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Determining efficacy of a thermally processed exogenous enzyme cocktail for broilers

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Determining Efficacy of a Thermally Processed Exogenous Enzyme Cocktail for Broilers

Kala Renea Beaman

Thesis submitted to the
Davis College of Agriculture, Natural Resources and Design
at
West Virginia University
in partial fulfillment of the requirements
for the degree of

Master of Science
in
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2010

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ABSTRACT

Determining Efficacy of a Thermally Processed Exogenous Enzyme Cocktail for Broilers

Kala R. Beaman

Feed ingredient price has influenced nutritionists to maximize diet nutrient availability through use of exogenous enzymes. Poultry are almost exclusively fed pelleted diets that entails feed being subjected to conditions of high moisture, temperature and pressure that could partially denature added enzymes. Exogenous enzyme efficacy may be decreased or completely lost if enzymes are not able to survive the pelleting process. The objective of the current studies was to properly assess the efficacy of a commercially available exogenous enzyme cocktail subjected to increasing steam conditioning temperatures during pelleting (82, 88, 93°C). Dietary treatments consisted of nutritionally adequate diets or Positive Control (PC), diets reduced in metabolizable energy (ME) and available phosphorous (AP) or Negative Control (NC), and NC diets with the exogenous enzyme. Study 1 established significant differences between the PC and NC diets ($P<0.05$). However, the exogenous enzyme cocktail did not improve performance. Study 2 was designed to improve the opportunity for the exogenous enzyme cocktail to demonstrate a benefit. This study utilized increased mixer-added fat addition in the diet formulation that may decrease frictional heat production in the pellet die, and a decreased metabolizable energy difference between the positive and negative control. Again, performance differences were observed between the PC and NC ($P<0.05$), with no beneficial effect demonstrated for the exogenous enzyme at any temperature ($P>0.05$). In Study 3, diet formulations were similar to Study 2; however, conditioning temperatures were decreased (71, 77, 82°C), a thinner die was used for pelleting and an additional unconditioned mash (UCM) treatment was added. Enzyme efficacy was demonstrated for live weight gain and feed conversion ratio (FCR) for UCM ($P<0.05$) as well as FCR for the 82°C diets ($P=0.07$). Performance observations in Study 3 were supported by nitrogen corrected apparent metabolizable energy (AMEn) data. Overall, these studies suggest that future evaluations of enzyme cocktail thermostability and subsequent efficacy should consider several manufacturing variables.

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Exogenous enzymes

In many animal production systems feed is the biggest single cost and profitability can depend on the relative cost and nutritive value of the feed available. In most production systems the pressure has primarily focused on two nutrients in particular: energy and phosphorus. Energy costs have elevated due to the increased demand of corn for ethanol production and the concomitant loss of availability of reasonably priced fats and oils that are being used as a feedstock for biodiesel [22]. Phosphorus (P) levels have also been a concern due to its limited availability and potential contribution of P in poultry excreta on eutrophication of surface water. The net result is that the increase in least cost formulations (LCF) price for energy and phosphorus has driven feed manufacturers to consider alternative options: such as feed enzymes [22]. The possibility of using exogenous feed enzymes in non-ruminant diets has provided nutritionist with a very important tool in improving feed digestibility, to moderate environmental contamination and to reduce feed cost.

In the past five years the market for poultry enzymes has changed significantly with phytases now leading followed by xylanases and then cellulases (glucanases) trailing in a distant third place. Other enzyme classes such amylases, proteases and mannanases are making up only a small proportion of the total feed enzymes [22]. One of the main reasons for using these enzymes in animal feeds is to break down anti- nutritional factors that are present in feed ingredients [2]. Various exogenous feed enzymes target ingredient substrates that are unavailable or that interfere with overall diet digestibility such as phytate bound phosphorus and non-starch polysaccharides. An additional use for exogenous enzymes is to increase the availability of starches, proteins and minerals that are not accessible to the animal's own digestive enzymes or bound up in a chemical form that the animal is unable to digest [2].

Various single enzymes are proposed to promote single effects on a particular chemical component of the diet. Phytase alone has been shown to improve performance of chicks fed diets deficient in P [19]. Phytase and xylanase, when used separately, have been shown to improve bird performance and enhance growth [19]. Under certain economic conditions, nutritionists are tempted to incorporate more than one type of enzyme in these diets to potentially maximize nutrient availability. These enzyme combinations can either be naturally occurring or mixed as a cocktail. Certain enzyme concoctions have been shown to degrade non-starch polysaccharides (NSP), which act as anti-nutritional factors by increasing digesta viscosity leading to decreased digestibility of starch, protein, and fat [5,23]. These enzyme concoctions have also been shown to improve bird performance, apparent metabolizable energy (AME), more specifically amino acid digestibility for some amino acids [5, 20, 23,].

One major dilemma with using exogenous enzymes is that enzymes are specific and are only effective when several conditions like temperature, humidity and pH are adequate [21]. Non-ruminant production animals are almost exclusively fed pelleted diets. The pelleting process entails complete diets being subjected to conditions of high moisture, temperature and pressure that may denature some or all of the enzymes contained in an exogenous enzyme cocktail. Although exogenous enzyme cocktails seem a feasible strategy to maximize diet nutrient availability, efficacy may be decreased or completely lost if enzymes are not able to survive the pelleting process.

Phytase

Phytases have been fed to poultry and pigs to degrade plant phytate which would otherwise pass through the digestive tract relatively intact with minimal release of P resulting in high levels

of bound P in manure. Moreover, since phytate is in the form of calcium and magnesium salts, and is able to chelate many cations, its hydrolysis has been associated with improved mineral utilization overall [22]. Consequently, most people feel feeding a phytase source can be effective for increasing phosphorus and mineral digestibility and retention, and as a result the use of inorganic P sources can be reduced. In the past, phytase was simply used to provide phosphorus to the bird, and as such a small savings in feed costs was obtained by decreasing the use of inorganic phosphate sources in the diet. Alternatively, new data derived from feeding second generation phytase sources have shown improvements beyond phosphorus with increases in retention of calcium, energy and amino acids [22] .

Phytic acid is the principal storage form of phosphorus in many plant tissues, especially bran and seeds. Phosphorus in phytate form is, in general, not bioavailable to monogastric animals because they lack the digestive enzyme phytase, which is required to separate phosphorus from the phytate molecule. The poor digestibility of phytate phosphorous increases the cost of production since additional sources of available phosphorous are needed in ration formulation to meet the nutritional requirements of the animal [2]. Further, undigested phytate is known to have a negative impact on mineral bioavailability and protein digestability. Phytic acid is a strong chelator of important minerals such as calcium, magnesium, iron, and zinc, and can therefore contribute to mineral deficiencies. Phytate has been shown to inhibit trypsin activity, which decreases protein digestibility. In addition protein in the phytate-bound form may be less susceptible to protease activity during intestinal passage [2]. An additional problem with undigested phytic acid is its negative impact on the environment. Undigested phytic acid is disposed in the manure which then leads to the contamination of fresh water and damage of other ecosystems.

Mode of action

Many factors have been found to influence the gut response to these different phytases, including the molecular properties. Some commercial phytases have been found to have limited stability in the gastric environment and low specific activity. It has been clearly shown that phytate solubilizes in the acidic region of the gut, thus this is the critical area of digestion where a highly effective phytase is needed [16]. The phytase needs to be able to withstand the acidic environment and be able to work quickly with a high affinity for the target substrate before being impacted by endogenous and exogenous proteases (i.e. pepsin). This low pH environment in the gastric phase is ideal for making phytate susceptible to hydrolysis by phytase. However, the low pH also places a stress on all proteins, including enzymes. For some fungal phytases this appears to be a greater problem than for other phytases. The acidic phase (pH 2.5-4.5) of the intestinal tract is the only part where phytate is truly soluble and most likely susceptible to degradation. It is essential that a phytase maintains maximum activity across the pH range where phytate is most susceptible to hydrolysis (pH 4.5 and lower) [22].

Thermal Stability

Phytase enzymes tend to be diverse in both their sources and their characteristic. Phytases originating from bacteria, fungi, yeast and plants have an optimum temperature range from 45 to 77°C. A phytase isolated from fungi *Aspergillus niger* was described to having activity at temperatures between 25 to 65°C, with the optimum temperature being 55°C. One study by Wyss and others studied the heat denaturation of two purified phytate isolates, *A. fumigatus* and *A. niger*. Both enzymes were denatured at 55°C. However, when submitted to a

temperature of 90°C the enzyme from *A. fumigatus* refolded into its active conformation on cooling, while that from *A. niger* did not [17]. While an additional study by Simons showed that pelleting at temperatures of 78 to 81°C did not lower enzyme activity. However, pelleting at temperatures of 84 to 87°C resulted in inactivation of the original enzyme activity [12]. A study by Gibson showed that two of three phytases pelleted at temperatures of 65 to 95°C suffered inactivation at 65°C. Only a commercially available stabilized preparation retained substantial activity with pelleting at 85°C and above [10].

Carbohydrases (NSP enzymes)

Recently, many nutritionists have renewed an interest in feeding non-starch polysaccharide (NSP) enzymes due to the potential cost savings they offer. The most popular being xylanase and glucanase followed by cellulases and mannanases. These enzymes were introduced many years ago due to their ability to target several substrate components within the plant material including fiber, starch and some plant proteins. These enzymes have shown to be most effective in diets based on barley and wheat [22]. However, diets based on barley and wheat has been known to have negative aspects, such as wet litter. Due to these negative aspects, nutritionists tend to favor corn - soy based diets. These enzymes have shown to be effective in wheat and barley diets but their responses with corn -soy diets can be quite variable. A response in corn-soy based diets requires the correct application adjustments. The response can be variable depending on what enzyme or cocktail of enzymes is used, the quality of feed ingredients or substrate used and if the enzymes are thermo-tolerant [22]. In turn this leads to the challenge that the nutritionist must know “that all NSP enzymes work differently” leading to different performances in the animal.

Mode of Action

It is thought that NSP enzymes function through a composite of three separate activities, the contribution of each activity varying with ingredients and individual birds. The two key functions for a corn-soy based diet would be plant cell wall destruction, and stimulation of beneficial bacteria with changes in the fiber composition.

The first main function for NSP enzymes is cell wall destruction. The cell wall material in the starchy endosperm of corn and sorghum is constructed mainly of small amounts of cellulose encrusted with hemicelluloses [15]. Since poultry do not possess the necessary enzymatic capacity to degrade plant cell walls, a lot of the content within this material can either bypass digestion or not be broken down until it reaches the lower gut by bacteria. This factor of encapsulation is based on the fact that some endosperm cells in corn and other ingredients manage to avoid physical breakdown during the activities of grinding and pelleting in feed manufacturing, and gizzard activity. Effective degradation of this material requires the addition of sufficient amounts of the appropriate NSP enzyme activity which created holes in the cell wall [22]. This allows water hydration and large enough amounts of pancreatic proteases and amylases to enter the cell wall and enable digestion of the starch and protein more rapidly. Xylanases, and to a lesser extent cellulases (B 1-4 glucanases) have proven most effective in the breakdown of corn cell wall material [11, 18]. Mannanases and pectinases have shown to target the soy more so than the corn fraction of the diet [9]. Research has shown that corn starch digestibility in the upper part of the digestive tract can vary depending on the source of corn. This variability could be due to differences in starch structure, protein matrix, and the handling of the corn post-harvest (drying and milling/processing techniques). These enzymes focus on

breaking down the cell wall material exposing more starch and protein for enzymatic digestion, reducing endogenous secretion and altering the lower gut microbial populations. This would expose more nutrients in the upper intestine, reducing the physical damage and lowering endogenous secretions by the gut villa [22]. This would then lower maintenance requirement spent on digestion and improve nutrient retention.

The second function in of NSP enzymes would be bacterial population stimulation. Exogenous NSP enzymes have proven to breakdown plant cell wall carbohydrates and reduce chain length producing smaller polymers and oligomers. At some point the fragments become small enough and numerous enough to act as a substrate for bacterial fermentation. These short chain oligosaccharides travel to the lower gut and become substrates for bacterial fermentation in the ileum and cecum [1]. This is beneficial in altering bacterial populations and altering volatile fatty acid production. Medium to long chain oligomers have shown to increase fermentation in the jejunum and ileum region. However, feeding a xylanase significantly decreases fermentation and shifts it to the cecum. If the diet is well digested in the upper part of the digestive tract by using NSP enzymes then small amounts of starch are present in the ileum, and more cell wall oligomers are present in the cecum [7].

Xylanase

Cellulose and hemicelluloses are known to convert soluble sugars by enzymes of mainly microbial origin. The hemicelluloses fraction of the cell walls of most plant species contains mainly xylan and mannan and requires more extensive enzymes to hydrolyze soluble sugars. The two main enzymes involved are endoxylanase and endomannanase. Xylanases are mainly produced by many species of fungi and bacteria [2]. Xylanase is specific for Beta 1-4 linkages

of linear polysaccharide xylan. Xylanase can either be classified as specific or non specific depending on its action. Specific xylanase are only active on Beta 1-4 linkages, while nonspecific hydrolyze Beta 1-4 linked xylan, Beta 1-4 mixed xylans and other Beta 1-4 linked polymers. Xylanase can also be classified as either debranching or non-debranching depending on its ability to release arabinose in addition to hydrolyzing the main chain [2].

Since xylanase is produced from a wide range of fungi and bacteria it has shown to have a variation in optimum conditions for activity. It has shown to have an optimum temperature range from 30 to 105°C and a pH range from 2 to 10. Research has shown a wide range in decreased activity at different temperature. A study by Bedford demonstrated a linear inactivation in enzyme activity between the temperature of 65 and 105°C [2]. Where broiler performance was best when feed was processed at 81-83°C. While the highest body weight and lowest feed conversion ratio were processed between temperatures of 80 to 85°C.

Beta-Glucanase

Endoglucanase is an enzyme that acts on glucan (a carbohydrate found in barley and oats). Endoglucanases specifically cleave the internal Beta-1,4 glycosidic bonds of amorphous, swollen and substituted celluloses as well as cello-oligosaccharides. These enzymes are inactive towards crystalline cellulose and cellobiose. It is speculated that microorganisms secrete multiple endoglucanases with a wide range of substrate specificities to effect efficient hydrolysis of complex cellulosic substrates [2].

There are a limited number of reports on the effect of heat treatment on Beta-glucanase enzyme that has been added to feed. The studies tend to show that Beta-glucanase has a relatively low feed conditioning stability. Eeckhout and others demonstrated that when pelleted

between 72 to 91°C there was a loss in activity at the lowest temperature and only 7% of activity remained at the highest processing temperature [6]. Bedford found an inactivation of Beta-glucanase after conditioning at a temperature as low as 75°C followed by pelleting [2]. Cowan and Rasmussen reported that a commercially available Beta-glucanase lost significant activity when pelleted at temperatures above 65°C, but when coated provided protection to 75°C [4].

Steam Conditioning and Pelleting

In most production systems, poultry are exclusively feed pelleted diets. The pelleting process requires thermo-mechanical processing which may impact broiler performance and nutrient availability. An essential part of the thermo-mechanical process is the addition of moist heat (steam) to mash feed through a process known as conditioning. This process requires steam to pass from the boiler into the conditioner, which is forced through a series of four different steam traps to help separate excess moisture from the steam. The steam is then reduced in pressure by use of a globe valve, and then throttled into the conditioner via a ball valve. The addition of the moisture has shown to be beneficial in the formation of a durable pellet [8]. It has also shown to ease the passage of a feed through the die by reducing friction and increase production rate [13]. The benefits of feeding pelleted diets have been widely researched; leading to such diets being favored over mash. Pellets have been shown to improve feed conversion ratio, reduced ingredient segregation and decreased energy used during feed consumption [24, 25]. While the influences of steam conditioning on pelleting are mostly beneficial, there are a few negative effects that have to be taken into consideration. One major negative effect associated with steam conditioning is case hardening. Case hardening is the product of excessive starch gelatinization on the pellets' exterior, while leaving the interior unaltered [8]. This occurs

when low amount of steam entering the system ultimately causing decreased lubrication leading to increased frictional heat in the die [14].

Pellet quality has been shown to improve FCR and LWG in broilers. Proudfoot and Sefton [26] define pellet quality as the pellets ability “to maintain structural integrity throughout conveying both at the mill, during transportation, on the farm, and during feeding by the bird.” Pellets of a lesser quality are usually associated with high percentages of fines; these fines are the result of poor bonding of the pelleted mash. Researchers have devised several methods of estimating pellet quality. The Pfast test entails tumbling a pre-weighed quantity of screened pellets for a set period of time. After tumbling, the test material is then subjected to another screening and the pellets are weighed separately from the fines. Fines should be sifted off from the pellets and recycled. The practice of feeding fines to poultry has been shown to negatively influence feed conversion and rate of gain and should be avoided [27].

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Determining Efficacy of a Thermally Processed Exogenous Enzyme Cocktail for Broilers

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Keywords: Exogenous enzyme cocktail, enzyme efficacy, feed manufacture, broiler performance

Primary Audience: feed manufacturers, nutritionists, researchers

Summary

Feed ingredient price has influenced nutritionists to maximize diet nutrient availability through use of exogenous enzymes. Poultry are almost exclusively fed pelleted diets that entails feed being subjected to conditions of high moisture, temperature and pressure that could partially denature added enzymes. Exogenous enzyme efficacy may be decreased or completely lost if enzymes are not able to survive the pelleting process. The objective of the current studies was to properly assess the efficacy of a commercially available exogenous enzyme cocktail subjected to increasing steam conditioning temperatures during pelleting (82, 88, 93°C). Dietary treatments consisted of nutritionally adequate diets or Positive Control (PC), diets reduced in metabolizable energy (ME) and available phosphorous (AP) or Negative Control (NC), and NC diets with the exogenous enzyme. Study 1 established significant differences between the PC and NC diets ($P<0.05$). However, the exogenous enzyme cocktail did not show improved performance. Study 2 was designed to improve the opportunity for the exogenous enzyme cocktail to demonstrate a benefit. This study utilized increased mixer-added fat addition in the diet formulation that may decrease frictional heat production in the pellet die, and a decreased metabolizable energy difference between the positive and negative control. Again, performance differences were observed between the PC and NC control diets ($P<0.05$), with no beneficial effect demonstrated for the exogenous enzyme at any temperature ($P>0.05$). In Study 3, diet formulations were similar to Study 2; however, conditioning temperatures were decreased (71, 77, 82°C), a thinner die was used for pelleting and an additional unconditioned mash (UCM) treatment was added. Enzyme efficacy was demonstrated for live weight gain and feed conversion ratio (FCR) for UCM ($P<0.05$) as well as FCR for the 82°C diets ($P=0.07$). Performance observations in Study 3 were supported by nitrogen corrected apparent

metabolizable energy (AMEn) data. Overall, these studies suggested that future evaluations of enzyme cocktail thermostability and subsequent efficacy should consider several manufacturing variables.

Description of Problem

In animal production systems feed is the largest single cost and profitability is specifically dependent on cost and nutritive value of available feed ingredients. One concern of diet formulation is the animal's inability to completely digest different constituent parts of feed ingredients. Exogenous feed enzymes target ingredient substrates that are unavailable or that interfere with overall diet digestibility such as phytate bound phosphorus, non-starch polysaccharides and large globular proteins. Nutritionists may address these anti-nutritional factors (ANF) with feed additive cocktails that contain several exogenous feed enzymes. Studies have shown that exogenous enzyme blends containing various combinations of amylases, proteases, xylanases, glucanase, cellulase, mannanase, and pectinase may improve AME, amino acid digestibility and bird performance in unconditioned mash diets [19, 20, 21]. However, non-ruminant production animals are almost exclusively fed pelleted diets. The pelleting process entails complete diets being subjected to conditions of high moisture, temperature and pressure that may denature some or all of the enzymes contained in an exogenous enzyme cocktail. Although exogenous enzyme cocktails seem a feasible solution to addressing ANF concerns, efficacy may be completely lost if enzymes are not able to survive the pelleting process.

Various exogenous feed enzymes have been shown to lose activity when exposed to high conditioning/pelleting temperatures. For example phytases have been shown to lose efficacy at 70°C [22] xylanases at 105°C [6] and glucanases at 75°C [9, 11].

The objective of the current studies was to properly assess the efficacy of a commercially available exogenous enzyme cocktail subjected to increasing steam conditioning temperatures during pelleting.

Materials and methods

Enzyme

A commercially available exogenous enzyme cocktail containing amylase, cellulase, phytase, xylanase, beta-glucanase, pectinase, and protease was tested for pelleting thermostability and feeding efficacy for starter phase broilers. The enzyme was added to diets at the manufacturers recommended level of 220.5 g/MT (200g/ton) [1].

Diet formulation

Dietary treatments consisted of PC and NC formulations as well as the NC formulation top dressed with the enzyme cocktail. The PC was based on Agristat data [2] and utilized least cost inclusions of corn, soybean and byproduct ingredients (Table 1). PC and NC formulations differed in AP by 1.0% and metabolizable energy by 90 kcal/kg and 41 kcal/kg in the first and next two studies respectively. Study 1 used cellulose as a nutrient diluent, while studies 2 and 3 used increased levels of byproducts. Studies 2 and 3 also utilized increased levels of dietary fat (3.0%) added at the mixer to reduce frictional heat production in the pellet die. Celite was added to all diets as an indigestible marker.

Feed Manufacture

All feed was manufactured at West Virginia University's pilot feed mill. In each of the three studies, two 907kg (2,000lb) allotments of feed were batched in accordance to the NC and

an additional 907 kg allotment of feed was batched in accordance to the PC. All batches were mixed for ten minutes dry, and an additional ten minutes post soybean oil addition using a single screw vertical mixer [3]. Each negative control batch was divided into two allotments of 453kg (1,000lbs), combined with the 453kg allotment from the other negative control batch and remixed. Each combined allotment represented the experimental unit to be steam conditioned and pelleted. Prior to pelleting, 4.5kg (10lbs) of mash were premixed with the exogenous enzyme cocktail at a concentration of 220.5g/tonne for five minutes, then placed back in the batch designated as NC with enzyme (NC w/E). All diets were placed back in the mixer and mixed an additional twenty minutes prior to pelleting. All steam conditioning treatments were applied to each batch. Pelleted treatments were passed through a 1.3m long conditioner with a diameter of 0.31m [8] and exposed to sequentially increasing temperatures, i.e. 82, 88, 93°C for the first and second studies and 71, 77, 82°C for the third study. Each conditioning temperature series included industry standard temperature of 82°C. As steam passed from the boiler into the conditioner, it was forced through a series of four different steam traps to help separate excess moisture from the steam. Steam was reduced in pressure by use of a globe valve, and then throttled into the conditioner via a ball valve. Mash temperature was taken following conditioning as an easily measured estimate of thermal processing. Measurements of hot pellet temperature were not taken due to the difficulties of measurement and confounding factors of flash off temperature and moisture, friction in the die that can vary based on conditioning temperatures. Once conditioned, mash was extruded through a pelleting die. The die had an effective thickness and diameter of 44.96mm x 4.7mm (1.75 x 3/16in) for studies 1 and 3, and study 3 used a thinner die with a similar diameter, 38.18mm x 4.76mm (1.5 x 3/16in). The pellets were cooled with ambient air utilizing a horizontal belt cooler [16]. Once the desired

conditioning temperature reached a steady state during pelleting, mash samples were taken from the feed screw auger that conveyed feed into the conditioner and corresponding pellet samples were taken from the cooler discharge. Approximately 136kg (300lbs) of feed were collected for potential feeding. Once the desired amount of feed was collected, the temperature was increased to the next desired conditioning temperature and the process was repeated.

Pellets were sampled in the studies 2 and 3 to examine moisture content immediately after pellet extrusion through the die, three, six and nine days post pelleting, as well as on the final day of feeding.

Pellet quality was assessed through durability testing to determine the structural integrity of the pellets [5]. However, prior to feeding, pellets were crumbled using a roller mill. Crumble size was based on 4 day old bird beak capacity and the desire to maintain similar crumble bulk density among treatments so that variations in feed form would not confound treatment effects. Thus, roller mill gap was manipulated to account for variations in pellet quality. Pellet quality and crumble density were recorded as descriptive data (Table 2). Crumbled feed was weighed into 11kg (24 lb) allotments and stored in buckets during the feeding study. Study 3 incorporated unprocessed mash as an additional treatment to explore effects of lack of thermal processing on enzyme stability and subsequent broiler performance and digestibility.

Performance data

The first two performance studies used 576 male Cobb 500 broiler chicks while the third study used 768 similar strain male chicks obtained from the same hatchery [14]. On day four all chicks were individually weighed and allotted into eight weight groups. One chick from each group was placed in each pen to create the experimental unit. Starting pen weight did not differ

significantly or numerically by more than 20 grams. Dietary treatments were randomly assigned to pens. Treatment assignment was blocked by location in the room. Initially chicks were exposed to continuous lighting. On day six, birds were placed on two hrs of darkness and every day after, darkness was increased by two hrs until a total dark period of 12 hrs was established. On day 21 the birds were fasted during the 12 hr photoperiod, then allowed to consume feed for a three hr period. This methodology was chosen so that chicks would have adequate digesta in the ileum. Between three and four hours after the start of the meal, the birds were killed by an intra-cardial injection of sodium pentobarbitone. After death, the birds were weighed by pen and the ileum of each bird was excised. Pen feed intake and mortality corrected FCR were calculated as well as per bird live weight gain.

Nitrogen Corrected Apparent Metabolizable Energy

The ileum was defined as the portion of the small intestine extending from the Meckel's diverticulum to the ileal-cecal junction. This section was then cut in half and the lower section was used for collection. The lower ileum was gently flushed with distilled water and the digesta washing was collected. The excreta collected was weighed and then freeze dried at - 40°C for 72 hours. Once dry, all excreta were ground with a mortar and pestle. Due to beneficial performance results due to enzyme, only excreta samples from Study three were analyzed for gross energy (GE), nitrogen (N) [4], and acid insoluble ash for AMEn determination. Celite concentration or acid insoluble ash (AIA) was determined according to the method of McCarthy and co-authors [15]. The AMEn values of the diets were calculated using the following formula.

$$\text{AMEn (kcal/g)} = \text{GE}_{\text{diet}} - [\text{ileal collection} \times \text{GE}_{\text{excreta}} \times (\text{AIA}_{\text{diet}}/\text{AIA}_{\text{excreta}})] + [((\text{N}_{\text{intake}} - \text{N}_{\text{excreta}}) \times 8220) \times (\text{AIA}_{\text{excreta}}/\text{AIA}_{\text{diet}})]$$

Statistical Analysis

The statistical analysis was based on a conditioning temperature x enzyme inclusion factorial design. Enzyme inclusion and conditioning temperature main effects as well as the enzyme inclusion x temperature interaction were determined. In the third study, an additional analysis was performed on the unprocessed mash diets separate from the factorial treatments. All data were statistically analyzed using the GLM procedure of the Statistical Analysis System [18]. Multiple comparisons with Fischer's LSD test were performed within all analyses. Alpha was designated as 0.05, and letter superscripts were used to denote differences among treatment means. Contrasts were also performed to obtain probability values for differences between the PC and NC treatments. In the third study an additional contrast was performed to obtain a probability value for the comparison of 82°C NC and 82°C NC w/ enzyme, due to the large numerical difference in FCR and the FCR ANOVA P-value of 0.1154.

Results and Discussion

Study 1

The enzyme cocktail did not improve male broiler 4-to-21 d performance. However, expected differences were obtained in live weight gain ($P < 0.05$) and FCR ($P < 0.05$) between the PC and NC diets (Table 4). We speculated that lack of enzyme effect may be due to heat induced denaturation of the enzyme associated with die friction caused by low fat inclusion of the negative control diet. Recent data has shown diets containing low mixer added fat may increase frictional heat in the pellet die and denature exogenous feed enzymes [10]. Without adequate fat addition at the mixer (~3%), frictional heat and pressure produced in the pellet die may increase to a point where enzyme and nutrient conformation may change and ultimately be

inactivated or unavailable to the bird [13]. In addition, enzyme denaturation may be associated with increased levels of added moisture, thus increased heat energy transfer efficiency, resulting from increased conditioning temperature. Study 1 supports this speculation due to the low level of mixer added fat in the NC i.e. 0.75% (Table 1) and the significant increase in FCR resulting from increasing conditioning temperature ($P=0.0487$, Table 4). In addition, we speculated that perhaps the ME difference between the PC and NC diets was too great for the enzyme cocktail to overcome.

Study 2

The primary differences between Study 2 and Study 1 were the increase in mixer added fat (from 0.75% to 3.0%), an increase in by-product ingredient use i.e. wheat middlings and DDGS, that precluded the use of cellulose as a nutrient diluent in diet formulations as well as decreased ME difference between the PC and NC diets. The increase in mixer added fat was to potentially reduce the frictional heat and pressure produced in the pellet die. This was based on the findings from Gehring and co-authors [10] that found that a low mixer added fat inclusion with high thermal processing of diets containing exogenous enzymes decreased broiler performance. The ME difference between diets were reduced due to the Study 1 speculations (from 90 kcal/kg to 41 kcal/kg). The enzyme cocktail again did not improve male broiler 4-to-21 d performance. However, significant differences were obtained in LWG ($P < 0.05$), feed intake ($P < 0.05$), and FCR ($P < 0.05$) between the PC and NC diets (Table 5). The study showed that despite increased mixer added fat in the formulation the enzyme cocktail was not stable at 82, 88 or 93°C conditioning temperatures. However, the diet conditioned at 82°C with the enzyme cocktail demonstrated a slight numerical improvement in FCR compared to the NC ($P > 0.05$). Enzyme

stability was likely reduced due to increased conditioning temperature most noticeable with 88 and 93°C treatments. A study conducted by Bedford and co-authors demonstrated a linear inactivation in xylanase enzyme activity between the temperatures of 65 and 105°C. Upon feeding these diets, the authors reported the highest body weight and lowest feed conversion ratio were achieved when feed was processed between temperatures of 80 to 85°C [7]. Another speculation was that certain enzymes (i.e. protease) in the cocktail could survive higher processing temperatures while other enzymes may not, thus potentially prohibiting full efficacy of the cocktail. Fuad Saleh and co-authors [17] have shown that certain enzymes in an enzyme preparation may digest other enzymes and reduce the effectiveness of the enzyme cocktail to improve digestibility of animal feeds. We speculated that conditioning temperatures of 88 and 93°C were too high for this particular enzyme cocktail and perhaps the thickness of the pellet die also contributed to increased frictional heat production and enzyme denaturation. Past research has shown that increased die thickness can contribute to decreasing nutrient availability in pelleted diets [28].

Study 3

The primary differences between Study 2 and 3 was decreased steam conditioning temperatures, use of a die with less effective thickness, and incorporation of an unconditioned mash treatment in the design. A thinner die was used during pelleting to reduce frictional heat production that could potentially denature the enzymes. The steam conditioning temperatures were decrease to 71 and 77°C due to the previous finding in Study 2 which suggested that the enzyme cocktail was not stable at high conditioning temperatures. Low conditioning temperatures and thin pellet die effects were apparent in reducing moisture content (Table 2)

ultimately resulting in decreased pellet quality (Table 3). The UCM treatment was incorporated to determine the efficacy of the enzyme cocktail alone. The enzyme cocktail was effective in improving live weight gain and FCR compared to the NC for unconditioned mash diets ($P < 0.05$). In fact, the enzyme cocktail decreased FCR compared to the PC for unconditioned mash diets ($P < 0.05$). This data demonstrates that the enzyme cocktail was effective in liberating nutrients and/or overcoming ANF associated with diet formulation. However, when diets were conditioned and pelleted, the enzyme cocktail did not demonstrate consistent efficacy. Diets containing the enzyme cocktail conditioned at 71 or 77°C did not illustrate performance benefits compared to the NC formulations. The diet containing the enzyme cocktail conditioned at 82°C tended to decrease FCR compared to the NC ($P = 0.07$). Enzyme cocktail efficacy in the 82°C diet was likely associated with increased mixer added fat, use of a thinner pellet die, and increased die lubrication associated with the 82°C conditioning temperature. Although increased conditioning temperature has been associated with an increased efficiency of heat transfer, it has also been associated with increased die lubrication [24], and likely reduced frictional heat in the pellet die. One speculation for not observing an enzyme cocktail benefit at conditioning temperatures of 71 and 77°C is that pellet case hardening may have occurred [23]. This may have been due to the low amount of steam entering the system causing decreased lubrication leading to increased frictional heat in the die. Mash moisture in Study 3 was reduced relative to Study 2 (Table 3). The increased frictional heat may have denatured the enzymes. The PC and NC diets did not differ in their effects on broiler performance ($P > 0.05$).

In Study 3, AMEn was calculated in order to better explain performance observations. The enzyme cocktail improved AMEn for the unconditioned mash diets ($P < 0.05$) following performance observations. Significant AMEn differences were also obtained between the PC

and NC diets for unconditioned mash. However, this difference did not translate to significant improvement in broiler performance. The AMEn data for diets conditioned at 71 and 77°C did not show improvement with use of the enzyme cocktail, similar to performance observations. Broilers fed the diet conditioned at 82°C with exogenous enzyme showed significant improvement over the NC. These data were supported with performance data. Nevertheless, significant AMEn differences between PC and NC diets conditioned at 82°C did not translate to performance differences. The main effect of increased conditioning temperature decreased AMEn ($P=0.0001$). These data demonstrate that increased thermal processing may alter nutrient conformation, decreasing their availability. Past research supports these observations [6, 27]. Significant alterations in AMEn did not consistently follow performance trends. A previous study by Kocher et al. [25] that evaluated commercial multi-activity enzyme added to a corn–canola meal-based diet also showed that performance was not affected by changes in AMEn.

In vitro enzyme assay were not preformed due to the proprietary composition of the multi-enzyme cocktail. However, recent research has shown that *in vitro* enzyme retention testing is not always a good predictor of enzyme efficacy [29]. *In vivo* testing seems essential in determining enzyme efficacy.

Overall, these data demonstrate that the particular enzyme cocktail used in the current study was sensitive to several manufacturing variables. The combination of increased mixer added fat, use of a thin pelleting die, and the 82°C conditioning temperature that provided increased moisture/ lubrication likely improved enzyme efficacy. Many of the performance observations were supported with AMEn data. Future evaluation of enzyme cocktail thermostability and subsequent efficacy should consider several manufacturing variables.

Conclusions and Applications

1. Thermal stability and consequent exogenous enzyme efficacy may be dependent on several feed manufacture factors.
2. The enzyme cocktail was not effective in improving broiler performance when diets were conditioned at 88°C or 93°C despite manipulations of mixer added fat.
3. The enzyme cocktail was not effective in improving broiler performance when diets were conditioned at 71 or 77°C despite use of increased mixer added fat and a thinner pellet die.
4. The enzyme cocktail was effective in improving performance when diets were not conditioned/pelleted and when diets were conditioned at 82°C using 3% mixer added fat and a 38.18 x 4.76m (1.5 x 3/16in) pellet die.

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manner, with the exception of adding five 13-mm hex nuts to the pretumbled sample to obtain added pellet agitation.

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Table 1. Diet Formulations¹

Ingredients	Study 1		Study 2 and 3	
	Positive control diet (%)	Negative control diet (%)	Positive Control diet (%)	Negative Control diet (%)
Corn	49.33	46.04	50.17	43.32
Soybean meal (48%)	25.82	31.55	29.34	32.13
Wheat middlings	5.00	5.00	3.00	6.00
Corn DDGS ^A	5.00	5.00	5.00	9.59
Porcine meat and bone meal	4.97	0.50	4.50	0.25
Soybean oil	3.10	0.75	3.10	3.10
Cellulose	2.00	5.62	----	-----
Celite	2.00	2.00	2.00	2.00
Monocal	0.99	1.16	0.76	0.84
Limestone	0.52	1.05	1.00	1.58
DL – methionine	0.34	0.34	0.32	0.31
NB3000 ^C	0.25	0.25	0.25	0.25
Lysine	0.23	0.21	0.21	0.25
Salt	0.18	0.25	0.29	0.30
Threonine	0.15	0.15	0.07	0.07
Coban 60 ^D	0.08	0.08	----	----
BMD ^E	0.05	0.05	----	----
Calculated nutrients				
ME (kcal/kg)	3072	2982	3072	3031
Crude Protein	21.94	22.00	22.00	22.00
Lysine (%)	1.28	1.28	1.28	1.28
Methionine + cystine (%)	0.98	0.98	0.98	0.98
Threonine (%)	0.85	0.85	0.85	0.85
Tryptophan (%)	0.26	0.28	0.28	0.30
Calcium (%)	0.91	0.91	0.91	0.91
Available P (%)	0.45	0.35	0.45	0.35
Sodium (%)	0.20	0.20	0.20	0.20

¹ The positive control diet was formulated to industry recommendations (starter phase), the negative control diet was formulated to be 0.1% lower in available phosphorus and 90 kcal/kg lower in metabolizable energy for Study 1. For Studies 2 and 3 the metabolizable energy was 41kcal/kg.

^A Distiller's dried grains with soluble

^B Celite (indigestible marker) – naturally occurring soft, chalk-like rock

^C Vitamin Mineral Premix - Supplied per kg of diet: manganese, 0.02%; zinc, 0.02%; iron, 0.01%; copper, 0.0025%; iodine, 0.0003%; selenium, 0.00003%; folic acid, 0.69mg; choline, 386mg; riboflavin, 6.61mg; biotin, 0.03mg; vitamin B₆, 1.38mg; niacin, 27.56mg; pantothenic acid, 6.61mg; thiamine, 2.20mg; manadione, 0.83mg; vitamin B₁₂, 0.01mg; vitamin E, 16.53 IU; vitamin D₃, 2133 ICU; vitamin A, 7716 IU

^D Active drug ingredient Monensin Sodium 60gpb (90g/ton inclusion).- Elanco Animal Health, Indianapolis, IN. As an aid in the prevention of coccidiosis caused by *Eimeria necatrix*, *Eimeria tenella*, *Eimeria acervulina*, *Eimeria brunette*, *Eimeria mivati*, and *Eimeria maxima*

^E Bacitracin Methylene Disalicylate 50g/lb (50g/ton inclusion)- Alpharma, Fort Lee, NJ. For increased rate of weight gain and improved feed efficiency.

Table 2. Feed Manufacture Variables Associated with Variation in Conditioning Temperature and Diet Formulation.

Treatment	Pellet bulk density (kg/m³)	Crumble Bulk Density (kg/m³)	PDI(%)^a	MPDI(%)^b
Study 1				
82°C PC ^A	620	608	82	71
82°C NC ^B	642	595	96	93
82°C NC w/ E ^C	640	600	95	93
88°C PC	620	605	88	80
88°C NC	636	592	96	95
88°C NC w/ E	640	594	97	95
93°C PC	615	608	95	93
93°C NC	640	595	97	96
93°C NC w/ E	636	599	97	96
Study 2				
82°C PC	632	599	83	75
82°C NC	639	595	84	73
82°C NC w/ E	624	578	83	73
88°C PC	631	599	88	80
88°C NC	632	584	88	81
88°C NC w/ E	632	581	86	79
93°C PC	631	603	93	89
93°C NC	636	591	90	85
93°C NC w/ E	634	592	92	87
Study 3				
UCM PC ^D	679	----	----	----
UCM NC	669	----	----	----
UCM NC w/ E	673	----	----	----
71°C PC	632	584	67	44
71°C NC	616	565	70	45
71°C NC w/ E	628	571	69	44
77°C PC	634	583	65	48
77°C NC	612	570	72	54
77°C NC w/ E	618	575	71	52
82°C PC	634	579	68	42
82°C NC	616	568	73	55
82°C NC w/ E	612	573	71	53

^a Pellet durability index was determined by placing 500 grams of sifted pellets into a Pfast tumbler. Samples were tumbled for 10 minutes at 50 rpm. The sample was then sifted again and weighed. Pellet durability index was calculated as the percent of sifted pellets retained after tumbling.

^b Modified pellet durability index was measured similar to the previous description, with the exception of the addition of five 13-mm hexagonal nuts to the 500 g sample prior to tumbling.

^A PC = positive control diet

^B NC = negative control diets

^C NC w/E = Negative control diet containing the exogenous enzyme cocktail

^D UCM = unconditioned mash treatment

Table 3: Moisture Percentages for Studies 2 and 3¹

Treatment	Mash ^A	Hot Pellet^B	Day three^C	Day six^D	Day nine^E	Last day^F
Study 2						
82°C PC	11.09	15.09	13.62	11.89	12.04	10.79
82°C NC	11.12	15.51	12.68	11.77	11.61	9.77
82°C NC w/ E	11.13	15.49	11.36	11.39	11.22	10.66
88°C PC	11.12	15.96	13.41	11.59	12.27	10.50
88°C NC	11.06	15.95	13.43	11.32	12.21	10.94
88°C NC w/ E	10.97	15.59	13.00	11.68	11.36	10.78
93°C PC	11.34	15.80	13.49	11.72	11.88	10.66
93°C NC	11.00	15.44	12.64	11.68	12.06	10.15
93°C NC w/ E	11.04	15.85	13.00	12.92	11.89	10.73
Study 3						
71°C PC	8.84	12.97	12.15	12.08	10.88	8.58
71°C NC	8.87	12.62	12.19	12.18	10.73	9.82
71°C NC w/ E	9.02	13.31	12.28	11.95	10.36	9.76
77°C PC	8.77	13.64	12.50	12.52	11.65	10.27
77°C NC	8.89	13.14	12.18	12.54	11.15	9.79
77°C NC w/ E	8.63	12.86	12.02	12.30	10.92	10.64
82°C PC	9.28	13.52	12.42	12.50	11.16	10.02
82°C NC	8.98	13.79	11.48	12.25	10.77	9.45
82°C NC w/ E	9.12	13.53	11.76	11.93	11.61	9.78

¹ - Samples were retained and analyzed for moisture after a lack of enzyme cocktail effect was demonstrated in Study

^A - Mash – sample taken prior to pelleting

^B - Hot Pellet – sample taken directly after pelleting

^C - Three day – sample taken 3 days after pelleting

^D - Six day – sample taken 6 days after pelleting

^E - Nine day – sample taken 9 days after pelleting

^F - Last day – sample taken the last day of feeding

Table 4: Study 1: Performance Data for 4-to-21-day male broiler chicks.

Treatment	Starting Pen Weight ± SD (kg/pen)	Live Weight Gain ± SD (kg/bird)	Feed Intake ± SD (kg/pen)	Feed Conversion ^A ± SD (kg/kg)
Positive Control 82°C conditioning	0.593 ± 0.006	0.664 ^{ab} ± 0.055	7.536 ± 0.525	1.44 ^c ± 0.063
Negative Control 82°C conditioning	0.591 ± 0.006	0.629 ^{cd} ± 0.049	7.446 ± 0.611	1.52 ^b ± 0.118
Negative Control + E 82°C conditioning	0.593 ± 0.009	0.613 ^{cd} ± 0.074	7.716 ± 0.811	1.58 ^a ± 0.053
Positive Control 88°C conditioning	0.593 ± 0.006	0.672 ^a ± 0.076	7.831 ± 0.798	1.46 ^c ± 0.042
Negative Control 88°C conditioning	0.593 ± 0.006	0.619 ^{cd} ± 0.061	7.946 ± 0.646	1.61 ^a ± 0.047
Negative Control + E 88°C conditioning	0.590 ± 0.007	0.596 ^d ± 0.057	7.518 ± 0.696	1.60 ^a ± 0.044
Positive Control 93°C conditioning	0.594 ± 0.009	0.695 ^a ± 0.059	7.896 ± 0.603	1.42 ^c ± 0.047
Negative Control 93°C conditioning	0.591 ± 0.005	0.636 ^{bc} ± 0.047	7.946 ± 0.488	1.57 ^{ab} ± 0.049
Negative Control + E 93°C conditioning	0.591 ± 0.006	0.618 ^{cd} ± 0.047	7.861 ± 0.667	1.59 ^a ± 0.052
P-value	0.9540	0.0001	0.1044	0.0001
LSD ^B	-----	0.033	-----	0.052
SEM ^C	0.002	0.012	0.150	0.018
P-values for main effects and the interaction excluding positive control treatments				
Enzyme inclusion	0.9432	0.0734	0.5184	0.2242
Conditioning temperature	0.9257	0.2944	0.1413	0.0487
Enzyme inclusion x Conditioning temperature	0.6743	0.9506	0.1167	0.2886
Contrasts (P-values)				
82°C Positive Control vs 82°C Negative Control	0.5201	0.0358	0.6706	0.0037
88°C Positive Control vs 88°C Negative Control	0.9407	0.0022	0.5914	0.0001
93°C Positive Control vs 93°C Negative Control	0.3216	0.0006	0.7496	0.0001

^{a-b}Means within a column without a common superscript differ significantly (P≤0.05)

^AFeed conversion was calculated using mortality weight. There were 4 mortalities, 2 in the Negative Control 82°C conditioning treatment, 1 in the Positive Control 82°C conditioning treatment and 1 in the Negative Control + SSF 88°C treatment.

^BFischer's Least Significant Difference Value

^CStandard Error of the Mean

Table 5: Study 2: Performance Data for 4-to-21-day male broiler chicks.

Treatment	Starting Pen Weight ± SD (kg/pen)	Live Weight Gain ± SD (kg/bird)	Feed Intake ± SD (kg/pen)	Feed Conversion ^A ± SD (kg/kg)
Positive Control 82°C conditioning	0.603 ± 0.005	0.681 ^a ± 0.035	7.920 ^{bc} ± 0.405	1.34 ^c ± 0.039
Negative Control 82°C conditioning	0.602 ± 0.006	0.619 ^b ± 0.041	7.718 ^{bcd} ± 0.274	1.41 ^{abc} ± 0.037
Negative Control + E 82°C conditioning	0.602 ± 0.010	0.614 ^{bc} ± 0.031	7.511 ^d ± 0.364	1.40 ^{bcd} ± 0.038
Positive Control 88°C conditioning	0.600 ± 0.005	0.671 ^a ± 0.025	8.029 ^{ab} ± 0.329	1.36 ^{cde} ± 0.052
Negative Control 88°C conditioning	0.603 ± 0.008	0.599 ^{bcd} ± 0.0258	7.59 ^d ± 0.327	1.41 ^{abc} ± 0.033
Negative Control + E 88°C conditioning	0.605 ± 0.009	0.590 ^{cd} ± 0.024	7.698 ^{cd} ± 0.458	1.45 ^{ab} ± 0.091
Positive Control 93°C conditioning	0.605 ± 0.005	0.695 ^a ± 0.019	8.314 ^a ± 0.430	1.35 ^{de} ± 0.054
Negative Control 93°C conditioning	0.605 ± 0.008	0.584 ^d ± 0.035	7.528 ^d ± 0.573	1.45 ^{ab} ± 0.034
Negative Control + E 93°C conditioning	0.606 ± 0.006	0.583 ^d ± 0.014	7.633 ^{cd} ± 0.354	1.46 ^a ± 0.112
P-value	0.5508	0.0001	0.0001	0.0002
LSD ^B	-----	0.0388	0.5085	0.0966
SEM ^C	0.002	0.008	0.111	0.021
P-values for main effects and the interaction excluding positive control treatments				
Enzyme inclusion	0.6390	0.4933	0.9966	0.5949
Conditioning temperature	0.3017	0.0023	0.8580	0.1440
Enzyme inclusion x Conditioning temperature	0.9282	0.9143	0.3398	0.6070
Contrasts (P-values)				
82°C Positive Control vs 82°C Negative Control	0.7608	0.0001	0.2041	0.0123
88°C Positive Control vs 88°C Negative Control	0.3191	0.0001	0.0081	0.0656
93°C Positive Control vs 93°C Negative Control	0.7279	0.0001	0.0001	0.0019

^{a-b} Means within a column without a common superscript differ significantly (P≤0.05)

^AFeed conversion was calculated using mortality weight. There

^BFischer's Least Significant Difference Value

^CStandard Error of the Mean

Table 6: Study 3: Performance Data for 4-to-21 day male broiler chicks

Treatment	Starting Pen Weight $\pm$ SD (kg/pen)	Live Weight Gain $\pm$ SD (kg/bird)	Feed Intake $\pm$ SD (kg/pen)	Feed Conversion ^A $\pm$ SD (kg/kg)
Unconditioned Mash Positive Control	0.598 $\pm$ 0.005	0.573 ^{ab} $\pm$ 0.047	6.742 $\pm$ 0.352	1.47 ^a $\pm$ 0.049
Unconditioned Mash Negative Control	0.611 $\pm$ 0.033	0.546 ^b $\pm$ 0.032	6.518 $\pm$ 0.325	1.49 ^a $\pm$ 0.058
Unconditioned Mash Negative Control + E	0.600 $\pm$ 0.007	0.593 ^a $\pm$ 0.037	6.710 $\pm$ 0.304	1.42 ^b $\pm$ 0.061
P-value	0.3522	0.0203	0.2112	0.0268
LSD ^B	-----	0.0311	-----	0.0524
SEM ^C	0.0067	0.0102	0.0916	0.0172
Positive Control 71°C conditioning	0.602 $\pm$ 0.006	0.619 $\pm$ 0.043	6.848 $\pm$ 0.395	1.38 $\pm$ 0.037
Negative Control 71°C conditioning	0.600 $\pm$ 0.009	0.607 $\pm$ 0.038	6.991 $\pm$ 0.564	1.40 $\pm$ 0.109
Negative Control + E 71°C conditioning	0.599 $\pm$ 0.005	0.605 $\pm$ 0.019	6.702 $\pm$ 0.346	1.41 $\pm$ 0.059
Positive Control 77°C conditioning	0.596 $\pm$ 0.007	0.616 $\pm$ 0.041	6.673 $\pm$ 0.457	1.36 $\pm$ 0.105
Negative Control 77°C conditioning	0.597 $\pm$ 0.010	0.603 $\pm$ 0.045	6.756 $\pm$ 0.386	1.42 $\pm$ 0.033
Negative Control + E 77°C conditioning	0.599 $\pm$ 0.007	0.612 $\pm$ 0.047	6.875 $\pm$ 0.245	1.41 $\pm$ 0.068
Positive Control 82°C conditioning	0.593 $\pm$ 0.009	0.591 $\pm$ 0.082	6.858 $\pm$ 0.542	1.48 $\pm$ 0.175
Negative Control 82°C conditioning	0.598 $\pm$ 0.004	0.585 $\pm$ 0.036	6.841 $\pm$ 0.533	1.49 $\pm$ 0.110
Negative Control + E 82°C conditioning	0.594 $\pm$ 0.009	0.595 $\pm$ 0.035	6.676 $\pm$ 0.451	1.41 $\pm$ 0.039
P-value	0.3202	0.7933	0.9700	0.1154
LSD	-----	-----	-----	-----
SEM	0.0026	0.0151	0.1697	0.0327
P-values for main effects and the interaction excluding positive control treatments				
Enzyme inclusion	0.7606	0.5324	0.7339	0.1933
Conditioning temperature	0.6570	0.1926	0.9068	0.2254
Enzyme inclusion x Conditioning temperature	0.3767	0.8236	0.6709	0.1720
Contrasts (P-values)				
71°C Positive Control vs 71°C Negative Control	0.5082	0.5925	0.8195	0.7670
77°C Positive Control vs 77°C Negative Control	0.7857	0.5295	0.7323	0.1813
82°C Positive Control vs 82°C Negative Control	0.1148	0.7717	0.7558	0.8293

^{a,b}Means within a column without a common superscript differ significantly ($P \leq 0.05$)

^AFeed conversion was calculated using mortality weight.

^BFischer's Least Significant Difference Value

^CStandard Error of the Mean

^D a separate contrast was performed between 82°C NC and NC w/ E diets ($P = 0.07$) not shown in the table

Table 7: Study 3: Nitrogen Corrected Apparent Metabolizable Energy Data for 4-to-21 day male broiler chicks.

Treatment	AMEn + SD (kcal/g)
Unconditioned Mash Positive Control	2975 ^a ± 136.8
Unconditioned Mash Negative Control	2528 ^c ± 221.7
Unconditioned Mash Negative Control + E	2774 ^b ± 105.3
P-value	< 0.0001
LSD ^B	137.38
SEM	45.29
Positive Control 71°C conditioning	2931 ^{ab} + 142.6
Negative Control 71°C conditioning	3037 ^a + 143.8
Negative Control + E 71°C conditioning	3008 ^{ab} + 149.2
Positive Control 77°C conditioning	2773 ^{cd} + 133.6
Negative Control 77°C conditioning	2739 ^d + 187.5
Negative Control + E 77°C conditioning	2800 ^{cd} + 172.9
Positive Control 82°C conditioning	2892 ^{bc} + 99.9
Negative Control 82°C conditioning	2581 ^e + 193.6
Negative Control + E 82°C conditioning	2729 ^d + 124.8
P-value	< 0.0001
LSD ^B	121.4
SEM ^C	42.5
P-values for main effects and the interaction excluding positive control treatments	
Enzyme inclusion	0.1443
Conditioning temperature	< 0.0001
Enzyme inclusion x Conditioning temperature	0.2171
Contrast (P-values)	
71°C Positive Control vs 71°C Negative Control	0.0875
77°C Positive Control vs 77°C Negative Control	0.5766
82°C Positive Control vs 82°C Negative Control	< 0.0001

^{a-b}Means within a column without a common superscript differ significantly (P≤0.05)

^BFischer's Least Significant Difference Value

^CStandard Error of the Mean