of the mean with 269 degrees of freedom. Samples in Trial 2 were collected over 2 days, to account for this a blocking factor (day) was included in the statistical model, reducing the degrees of freedom to 241.

[4]T=Trial; signifies if results were from Trial 1 (T1) or Trial 2 (T2).

*Data were collected at 20d for birds in Trial 1 and 27-28d for birds in Trial 2.

[a,b]Means within a column with no common superscript are significantly difference ($P \leq 0.05$).

Appendix B

Change in the definition of xylanase activity

The research for this dissertation was conducted using a raw, dry mono-component xylanase product; the product did not contain any additional carriers or fillers. The xylanase inclusion levels in the early studies (Manuscripts I and II) were defined in milligrams of xylanase enzyme per kilogram of finished feed (mg/kg), or parts per million (ppm). As research progressed (Manuscripts III and IV), the product was defined by the units of xylanase activity per kilogram of finished feed. One unit of xylanase activity (XU) is defined as amount of xylanase needed for the release of 1 nanomol of reducing sugars per second from 0.5% beechwood xylan at 50^0C in 50 mM trisodium citrate buffer at pH 6.0. The inclusion level of 20,000 XU/kg feed utilized in Manuscripts III and IV is equivalent to 30 ppm xylanase. Therefore, 1 ppm xylanase inclusion provided about 667 XU/kg of finished feed.