

ABSTRACT

POPE, JEFFREY TAYLOR. Non-conditioning factors affecting enzyme thermostability during feed processing (Under the direction of Dr. Adam C. Fahrenholz and Dr. Jesse Grimes).

A series of experiments were conducted to determine the impact of non-conditioning factors utilized to improve physical feed quality on nutritional feed quality, with enzymes being a marker of nutritional status. The first experiment examined the effect of the level of mixer-added fat (MAF; 1, 3, or 5%), the source of fat (coconut oil, poultry fat, or canola oil), and pellet mill die length-to-diameter ratio (LD; 8:1 or 10:1) on mixer-added xylanase thermostability when diets were conditioned and pelleted at 82°C in one-way arrangements. The samples collected included unconditioned mash (UCM), conditioned mash (CM), and pellets (P). The parameters monitored included the change in temperature between hot pellets and conditioned mash (ΔT), pellet durability index (PDI), and pellet mill energy consumption (PMEC).

The recovery of xylanase in P relative to CM (P:CM) was greatest in diets pelleted with 5% MAF when compared to 1 and 3% MAF. The recovery of xylanase in P:CM was greatest in diets pelleted with coconut oil and poultry fat when compared to canola oil. The recovery of xylanase in P:CM and P relative to UCM (P:UCM) was greatest in diets pelleted through an L:D 8:1 pellet mill die when compared to an L:D 10:1 pellet mill die. A negative correlation was observed between the all the parameters monitored and xylanase activity in P:UCM and P:CM.

A second experiment was conducted to determine the impact of mixer-added water (MAW) and mash conditioning temperature (CT) on the thermostability of a phytase and xylanase in a factorial arrangement. The levels of MAW included 0, 1, and 2% and the mash

CTs included 80, 86, and 92°C. The samples collected and parameters monitored were the same as described in the first experiment. As the mash CT increased from 80 to 92°C, phytase recovery in CM relative to UCM (CM:UCM) decreased from 104.4 to 81.9%. However, phytase recovery in P:CM decreased from 76.4 to 6.3% at the same temperatures, which indicated that the pellet mill die was denaturing greater amounts of phytase when compared with the conditioning chamber.

A third experiment was conducted to determine the effect of pellet mill throughput (PMT) and mash CT on phytase and xylanase thermostability in a factorial arrangement. The PMTs utilized included 227, 454, 908, and 1816 kg/hr and the mash CTs utilized included 75 and 86°C. The samples collected and parameters monitored were the same as those described in the first two experiments. The recovery of phytase in P:UCM was subjected to an interaction between PMT and mash CT. When feeds were pelleted at 86°C with a PMT of 227 kg/hr, only 13.5% of the phytase was recovered when compared to 92.0% at 75°C. When the PMT increased to 1816 kg/hr at 86°C, the recovery of phytase in P:UCM was statistically similar to diets pelleted at 75°C at all the PMT.

A fourth experiment was conducted to determine the impact of fat application site (FAS) and pellet mill die L:D on phytase thermostability in diets pelleted at 86°C that were fed to broilers from 0-35 d of age with NC and PC diets for comparison. The FASs included 3.5% MAF or 0.5% MAF with the remaining 3.0% being applied post-pellet (PPLA) and the pellet mill die L:Ds were 8:1 and 10:1 in a factorial arrangement. Diets pelleted with MAF contained 79% greater levels of phytase when compared to those pelleted with PPLA fat. Broilers receiving diets pelleted with MAF exhibited improved bone characteristics when compared to broilers that received diets manufactured utilizing PPLA fat.

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Non-conditioning Factors Affecting Enzyme Thermostability during Feed Processing

by
Jeffrey Taylor Pope

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APPROVED BY:

Dr. Adam C. Fahrenholz
Committee Co-chair

Dr. Jesse L. Grimes
Committee Co-chair

Dr. Charles R. Stark

Dr. Peter R. Ferket

DEDICATION

To a father that taught me how to walk, a mother that supported every step, and a wife that took every step with me.

BIOGRAPHY

Jeffrey Taylor (J.T.) Pope was born on March 30th, 1991 to Nancy Jo Richards and Jeffrey Boyd Pope in Chapel Hill, NC. He grew up in Hillsborough and Mebane, NC. He graduated from Orange Senior High School in Hillsborough, NC in 2009 and attended Pfeiffer University on a baseball scholarship, but transferred to NC State in 2011 to focus on academics. J.T. worked as an undergraduate research assistant at the NC State feed mill and Chicken Education Unit during his undergraduate studies and graduated *summa cum laude* with his Bachelor of Science from NC State in May of 2014. After briefly attending Pharmacy School at UNC, J.T. returned to NC State to pursue a Master of Science in Poultry Science under the direction of Dr. Adam Fahrenholz. After completion of his Master of Science, JT continued his research and coursework to fulfill the doctoral requirements at NC State in Animal and Poultry Science and Nutrition. Upon graduation, JT will work as a production nutritionist for a poultry integrator based in North Carolina.

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LITERATURE REVIEW

OVERVIEW OF BROILER INDUSTRY

Broiler production was a global industry with several participating partners that shared a common goal regardless of the region; produce a protein product to improve animal protein accessibility and affordability while maintaining company profitability. As more nations entered the industrialized or developing classification, the demand for animal based proteins increased. According to the Food and Agricultural Organization of the United Nations [1], there was a strong positive correlation between income and animal protein consumption. As nations became more urbanized and developed the infrastructure to trade in perishable goods, their ability to distribute and supply animal based proteins improved.

Broiler meat production had increased at an unprecedented rate since the 1960s, with more recent projections indicating that annual meat production will need to increase from 218 million tonnes in 1997 to 376 million tonnes by 2030 [1]. Broiler production will likely be part of the solution for increased global demand for meat products due to the absence of restrictions for consumption based on religion and overall efficiency of production.

The model of broiler production was regionally specific. The majority of the United States broiler production occurred in a vertically integrated model; the integrators controlled all aspects of production from hatching egg production through the distribution of processed meat products. This model may also be observed in other industrialized nations such as the United Kingdom and Brazil. In a fully integrated model, growers were contracted to rear broilers while the contracting company provided chicks, feed, and technical services. Other models of broiler production included partial integration and fully independent growers, which were responsible for supplying poultry products for the much of the global population.

Independent broiler growers purchased chicks and feed from their suppliers of choice, and thus generated a different set of demands for companies supplying chicks and feed when compared to vertically integrated models.

Regardless of the model of broiler production, feeding was essential for the efficient production of broilers. Global feed production surpassed one billion tonnes in 2017 [2] and projections for an increase in global meat demand indicated that feed production would continue to grow. Since feed costs represented nearly 70% of the live production costs associated with rearing broilers [3] it was imperative that the global resources of grains and feedstuffs be adequately utilized to improve production efficiencies, supply the world with high value protein products, and maintain the profitability of the industry so that further growth may ensue.

FEED PELLETING

Feed had been pelleted to further extract value from raw materials utilized in broiler rations. Pelleting was the process by which blended ingredients were pressed together so that the individual particles of the blended ingredients adhered to each other to form a cylindrical pellet. Pelleting may be separated into three main processes: conditioning, pressing, and cooling/drying [4]. Feed was determined to be conditioned once it had been subjected to steam treatment for some period of time, which had typically been between 30 and 60 s. Conditioning softened and lubricated the surface of the individual particles to prepare them for adhesion. Subsequent to conditioning, the individual particles were pressed through a pellet mill die via pressure exerted by rollers that rotated along the interior of the pellet mill die surface. Once the ingredients had been pressed through the die, the individual particles were adhered to one another and the formed pellets were dried and cooled to form a final

feed that was adequate for broiler consumption. The pelleting process was energy intensive and accounted for greater than 40% of the energy expended to transform raw materials into an edible ration [5].

Feeding pellets to broilers had become common practice due to several advantages afforded on a physical and nutritional basis when compared to feeds that were not pelleted, commonly referred to as mash feed. From a physical perspective, pellets when compared to mash exhibited improved flow properties, greater bulk density, and a fixed composition [4]. It was imperative that finished feed flowed better than raw materials so that bridging would not occur within bins or feeders to ensure that feed was always accessible to broilers. Furthermore, improving the flowability of feed improved conveying properties and allowed for quicker and more uniform distribution of feed throughout broiler rearing houses equipped with automated feeding systems. Increasing the bulk density of feed allowed for the transport of a greater tonnage of feed per a fixed volume and also improved the nutrient density associated with a given volume of feed. Ensuring that the feed remained in a fixed composition prevented segregation of materials, reduced selective feeding, and minimized dust to ensure that the intended formulation of ingredients and nutrients were available to each animal offered the ration.

Jensen and colleagues [6] reported that the physical properties of pelleted feed when compared to mash feed influenced poultry behavior. The experiment was conducted by feeding Broad Breasted Bronze poults and New Hampshire chicks mash or pelleted feed. The researchers observed that poults offered mash feed dedicated 18.8% of the day for feed consumption, while those offered pelleted feed dedicated 2.2% of their day for feed consumption. In chicks, those offered mash feed occupied the feeder pans for 14.3% of the

day when compared to 4.7% of the day for those offered pelleted feed. Within a commercial broiler facility, the reduction in feeding duration was also likely associated with improved feeder pan accessibility. Additionally, these data indicated that offering pellets to poultry when compared to mash would allow for poultry to rest for greater amounts of time, thus reducing the energy expended for feeding.

McKinney and Teeter [7] later reported on the nutritional benefit associated with the physical properties of pelleted feed. The researchers conducted two experiments to determine the impact of feed form on broiler growth. Experiment 1 consisted of feeding broilers four rations that differed in caloric density with energy and protein being fixed in a constant ratio. The data collected, which included BW, feed intake, and FCR adjusted for mortality were used to generate mathematical models to explain the relationship between dietary energy and the parameters measured. Experiment 2 consisted of feeding broilers identical rations with different pellet qualities and a mash diet that served as a negative control to determine the association between pellet quality and caloric density, with pellet quality being defined as the ratio of pellets to pellet fines in the feeder pan. The pellet qualities fed included 20, 40, 60, 80, and 100% and the pellet qualities were achieved by screening a pelleted diet and then blending fines back into the diet at the appropriate ratio of pellets-to-fines for each treatment. The broilers were fed the different pellet quality treatments from 38 to 45 d of age and then BW, feed intake, and FCR were recorded and input as terms to the model generated in experiment 1 to determine the effect of pellet quality on caloric density.

The researchers determined that there was a positive correlation between pellet quality and resting frequency, which impacted the effective caloric value of the diets. Improving pellet quality from 20 to 100% was associated with a 111 kcal/kg improvement in

energy density. The greatest improvement in energy density was observed when pellet quality was improved from 60 to 70%, which represented 33 kcal/kg. The increase in the effective caloric value of the diets resulted in improved FCR for broilers fed diets with greater pellet qualities. Additionally, numerous other reports [8-10] concluded that improved pellet quality was associated with an improved FCR, likely as a result of the effective energy afforded due to improved feed form. Although the McKinney and Teeter [7] report definitively described the impact of pellet quality on broiler growth and efficiency, feed processing methods utilized to achieve different levels of pellet quality may differentially impact nutrient availability, which were failed to be determined in the report. However, these data definitively defined the nutritional benefit of improved pellet quality and influenced feed manufacturers to constantly strive for better pellet qualities so that broilers would be most efficient with the input raw materials. The broiler live performance efficiencies afforded by offering broilers pellets led to more than 80% of monogastric rations being pelleted in the United States [11].

FACTORS AFFECTING PELLETING AND PHYSICAL FEED QUALITY

The factors determined to influence pellet quality included feed formulation, mash conditioning, pellet mill die specifications, dietary ingredient particle size, and pellet cooling [11]. Ferket and Stark [12] later determined that pellet mill throughput, or pellet production rate, should also be considered as a factor that impacted pellet quality. These factors may be manipulated in various manners based on pellet quality objectives for various species, the model of animal production, and the region in which the animals would be reared.

Physical feed quality was typically determined through pellet durability testing with the results being reported as pellet durability index (PDI). There were two commonly utilized

methods to test pellet durability: the KSU tumble box method [13] and the New Holmen Pellet Tester (NHPT) method. The tumble box method relied upon mechanical agitation of the pellets by rotating a cubical testing chamber for 10 mins and may be modified by adding objects into the testing chamber, such as hex nuts. The NHPT relied upon pneumatic agitation of the pellets by forcing a stream of air through a pyramidal testing chamber for a period of 30, 60, 90, or 120 s. For both testing methods, pellets must be screened prior to entering the testing chamber. Fahrenholz [14] determined that the linear correlation between the standard tumble box method and the NHPT method was highly significant with an $R^2=0.93$, which indicated that both methods would predict differences in pellet durability in a similar manner, although the obtained raw values may differ based on testing method.

The resulting parameter of testing, PDI, was used as an indicator of the ability of pellets to withstand abrasion and fragmentation, but did not directly relate to pellet quality. Since pellet quality, or the ratio of pellets-to-fines in the feeder pan [7], was the parameter directly related to broiler live performance, PDI testing should have been implemented to monitor changes in physical feed quality as affected by the aforementioned factors, but not to directly estimate the impact of physical feed quality on animal growth performance. However, researchers had concluded that there was a positive correlation between PDI and reduction of fines in finished feed with an $R^2=0.58$ [15], so PDI testing offered some insight to pellet quality. The forces necessary to bind pellets were well described in a review by Thomas *et al.* [4] and included solid-solid interactions, capillary forces, liquid necking, adhesive and cohesive forces, and interactions between particles due to folding and plying. The forces described may be impacted by the known factors that affected pellet quality and durability.

Feed Formulation

Feed formulation was determined to be the most influential factor affecting pellet quality [16]. When diets were formulated to achieve improved pellet quality, dietary fats must be considered. The addition of fats or oils in a compound feed prior to pelleting had typically resulted in decreased pellet quality, pellet hardness, and pellet durability [14, 17-20]. Pope *et al.* [20] pelleted diets with 0.5 or 4.0% mixer-added fat (MAF) and determined that the additional fat present during pelleting reduced PDI by 33 percentage-points when the modified tumble box method was utilized. Added fats, especially those that contributed to dietary fat composition that were not bound within the matrix of cell walls, impeded the binding of water solubilized starches, proteins, and fibers through hydrophobic effects that prevented the inherent binding properties of those nutrients [21]. However, added dietary fats markedly influenced other parameters of the pelleting process.

Some formulations, depending on energy content and pricing of raw materials, forced fat into diets at rates that would be unsuitable for proper ingredient binding. While the lubricating properties of fat improved pellet mill throughput [22, 23] and thus the efficiency of pellet mill operations, a balance between the level of fat and desired pellet quality must be considered. One solution for providing dietary fat quantities in excess of those that optimized particle binding had been through applying dietary fats subsequent to pelleting so that pellet quality could be maintained in high fat rations. The use of post-pellet liquid application (PPLA) systems had become more common so that proper levels of dietary energy could be achieved without adversely affecting pellet quality and durability.

Dietary fiber had also been noted to impact pellet quality and durability. Soluble fibers, or those that swelled upon hydration such as glucans, arabinoxylans, and pectins,

increased the viscosity of diets and upon pressing resulted in reduced feed porosity and improved pellet hardness [24]. In diets with relatively low levels of dietary fiber, such as corn-soy based rations, including a viscous grain, such as wheat, has been considered a solution to improve pellet quality and durability due to the inherent binding properties of wheat fibers. Fahrenholz [25] demonstrated that adding wheat middlings to diets from 0 to 45% improved pellet durability of swine diets. Insoluble fibers, or those that did not hydrate upon exposure to moisture, may have improved pellet binding by entangling particles, but their resiliency to compress may have reduced the contact of the fibers with other particles. One such insoluble fiber used to improve pellet quality was lignin in the form of calcium lignosulfonate [26, 27]. Pope [27] demonstrated that diets amended with 0.5% calcium lignosulfonate improved PDI by 22.5 percentage-points in rations containing 3.0% MAF when compared to diets that were not supplemented with calcium lignosulfonate. Calcium lignosulfonate had been demonstrated to improve physical feed quality, likely via improved hydrogen bonding on the surface of particles, in a myriad of pelleting conditions that were understood to negatively impact pellet quality, such as high fat rations and low conditioning temperatures [27].

Dietary protein and starch were also constituents of broiler diets that impacted pellet quality and pellet processing. Starch had been characterized as an adhesive or binding agent [28] and was determined to impact binding properties once gelatinized upon exposure to heat and water. Although mash conditioning temperatures commonly exceeded the temperature at which starch gelatinization occurred for common feedstuffs [28], water was a limiting factor for starch gelatinization in compound animal feeds. To fully gelatinize starch, a water-to-starch ratio of 1.5:1 was required, which was unlikely to be accomplished in practical

pelleting scenarios [29]. Protein was also determined to be a potential binding agent in broiler feeds [24]. Raw proteins remained primarily in their native quaternary or tertiary structures, while the denaturing of proteins was described as the breakdown of the more complex protein structures into their linear or lower levels of amino acid organization. Wood *et al.* [30] determined that dietary protein and starch impacted physical feed quality. When raw starch was replaced with pregelatinized starch from 0 to 40%, researchers noted that PDI was improved by ~30 percentage-points in the presence of denatured proteins. Additionally, the inclusion of raw protein when compared to denatured protein improved PDI by ~65 percentage-points in diets containing raw starch.

Mash Conditioning

Mash conditioning parameters were determined to be the second most influential factor in determining physical feed quality [16]. Mash feed was noted to be conditioned upon exposure to heat and moisture for some duration of time to facilitate particle binding and compaction [31]. The mash conditioning parameters subjected to manipulation during feed pelleting included the mash conditioning temperature, pressure of steam, duration of residence within the conditioning chamber, and the level of moisture added via steam, water, or inherent in feedstuffs [31].

The relationship between mash conditioning temperature and physical feed quality had been well investigated. Several reports had indicated a positive correlation between mash conditioning temperature and pellet durability [14, 27, 32-34]. Loar *et al.* [34] demonstrated that increasing mash conditioning temperatures from 74 to 85 and 96°C improved PDI from 73.3 to 82.7 and 93.8%, respectively. As mash conditioning temperatures increased, via increased steam flow rate into the conditioning chamber, pellet durability improved. The heat

and water associated with steam addition likely induced physicochemical changes that positively affected the binding capacities of starches and proteins [31]. The pressure of steam entering the conditioning chamber was also investigated, but the results were largely inconclusive with no differences in PDI or pellet quality being observed when steam was applied at 552 or 138 kPa [33].

The residence time of feed within the conditioning chamber was manipulated by conditioner paddle pitch, conditioner shaft rotation speed, and conditioner length [35]. Briggs *et al.* [15] conditioned feed with conditioner paddles in a standard or parallel pitch and demonstrated that parallel paddle pitch configurations improved conditioner retention time, which resulted in a 5 PDI point improvement when compared to standard paddle pitch configurations. Prolonged exposure of ingredients to heat and moisture likely further plasticized the ingredients via greater moisture penetration and heat transfer when compared to short term exposure to heat and moisture, which improved their binding characteristics and thus the physical quality of finished feed [36].

Since steam encompassed both heat and moisture, the impact of moisture must also be considered when determining the impact of steam conditioning on physical feed quality. Fairchild and Greer [37] demonstrated that increasing the moisture content of mixed feeds prior to pelleting improved pellet durability, with the research of Moritz *et al.* [38] drawing similar conclusions. Water may be added to rations independently of steam either at the mixer or in the conditioning chamber. Adding water to rations may also be associated with decreased pellet mill energy consumption (PMEC) [37] which may have facilitated an energy savings or improved throughput. While adding water to rations was not considered as a common practice, it had been desired when low unprocessed mash moistures did not

facilitate the full binding potential of ingredients. However, water added via steam was considered superior to conditioning with water alone since heat was also necessary to modify the physicochemical properties of nutrients to induce particle adhesion [31].

Particle Size

The particle size of ingredients was considered to be the third most influential factor in determining pellet quality [16]. Grains were typically ground prior to mixing and pelleting to improve mastication characteristics and increase the surface area available to digestive secretions [39]. Although particle size was assumed to have an impact on pellet quality, reports had been inconclusive as to exactly how particle size influenced binding characteristics and durability. Some reports indicated that a larger particle size was detrimental to pellet durability, such as when grain was ground through a 6-mm screen when compared to a 3-mm screen [40], while others concluded that relatively larger particles of grain had no impact on pellet durability [41] or even improved pellet durability [42]. Pope [27] demonstrated that pelleting diets with an average particle size of 472, 576, and 691 μm did not impact pellet durability when utilizing both the tumble box and NHPT methods. As reports concerning poultry live performance had indicated that providing dietary structure, such as through the inclusion of coarse particles of grain, was beneficial to gastrointestinal tract development and nutrient digestibility [39, 43], poultry feed manufactures had been encouraged to increase the particle size of ground ingredients. Though grain particle size, as it related to pellet quality, had long been a subject of debate for feed manufacturers, conclusive evidence that particles of grain negatively impacted pellet quality to the extent that broiler performance diminished did not exist.

Pellet Mill Die and Throughput

The pellet mill die was considered to be the fourth most influential factor in determining pellet quality [16]. Pellet mill throughput, which was invariably associated with pellet mill die specifications, was also determined to be a consideration when achieving improved physical feed quality was desired [12]. Pellet mill die specifications were typically described by the effective thickness of the die relative to the diameter of the pellet mill die hole. These two measurements were typically expressed in a ratio of pellet mill die hole length-to-diameter (L:D). The aforementioned pellet mill die specifications determined the duration of feed retention within the pellet mill die, and thus influenced pellet durability and physical feed quality.

As pellet mill die L:D increased, PDI and pellet quality increased [31]. Stark and Ferket [12] held the pellet mill die hole diameter constant at 4.4 mm and increased the effective thickness of the pellet mill die from 29 to 35 and 44 mm and demonstrated that pellet durability improved from 32 to 35 and 60%, respectively. This most likely occurred because the raw materials encountered a greater amount of friction and shear within the pellet mill die, increasing compaction and reducing elasticity, which likely made the feed more malleable and improved adhesion [44]. Additionally, the pellets were retained within the pellet mill die for a greater duration, which increased the backpressure of the die and reduced the porosity of the pellets upon exiting the pellet mill die [31]. Stark and Ferket [12] also reported that as throughput increased, pellet durability decreased. Holding the pellet mill die dimensions constant, the researchers pelleted diets at 500, 1000, and 1500 kg/hr and demonstrated that pellet durability decreased from 55 to 41 and 30%, respectively. This likely occurred due to a decreased pellet mill die residence duration. Therefore, pellet mill

die L:D and its relationship to pellet durability and quality should be considered relative to throughput since both of the specifications altered feed residence time within the pellet mill die. Pellet mill die L:D and throughput were some of the least commonly investigated factors known to impact physical feed quality.

Pellet Cooling

Pellet cooling was considered the least influential factor in determining pellet quality [16]. After exiting the pellet mill die, the moisture of pellets often exceeded 16% and the temperature of pellets often exceeded 80°C, which necessitated an additional process during pelleting to remove heat and moisture from pellets prior to feeding [45]. Pellet coolers operated by drawing ambient air through a bed of pellets. The ambient air cooled the pellets at the air inlet and the heated air then removed moisture from the pellets proximal to the pellet mill die. The adjustable operational parameters of coolers included air volume, velocity, and bed depth, or residence time within the cooler [31]. The improper drying and cooling of pellets may have resulted in poor pellet quality [45]. If pellets were cooled and dried with excessive air velocity, cracks at the surface of the pellet may have been induced and poor pellet quality may have ensued. Retaining pellets in a cooler for a longer duration would likely result in greater moisture and heat loss, but may not have been an option depending on pellet production rate [31].

ENZYMES

Enzymes had been defined as proteins that catalyzed the conversion of specific compounds, or enzyme substrates, into different compounds, or enzyme products [46]. Enzymes were neither consumed nor permanently altered through their participation in reactions. Enzymes had been classified based on the type of reaction they catalyzed, but the

feed industry primarily relied upon hydrolases, which catalyzed the hydrolytic cleavage of specific covalent bonds [46]. Enzyme products utilized in poultry feeds were defined as “a processed, standardized enzyme-containing material which has been produced with the intention of being sold for use in animal feed and feed ingredients” by the Association of American Feed Control Officials [47]. Since several entities were involved with the manufacture and distribution of enzyme products for use in poultry feeds, enzymatic activity was standardized and defined as “the catalytic activity required to convert a given amount of assay substrate to a given amount of product per unit time under the standard conditions set forth in the assay procedure” [47]. Enzyme assays, which were used to quantify enzymatic activity, were implemented in the feed industry to address whether adequate distribution of the enzyme product throughout feed was achieved and to determine whether it could withstand the rigors of pelleting to ensure that the product would catalyze substrate to provide the intended benefits associated with feeding the enzyme [48].

The efficacious use of enzymes required that the moisture content, temperature, pH, and enzyme and substrate concentration be optimized to realize the full potential of enzymes [49]. Enzymes required an aqueous environment to ensure free interaction with substrate and to promote catalytic activity. While candidate enzymes needed to withstand the rigors of feed pelleting, in which temperatures often exceeded 80°C [49], their activity needed to be optimized at the body temperature of the animal being fed, or ~41°C for broilers [50]. Enzymes were also sensitive to denaturation in acidic or alkaline environments, but needed to be stable in regions of the broiler gastrointestinal tract with large differences in pH, such as the crop and the proventriculus, which had a pH range of 6.0 to 2.0, respectively [43]. Lastly, it was imperative that the level of enzyme provided was properly paired with substrate

concentration so that the interaction between them was optimized without over supplying the enzyme, which was costly, or incompletely hydrolyzing the substrate, which may have had negative consequences for nutrient delivery and broiler health.

Types of Enzymes, Their Benefits, and Modes of Action

The classification of enzymes approved for use in poultry feeds in the United States that were commercially implemented included carbohydrases, proteases, and phosphatases, which catalyzed the hydrolysis of carbohydrates, proteins, and phytate, respectively [47]. The most commonly used enzymes had been phytases and non-starch polysaccharide degrading carbohydrases (NSPase) [51]. In general, enzymes had been utilized in poultry feeds to augment the digestion of nutrients for which the bird produced endogenous enzymes, to digest and release nutrients for which the bird did not produce enzymes, and to destroy or overcome anti-nutritional factors (ANF) that negatively affected the digestion or absorption of other nutrients [49].

The benefits of utilizing enzymes in poultry feeds were numerous. (1) Enzymes improved the feeding value of raw materials by improving their digestibility. This resulted in improved formulation flexibility, allowing for the inclusion of ingredients known to contain indigestible components at greater rates, and reduced the costs of feed formulation since nutrient constraints in feed formulation were partially met by the inclusion of an enzyme instead of a raw material [49, 51-54]. (2) Enzymes reduced the variability in nutrient content of ingredients by uplifting the nutrient values of batches of ingredients that would have otherwise been less digestible, thus improving the precision of rations [49, 52]. (3) Enzymes, specifically NSPases, reduced the incidence of wet litter in regions where the use of viscous grains was inevitable, which included wheat, rye, and barley [49, 52]. (4) Enzymes were

determined to shift the gut microflora towards microbial species associated with improved gut health [49]. (5) Enzymes reduced the environmental footprint of poultry production by capturing more value from raw materials and thus reduced their inclusion in rations and reduced the amount of total excreta and nitrogen and phosphorous excretion into the environment [49]. In integrated broiler production systems, the use of enzymes was rapidly implemented when raw material prices, especially corn and phosphates, increased which made the use of enzymes inevitable to remain competitive with regards to feed prices, and thus the overall profitability of broiler producers. Enzymes effectively improved the global accessibility to meat products through reduction in feed costs while making the broiler industry more sustainable through the use of less raw materials and reduced environmental outputs.

There were several considerations for predicting the impact of enzymes on digestion [55]. Since enzymes enhanced the digestibility of diets, rations formulated with highly digestible ingredients that were amended with enzymes may not have shown as great a response when enzymes were utilized when compared to rations formulated with poorly digested ingredients. Furthermore, in rations formulated to meet or exceed the nutritional requirement of an animal, the effect of the enzyme was less apparent. Zhou *et al.* [56] fed rations formulated to five levels of dietary energy with or without a blend of enzymes to broiler chicks. The researchers determined that the enzymes improved the energy digestibility in diets formulated to 11.55 and 11.8 MJ/kg, but not in diets formulated to greater energy densities. Therefore, it was deemed necessary to assign nutritional credits to enzymes so that their benefit could be realized as opposed to adding enzymes ‘on top’ of the

formulation, or when there was not otherwise a reduction in dietary energy attributed to the enzyme inclusion.

Enzymes also presented sharp diminishing returns [55]. As the concentration of the enzyme in the diet increased, or as the digestibility of the diet increased, the effectiveness of the enzyme per unit inclusion decreased [57]. Therefore, it was imperative that enzyme and substrate concentration be properly matched to ensure that the enzyme was able to deliver the nutrients for which it was credited in formulation programs [58]. Ravindran [49] also commented that enzymes were most efficacious in young chicks in which physical limitations to feed intake and immature endogenous enzyme systems afforded the greatest opportunity for enzymes to enhance digestibility.

Phytase

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate; IP₆) was the primary form of phosphorous storage in plants and contained six phosphate molecules [59]. The average poultry diet contained 10 g/kg of IP₆ [60], most of which was indigestible with innate enzyme secretions. Phytase was the enzyme necessary to cleave phosphates from IP₆ [61], and despite some endogenous phosphatase secretions [55], phytase presented nutritionists with an opportunity to improve the utilization of phosphorous within feedstuffs as opposed to relying completely on mined rock phosphates to meet the phosphorous requirements of broilers [59]. Utilizing phytase enzymes may have replaced between 5 and 7 kg of inorganic phosphates per tonne of feed, which represented a significant cost saving for feed manufacturers [55]. Additionally, phytase reduced environmental phosphorous excretion and became a mandatory feed additive in environmentally sensitive regions with estuaries such as the Netherlands and the Chesapeake Bay region of the United States [59]. The use of

phytase was first reported in the 1960s, although the commercial implementation of phytase occurred in the 1990s to mitigate environmental phosphorous pollution [59].

In addition to containing indigestible phosphorous, phytic acid was also considered an ANF [48, 55, 59] due to its polyanionic characteristics which allowed it to chelate positively charged molecules. Phytic acid was demonstrated to reduce the absorption of nutrients other than phosphorous, such as the divalent cations calcium, magnesium, and zinc, in addition to starch and amino acids. Phytic acid chelated these nutrients, making them unavailable to the broiler and thus requiring over-formulated rations to meet the nutrient requirements of the broiler. Thus, phytase manufacturers recommended the assignment of matrix credits for energy, protein, and certain trace minerals, in addition to phosphorous, to capture the full value of phytase. These matrix credits for nutrients other than phosphorous, or the benefits associated with feeding phytase unrelated to the release of phosphorous, were termed the ‘extra-phosphoric’ effects related to destroying the ANF component of phytate [55]. Additionally, phytase may have provided a nutrient sparing effect associated with endogenous losses of mucins, intestinal cells, and pancreatic secretions [55]. Phytase offered an economical solution to reduce the levels of phosphate, energy, and amino acids in rations that would have otherwise been supplied by raw materials.

Phytases were expressed in several source organisms, including *Aspergillus niger*, *Pentophora lycii*, *Schizosaccharomyces pombe* and *Escherichia coli* [48]. There were several classifications of catalytic mechanisms for phytases, but the majority of phytases belonged to a family of histidine acid phosphatases [48]. One of the primary classifications of commercially available phytases was based on their preference for the position of the first phosphate moiety to be released. The 3-phytase family of phytases, mainly derived from

Aspergillus sp., cleaved the phosphate moiety at the 3-carbon position of the IP₆ ring and functioned optimally at pH 3 [55]. The 6-phytase family of phytases, derived from *E. coli* and *P. lycii*, cleaved the phosphate moiety at the 6-carbon position of the IP₆ ring and functioned most effectively between pH 4-5 [55]. Phytases cleaved phosphate moieties in a stepwise manner from IP₆ to its lower esters. Most of the phytase activity *in vivo* was characterized in the anterior portions of the poultry gastrointestinal tract, which included the crop, proventriculus, and gizzard, due to the favorable pH and lack of endogenous proteolytic enzymes in the small intestines [62].

The *in vitro* mechanisms of phytase were studied extensively and were considered to be well characterized. However, the *in vivo* mechanisms were not as well defined and Bedford *et al.* [63] noted that all the consequences of phytase application had yet to be identified. Despite this incomplete knowledge of phytase action *in vivo*, several reports identified the benefits associated with feeding phytase.

Applegate *et al.* [64] fed turkeys four levels of non-phytate phosphorous (NPP) from 0.47 to 0.79% by incrementally increasing the monocalcium phosphate concentration in the ration. Additionally, the negative control diet (0.47% NPP) was amended with four levels of phytase (250, 500, 750, 1000 units/kg) to determine whether phytase could spare dietary phosphorous. Since phosphorous was heavily incorporated into bones, the researchers utilized a tibia bone ash assay to determine the utilization of dietary phosphorous. As the NPP and phytase inclusion increased, the tibia ash increased. The tibia ash data collected from incrementally increased levels of inorganic phosphate inclusion was paired with tibia ash data from phytase inclusion to quantify the phosphorous sparing effect of phytase. The researchers reported that feeding 250, 500, 750, and 1000 phytase units/kg spared 0.170,

0.220, 0.236, and 0.244% NPP, respectively. These data, as well as other reports [62], demonstrated the phosphorous sparing effect of phytase attributed to phosphate release from phytate through *in vivo* assays.

Cowieson *et al.* [65] conducted a systematic review of publications between 1996 and 2016 that investigated the impact of phytase on amino acid digestibility in broilers. The meta-analysis included 24 reports that represented a total of 745 individual observations for amino acid digestibility that spanned several formulation conditions, which included the use of corn, soy, wheat, corn DDGS, sorghum, and rice bran. The researchers determined the amino acid digestibility with and without phytase, which included six commercially available phytases, to determine the average increase in amino acid digestibility associated with feeding phytase. The report indicated that amino acid digestibility increased, on average, by 4.1% with values ranging from a 1.3% increase in methionine digestibility to a 7.2% increase for cysteine. A negative correlation between feed phytate concentration and amino acid digestibility was determined to exist for control diets that did not receive phytase. Therefore, the improved amino acid digestibility associated with feeding phytase was related to the disappearance of phytate as an ANF. These data supported the ‘extra-phosphoric’ effect for amino acid digestibility associated with phytate destruction as evidenced from feeding phytase.

Ravindran *et al.* [66] investigated the impact of phytase on apparent metabolizable energy (AME) in broilers. The control diet was considered adequate in NPP and phytase was added at 125, 250, 375, 500, 750, and 1000 units/kg. There was a significant positive correlation between AME and phytase inclusion up to 750 units/kg. While the energy sparing effects of phytase were not well elucidated, the authors attributed the increase in energy

digestibility to sparing amino acids, which contributed to dietary energy, in addition to improved starch digestibility since phytate was determined to chelate starch through direct phosphate bonds or indirectly through chelating the proteins associated with starch [66]. Additionally, phytate was described as a potent inhibitor of amylase which may have also negatively impacted starch digestibility [66]. Another theory for the effect of phytase on energy digestibility was related to the chelation of minerals by phytate [67]. Lipid utilization was determined to be constrained through the formation of insoluble phytate-metallic soaps, which reduced energy digestibility in broilers [67]. Since phytase was a known chelator of minerals [59], Ravindran *et al.* [67] proposed that phytase may have reduced the degree of soap formation in the gastrointestinal tract, and thus improved the energy digestibility of rations. The ‘extra-phosphoric’ effect of feeding phytase was determined to extend beyond improved amino acid digestibility to include energy digestibility as well, both of which were associated with the reduction of phytate as an ANF. The benefits of feeding phytase with respect to nutrient digestibility eventuated in improved body weight (BW) gain and feed conversion in broilers as evidenced through other reports [17, 68].

Carbohydrase

The primary NSPases utilized in broiler rations have been xylanase and β -glucanase which hydrolyzed arabinoxylans and β -glucans contained in fibrous feedstuffs, respectively [47, 69]. Several other carbohydrases had also been utilized, such as hemicellulase, alpha-galactosidase, and amylase, but often to a lesser extent [69]. The primary role of carbohydrases was to hydrolyze fibers that were not hydrolyzed through endogenous gastric enzyme secretions, such as the ANF non-starch polysaccharides (NSP) [70]. The primary concern with NSPs was their soluble fiber content, which hydrated upon exposure to water,

and the resulting viscous nature of the digesta when feeding grains such as barley, rye, and wheat [52].

The benefits associated with feeding carbohydrases, specifically NSPases, were increased feeding value of raw materials, reduced variation in nutrient quality, and reduced incidence of wet litter [52]. The observed benefits associated with feeding NSPases were realized through increased rates of digestion, increased feed passage rates, augmented endogenous secretions, and controlled microbial populations [52, 63, 69-71]. The soluble arabinoxylans and β -glucans were identified as the ANF components of fiber through isolation and re-feeding trials, which resulted in the same complications associated with feeding the intact grains [72, 73].

The primary mechanism by which NSPs exerted their anti-nutritional effects was through reduced rates of digestion [52]. The NSPs solubilized to form a viscous gel in the digesta, which impeded the diffusion of enzymes and substrate throughout the aqueous digesta and effectively reduced digestion rates [48, 74]. The high molecular weight carbohydrate (HMC) fraction of feeds was determined to be the constituent that impeded the free movement of endogenous enzymes and substrate [75]. A dose response study, which utilized rye concentrations from 0 to 60% and xylanase concentrations from 0 to 1.6%, was conducted to investigate the impact of gut viscosity on broiler performance. The authors determined that a positive correlation existed between HMC concentration and both foregut and hindgut digesta viscosity. Also, the authors reported a linear decrease in weight gain and feed conversion efficiency as foregut viscosity increased. Therefore, it was determined that the slowed digestion rates due to the gelling and entanglement associated with feeding soluble fibers was responsible for the negative impact of soluble fibers on live performance

parameters, rather than a specific action exerted by the anti-nutritional polymers associated with fiber.

Feed passage rate was determined to be reduced when birds were fed rations that contained viscous grains [76]. When gut passage rate was reduced there was a significant decrease in feed intake which negatively affected and limited nutrient assimilation [71]. Additionally, when gut passage rate was reduced the flushing effect of digesta moving down the gastrointestinal tract was reduced which allowed bacteria to migrate towards anterior portions of the gastrointestinal tract. Some of these bacteria produced bile acid degrading enzymes [71, 77], which negatively impacted lipid digestion and ultimately led to fat soluble vitamin deficiencies [71]. Enzyme supplementation, as well as antibiotic supplementation, alleviated the poor AME values and rachitogenic effects associated with feeding viscous grains which indicated that the hydrolysis of NSPs improved the flushing effect of feed passage and thus reduced microbial loads in the anterior portions of the gastrointestinal tract [69].

Bedford [78] postulated that the effects of NSPases on microbial populations may be of more interest than the effects exerted directly on the cell wall of grains. While *in vitro* data confirmed that NSPases would open cell walls to allow endogenous enzymes better access to nutrients, it took up to 48 hrs to degrade cell walls. Thus, Bedford [78] proposed that NSPases released prebiotic xylo-oligosaccharides, which were fermented by bacterial species to produce volatile fatty acids which were then utilized as a source of energy by enterocytes and as a bacterial control mechanism to suppress the growth of pathogenic organisms. Regardless of the NSPase mode of action, their effects on AME and amino acid digestibility had been investigated.

Kiarie *et al.* [79] fed broilers wheat or corn based rations that were devoid of or supplemented with xylanase. At 21 d, birds were harvested to determine the impact of xylanase on fat, crude protein, and gross energy digestibility. The researchers reported that fat, crude protein, and gross energy digestibility of the diets was improved through xylanase supplementation, regardless of the source of grain. Additionally, birds supplemented with xylanase were reported to exhibit greater concentrations of acetic acid in the ceca in addition to reduced jejunal digesta viscosity, regardless of the grain type. Bedford and Campbell [80] fed broilers rations containing barley with or without β -glucanase and measured the apparent ileal digestibility of amino acids at three and six weeks of age. The researchers reported that amino acid digestibility was improved by a minimum of 10% at three weeks of age and while amino acid digestibility was still improved at six weeks of age, it was improved to a lesser extent. These data exemplified the ability of NSPases to improve endogenous enzyme access to nutrients, which improved the overall digestibility of rations that contained NSPs.

Protease

Of the enzymes utilized in commercial broiler diets, proteases were evaluated the least [48]. Proteases existed primarily to augment the digestive capacities of broilers, but also improved dietary protein digestibility in some instances, reduced endogenous protein flow, and proved beneficial when ANFs associated with protein products were present, such as trypsin inhibitors and lectin [48]. However, the mechanism of action for proteases remained unclear. Regardless of the mode of action, proteases were determined to improve litter conditions, the hind gut bacterial environment, and the sustainability of poultry production [81].

Several researchers reported improved live performance parameters when a protease was fed [82-84]. The clearest benefit from feeding protease was an observed improvement in FCR, which indicated that the protease was sparing or extracting nutrients beyond the endogenous capacities of the broilers. Peek *et al.* [84] reported that broilers challenged with *Eimeria* spp. benefited from dietary protease supplementation as apparent through improved BW gain, although there were no differences in coccidial lesions or oocyst shedding. Additionally, reports that demonstrated the beneficial effects of feeding protease on FCR or BW gain failed to elucidate the exact mechanism of nutrient sparing. None of the reports indicated improved protein or energy digestibility [82, 84]. However, the inability to determine the cause of improved FCR may have been related to overall amino acid digestibility.

In a meta-analysis, Cowieson and Roos [85] reviewed reports to determine the effect of protease on apparent ileal amino acid digestibility in poultry and swine diets. The meta-analysis included 25 independent experiments and a total of 804 individual data points. It was determined that protease supplementation improved amino acid digestibility by 3.74%. However, the response was primarily based on control diet digestibility. When the inherent digestibility of control diets was less than 70%, protease supplementation improved amino acid digestibility by 10%. If the amino acid digestibility of the control diet was determined to be greater than 90%, which was more likely for commercial formulations in the United States, the protease only improved digestibility by 2%. Thus, it was concluded that the inherent amino acid digestibility of a ration was the main determinant for protease efficacy. Protease remained to be the least well understood commercially implemented enzyme and warranted further investigation into the direct benefits associated with its feeding [48].

FEED PELLETING AND ENZYME THERMOSTABILITY

One of the primary concerns for enzyme use was their ability to withstand the rigors of feed pelleting. Proteins, especially those that were to remain functional after feed processing, were subjected to denaturation upon exposure to the heat, moisture, and pressure associated with the conditioning and pressing of feeds [24]. Feed enzyme efficacy was typically determined through *in vivo* assessments of live performance parameters in addition to enzyme specific parameters such as tibia/toe bone ash for phytase or digesta viscosity for NSPases. Additionally, *in vitro* assays were routinely performed to quantify enzyme concentrations in feed to ensure that the enzyme survived pelleting.

Enzyme Thermostability Issues

Spring *et al.* [32] pelleted barley-wheat-soy rations which contained cellulase, pentosanase (xylanase), bacterial amylase, and fungal amylase at 60, 70, 80, 90, and 100°C through an experimental pellet mill with a pellet diameter of 2.5 mm. The authors reported that all the enzymes were thermostable to 80°C and the bacterial amylase remained thermostable to 90°C. The authors also determined that increased pelleting temperatures resulted in greater feed viscosity, although digesta viscosity was not directly measured.

In a similar trial, Inbarr and Bedford [86] pelleted barley-based diets with or without β -glucanase. The diets were conditioned for either 30 s or 15 min at 75, 85, or 95°C. The authors reported that 30 s conditioning at 75, 85, and 95°C resulted in a 66, 56, and 16% recovery of β -glucanase, respectively. When mash was conditioned for 15 min at the same temperatures, the recoveries were 49, 31, and 11%, respectively. As β -glucanase activity decreased, digesta viscosity increased in a linear manner and a decrease in live performance

parameters ensued. However, the temperature of pelleting was determined to be more influential on the denaturation of β -glucanase when compared to conditioning time.

Carbohydrases were not the only enzymes that exhibited thermostability issues. Wilkinson *et al.* [87] pelleted diets containing two different types of phytase, which exhibited different thermostabilities, at 75, 80, 85, or 90°C. For both enzymes, a negative linear effect of pelleting temperature on phytase denaturation was observed as evidenced through *in vitro* testing in feed. However, tibia bone ash was not affected by pelleting temperature. This may have indicated that *in vitro* testing of phytase activity was not directly related to *in vivo* phytase action. Conversely, *in vitro* feed assays may be pertinent to predicting phytase activity *in vivo*, but the authors may not have reduced the dietary NPP levels to an extent that broilers would have benefited from greater concentrations of phytase in finished feed.

Loop *et al.* [68] tested nine variants of *E. coli* derived phytases for thermostability and subsequent broiler performance and tibia bone ash and breaking strength to 21 d of age. The diets were conditioned for 10 s at 71, 77, and 82°C prior to pelleting through a 38 x 4.4 mm pellet mill die. Although the variant manipulations were not described in the report and the feed manufacturing was not replicated, all the phytases were recovered in excess of 60% after pelleting, with the majority of the variants being recovered in excess of 80%. Despite the consistent recoveries, tibia bone ash and breaking strength varied between phytase variants, which indicated that phytase activity within the gastrointestinal tract was dependent upon more factors than thermostability during pelleting, such as pH and resistance to proteolysis. Therefore, *in vitro* feed assays should be used judiciously, although depletion of the enzyme in excess of that reported herein would likely impact phosphorous utilization for broilers.

Feed manufacturers had continuously increased their conditioning temperatures for various reasons, and often operated their conditioners at temperatures known to denature enzymes [51]. Increased pelleting temperatures were associated with improved feed hygiene, as means to control *Salmonella* and *Campylobacter*, in addition to improved starch digestibility and physical feed properties, such as decreased fines and improved pellet hardness and durability [51]. However, increased conditioning temperatures were associated with decreased enzyme recoveries, which was problematic for manufacturers that relied upon enzymes to deliver nutrients to broilers.

Enzyme Deactivation and Resolutions

Enzyme denaturation was typically investigated through analytical techniques that determined both changes in physical properties as well as enzyme-substrate kinetics to determine residual enzyme activity subsequent to chemical or thermal denaturation [88-90]. Fluorescence and circular dichroism (CD) spectroscopy were common methods utilized to detect conformational changes in enzyme structure [89]. Enzyme activity was frequently quantified through the incubation of enzymes with substrate in specified buffers at a specified pH, temperature, and duration, and then substrate hydrolysis was quantified through absorbance at a wavelength specific for the enzyme product [89]. Experiments conducted utilizing such techniques provided insight into the unfolding of enzymes and the resulting activities after denaturation.

Wyss *et al.* [89] studied the denaturation and activity characteristics of three phytases derived from *Aspergillus* spp; *A. fumigatus*, *A. niger*, and *A. niger* pH 2.5 acid phosphatase. The experiment was conducted by exposing the enzyme to temperatures of 30, 50, 70, 80, and 90°C in a buffered solution to observe conformational changes utilizing the analytical

methods previously mentioned. Additionally, the enzymes were exposed to temperatures of 37, 40, 45, 50, 55, 60, 65, 70, 80, and 90°C in a buffered solution with phytic acid for 15 mins and then the reactions were stopped utilizing trichloroacetic acid before quantification of liberated phosphate molecules utilizing a spectrophotometer. The enzymes were also pelleted after conditioning at 75 and 85°C to determine their ability to withstand pelleting.

The authors detected conformational changes in the arrangement of α -helices and β -sheets of phytases derived from *A. fumigatus* and *A. niger* via CD between 50 and 70°C, while the *A. niger* pH 2.5 acid phosphatase was not structurally altered until 80°C, which indicated a greater thermostability. Upon exposure to lower temperatures, the *A. fumigatus* and *A. niger* phytases were able to refold into their native conformation, both of which were monomeric proteins. However, upon deactivation of the phytase derived from *A. niger* pH 2.5 acid phosphatase above 80°C, the phytase was not able to refold into its native confirmation. The *A. niger* pH 2.5 acid phosphatase was an oligomeric protein and the authors suggested that upon conformational change the tetramer was not able to reorganize into its native state. The *A. niger* pH 2.5 acid phosphatase was considered to be more thermostable based on *in vitro* analysis, although it was not able to refold upon denaturation.

When the enzymes were subjected to feed pelleting, different conclusions were drawn as to the thermostability of the different phytases. At 85°C, the most stable enzyme in the laboratory setting, *A. niger* pH 2.5 acid phosphatase, was reported to contain the least residual phytase activity once pelleted. The authors suggested that the poor retained activity when compared to *A. fumigatus* and *A. niger* was due to its inability to refold and remain functional after exposure to heat. Other reports also suggested that conformational changes may have occurred in enzymes without negatively impacting the active site of enzymes or

their ability to reassemble to their active structures [88, 90]. One pertinent stressor that enzymes experienced during feed pelleting that was often withheld from laboratory testing was pressure. Many reports on enzyme thermostability via *in vitro* analytics and conformational changes stressed enzymes to heat, but rarely to pressure, which may also exacerbate enzyme denaturation [31].

Enzyme thermostability issues were resolved through various mechanisms. These mechanisms included (1) screening for more thermotolerant organisms to express phytase, (2) genetic manipulation of organisms expressing phytase via site-directed mutagenesis of specific amino acid sequences to enhance thermostability, (3) coating enzymes to protect them from heat, and (4) applying the enzymes to pellets subsequent to pellet manufacture [48, 51, 91]. Screening for thermotolerant organisms to express feed enzymes was the most common method to improve enzyme thermostability before genetic manipulation techniques were refined [48, 92]. The challenge with screening for new organisms to express feed enzymes was typically related to their output of the desired enzyme on a commercial scale in quantities desirable for inclusion in animal feeds [48]. Genetic mutation of organisms expressing the desired enzymes was performed via gene site saturation mutagenesis, a high throughput method, that rapidly detected regions related to enzymatic performance [93]. When coupled with a heat step, gene site saturation mutagenesis was considered an efficient way to improve enzyme performance while maintaining or improving thermostability. Coating enzymes, most often with a peptidoglycan or carbohydrate-lipid complex, was believed to be an alternative to screening and genetic manipulation to improve thermostability [48, 68]. While many patents for enzyme coatings existed, few peer-reviewed publications indicated whether coatings were suitable for enzyme protection while retaining

the ability to solubilize to interact with substrate within the gastrointestinal tract [48]. Slominski [61] reported no substantial differences in phytase recoveries when granulated and powdered forms were compared in commercial testing, which indicated that more research was necessary to determine the value of coating technologies. The PPLA of enzymes was the simplest method to prevent enzyme denaturation, but the equipment necessary to apply enzymes via PPLA and the inaccuracies encountered when metering minute volumes of liquid made mixer-added enzymes more manageable for the manufacture of commercial feeds [51, 68]. Regardless of the methods utilized to improve enzyme thermostability, it was necessary to advance *in vitro* discoveries into practically applied enzymes that could withstand the rigors of pelleting and solubilize in the gastrointestinal tract to perform its intended hydrolysis.

HEAT, PRESSURE, AND ENZYMES DURING FEED PELLETING

Considerations that were sparsely investigated in previous research that pertained to enzyme denaturation during feed pelleting were the factors that affected frictional heat transfer between pellets and conditioned mash and pressure within the pellet mill die. The majority of the literature that described enzyme denaturation during feed pelleting focused solely on the temperature at which mash feed was conditioned [32, 68, 86, 87]. Previous research also failed to separate feed pelleting into two separate processes, conditioning and pressing, by failing to collect samples after feed was conditioned but before pressing and pellet formation. The researchers were warranted in their justification to focus solely on mash conditioning temperature; it was understood to directly influence enzyme denaturation and was a focus for feed mills to improve physical feed quality. However, feed manufacturers

and nutritionists manipulated and monitored several processes during feed pelleting that may have influenced the exposure of a mixer-added enzyme to heat and pressure.

Pope *et al.* [20] demonstrated one of these effects when rations were pelleted with 0.5 or 4.0% mixer-added fat (MAF). The authors measured frictional heat transfer as feed transitioned from the conditioning chamber through the pellet mill die (ΔT) as well as the amount of energy required to pellet a given mass of feed (PMEC) in addition to PDI. The researchers determined that diets pelleted with 0.5% MAF resulted in a 74% increase in ΔT from 5.83 to 10.17°C and a 42% increase in PMEC from 8.66 to 12.34 kWh/T when compared to diets pelleted with 4.0% MAF, which was an indicator of pellet mill die backpressure [31]. The increased level of fat acted as a lubricant which decreased friction and backpressure within the pellet mill die [24], which resulted in decreased transfer of heat, energy required to press the feed, and PDI.

Gehring *et al.* [17] pelleted diets containing phytase, carbohydrase, and protease that contained either 1, 2.5, or 4.0% MAF at 82 or 85°C and fed broilers from 11 to 38 d of age before determining carcass parts yields. The authors concluded that broilers fed rations pelleted with 4.0% MAF at 85°C converted the most nutrients to salable carcass based on carcass yield data and they suggested that the increased MAF concentration in the 4.0% MAF rations improved exogenous enzyme recovery, although they did not measure the recovery of enzymes before or after pelleting nor did they perform *in vivo* analysis of parameters known to correlate with enzyme concentration or recovery, such as tibia ash or digesta viscosity. Although greater concentrations of fat were determined to decrease frictional heat and pressure within the pellet mill die as suggested by Pope *et al.* [20], the

report of Gehring *et al.* [17] failed to determine whether frictional heat or pressure was correlated with enzyme recovery.

The level of MAF was not the only factor known to impact frictional heat, pressure, and pellet durability and quality during the manufacture of pellets. The pellet mill die was also considered to play a role in frictional heat and pressure imposed on feed during the manufacture of pellets [31]. As the L:D of a pellet mill die increased, the feed would be expected to be exposed to more shear pressure, and thus greater amounts of frictional heat may have been generated. Additionally, as the die hole wall lengthened, the surface area for feed contact increased which resulted in greater friction and pressure within the die. The lengthened contact time between feed and the pellet mill die hole wall relaxed elastic components of feed, which influenced physical feed quality in a positive manner, but was not investigated with respect to nutritional feed quality. Feed was retained within the pellet mill die for various durations based on the length of the die, in addition to the pellet mill die throughput. *In vitro* experiments determined that prolonged exposure to steam negatively impacted enzyme activity [86], but changes in formulation, pellet mill die specifications, and pellet mill throughputs that influenced the extent and duration of heat exposure were not investigated in previous work. To be efficient with raw materials, it was imperative that nutritionists and feed manufactures better understood the impact of feed pelleting on enzyme denaturation so that value extraction from raw materials was optimized without sacrificing animal wellbeing. Thus, the objectives of the body of work herein were to determine:

1. The impact of non-conditioning factors during pelleting on enzyme thermostability.
2. The impact of the conditioning chamber and pellet mill die on enzyme thermostability, independently.

3. The correlation between parameters monitored during feed pelleting and enzyme thermostability.
4. The relationship between practices implemented to improve physical feed quality and its impact on nutritional feed quality, with exogenous mixer-added enzymes being the marker of nutritional status.

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CHAPTER I

The effect of the level of mixer-added fat, fat source, and pellet mill die L:D on parameters monitored during pelleting and xylanase thermostability

SUMMARY

Exogenous enzymes have been regularly used in broiler diets to improve the value of raw ingredients by enhancing their digestibility. Broiler feed has also been most commonly pelleted, which has exposed exogenous enzymes to great amounts of pressure and heat. These harsh conditions have often resulted in exogenous enzyme destruction and inactivation. The present experiments were intended to define some of the variables involved in feed formulation and manufacturing that may impact exogenous enzyme stability during thermal processing utilizing a xylanase feed enzyme for the experimental model. Additionally, parameters typically monitored during feed manufacturing were also observed so that their relationship to exogenous enzyme stability throughout thermal processing would also be characterized. It was determined that the concentration of mixer-added fat (MAF), source of MAF, and pellet mill die length-to-diameter ratio impacted xylanase activity in pellets relative to conditioned mash. Additionally, parameters typically monitored during feed manufacturing were robust predictors of xylanase activity in pellets relative to unconditioned and conditioned mash. When the controlled factors and monitored parameters of the experiments were combined in a predictive model, xylanase recovery was very accurately predicted. In conclusion, practices utilized in feed formulation and manufacturing impacted the stability of the xylanase enzyme used in the current experiments, and such impacts may be predicted through monitoring parameters of feed manufacturing.

DESCRIPTION OF PROBLEM

Exogenous enzymes have been regularly used in broiler diets, with phytases and non-starch polysaccharide degrading enzymes being the most common [1]. The overarching reason for feed additive enzyme use has been to increase the feeding value of raw materials

through reduced variability in nutrient contents of seemingly similar ingredients. Exogenous enzyme use has resulted in more consistent performance within flocks as well as from flock to flock [2]. Despite an incomplete knowledge of all the factors affecting exogenous enzyme efficacy, their value as additives that reduce feed formulation costs and improve animal performance has been reasonably well established [3, 4, 5].

While the use of exogenous enzymes has become more common, the predilection to produce more “sterile feed” to control pathogens [6, 7] and to improve pellet quality [8, 9, 10] has also become common practice. This has been achieved by increasing mash-conditioning time, through the use of double pass conditioners and hygenizers, in addition to increasing mash conditioning temperatures. While these practices have successfully mitigated the direct transfer of pathogens from raw ingredients and improved pellet quality, they have also contributed to the loss of available nutrients to the bird and increased intestinal digesta viscosity, both of which have negatively impacted overall animal performance and potentially increased the amount of nutrients available for pathogens in the lower gastrointestinal tract [11, 12, 13, 14]. Additionally, these practices have negatively impacted exogenous enzyme activity, as demonstrated by *in vitro* assay [3,15]. However, despite a severe loss of enzyme activity as determined by *in vitro* assay at high pelleting temperatures, enzymes were still found to be effective *in vivo* as evidenced by improved broiler live performance [16].

Exogenous enzymes, being functional proteins, have been reported to be subject to denaturation and loss of function during thermal processing. Their denaturation eventuated from exposure to pressure and heat in the humid environment present during pelleting [17]. During the pelleting process, three levels of control have existed that determined the overall

exposure of enzymes to harsh pelleting conditions. The levels of control have included the mash-conditioning chamber, the pellet mill die, and the pellet cooler [18]. Loss of enzyme activity in the conditioning chamber, frequently addressed in prior publications, was primarily a function of conditioning temperature and time, with an increase of either negatively affecting enzyme activity post-pelleting [15]. Pellet cooling most likely affected enzyme activity minimally if all measures to reduce pellet temperature and moisture were implemented properly. The pellet mill die, location of the greatest pressure and temperature within the pelleting system, has been the subject of little evaluation with respect to enzyme activity post-pelleting in preceding research.

The interaction between feed formulation and the pellet mill die has been characterized by various parameters, which have included change in temperature between hot pellets and conditioned mash (ΔT), pellet durability index (PDI), and pellet mill energy consumption (PMEC). Some of the factors that have affected the aforementioned pelleting parameters included, but were not limited to, percentage mixer-added fat (MAF), pellet mill die length-to-diameter (L:D) ratio, and the temperature of feed entering the pellet mill [17,19]. It was suggested that diets containing greater concentrations of MAF might exhibit improved exogenous enzyme stability during thermal processing based on the results of an *in vivo* assay [20]. This may have been achieved through reduced pressure within the pellet mill die during pelleting that would also have reduced the frictional heat transferred into the feed as it passed through the pellet mill die [17].

It was proposed that grain type, mash conditioning time and temperature, moisture, and age of birds should be considered when attempting to model the broiler response to feeding an enzyme [1]. The current authors would also suggest that other factors, such as

feed formulation and pellet mill die specification, may also play an important role in exogenous enzyme stability during thermal processing, thus affecting the efficacy of the enzyme *in vivo*. In the present experiments, factors were manipulated to generate a range in the severity of heat and pressure that an exogenous enzyme would experience at the pellet mill die during thermal processing. These factors included varying concentrations of MAF, MAF sources with varying degrees of saturation, and pellet mill die configurations that differed in L:D. Additionally, these factors were intended to establish a range of values for PDI, ΔT , and PMEC, which would be used to evaluate their relationship to enzyme activity post-pellet. While it has been suggested that the most appropriate way to determine the effect of a feed manufacturing factor on exogenous enzyme stability was through an *in vivo* assay, the goal of this research was to determine how these factors may affect the loss of exogenous enzyme activity in a pelleted diet relative to the conditioned and unconditioned mash utilizing an *in vitro* assay [20, 21].

MATERIALS AND METHODS

Experiment 1

The experiment was conducted by pelleting six 227 kg batches of broiler grower diets (Table I-1) with or without xylanase that differed in percentage MAF. Individual batches were sourced from a common basal to reduce variation in ingredient composition between batches. The experiment was a 3 x 2 factorial arrangement utilizing three percentages of MAF and two xylanase inclusions. The MAF was included at 1, 3, or 5% and the xylanase was included at 0.01% or withheld to serve as a control.

All feed was manufactured following the guidelines for Current Good Manufacturing Practice (CGMP). All corn was ground utilizing a hammermill [22] equipped with a 2.4 mm

screen on the impact side and a 3.2 mm screen on the release side. The basal diet was batched utilizing a twin shaft counterpoise ribbon mixer [23] for 240 s of dry mixing time. The basal was devoid of fat and xylanase. After batching the basal, individual 227 kg batches were blended in a smaller horizontal double ribbon mixer [24] where the basal was amended with MAF and xylanase as appropriate. The individual batches were blended for 120 s of dry mixing time, followed by an additional 90 s of wet mixing time. For diets containing less than 5% MAF, the diets received corn to replace fat so that the xylanase concentration would remain constant when added. For diets receiving xylanase, the enzyme was mixed with 908 g of the basal diet in a tabletop mixer [25] for 180 s of mixing time to dilute the enzyme before adding it to the 227 kg batch. Upon discharge, 5 mash samples of each individual batch were collected.

All diets were conditioned at 82°C for 30 s in a single pass conditioner [26] and then pelleted utilizing a 30 HP CPM pellet mill [27] equipped with a 4.4 x 35.2 mm die with 548 cm² of working surface area at a production rate of 908 kg/hr. The steam pressure was 207 kPa. The pellet mill die was warmed with 455 kg of feed before proceeding with the experimental batches. The individual batches were pelleted from the greatest to the least percentage MAF, pelleting the control batch first, and then followed by the batch amended with xylanase. After pelleting a batch containing xylanase, and before transitioning to a lower percentage MAF, the mixer and pellet mill were flushed with 181 kg of feed.

Data and sample collection at the pellet mill included production rates, conditioned mash and hot pellet temperatures for determination of ΔT , conditioned mash samples to determine xylanase activity, pellet samples to determine xylanase activity and PDI, and PMEC data. Data collection started approximately 3 min after pellet mill motor load was

detected, which included a conditioned mash and pellet sample in 1 min intervals over a 9-min period of pelleting. Production rates and hot pellet samples were also collected. Pellets were cooled in custom manufactured 30 x 30 cm trays, which were placed in a custom manufactured pellet cooler resembling a vertical counterflow cooler. Conditioned mash samples were also collected in custom manufactured 30 x 30 cm trays and cooled over open grates with circulating air.

Pellet durability was determined by placing 100 g of pellets, screened with a #5 US sieve, into a pellet durability tester [28] for 30 s. Pellets were removed directly from the testing chamber and weighed to represent a proportion of the initial mass added to the testing chamber, with each sample being tested in duplicate. Three of the nine pellet samples collected were selected for durability analysis, with the three selected samples coming from the time intervals when the conditioning temperature was closest to 82°C. The PMEC data were collected by the feed mill automation system server [29] as a continuous input variable at the programmable logic controller.

Experiment 2

The experiment was conducted by pelleting six 227 kg batches of broiler grower diets (Table I-2) with or without xylanase that differed in the source of MAF. Experimental procedures were as in Experiment 1, except as described below. The experiment was a 3 x 2 factorial arrangement utilizing three sources of MAF and two xylanase inclusions. The sources of MAF ranged in degree of saturation and included coconut oil, poultry fat, and canola oil, which were 92, 31, and 7% saturated, respectively. The individual batches were pelleted from the greatest to the least degree of saturation for MAF source, pelleting the control batch first, and then followed by the batch amended with xylanase. After pelleting a

batch with xylanase, and before transitioning to a different MAF source, the mixer and pellet mill were flushed with 181 kg of feed.

Experiment 3

The experiment was conducted by pelleting four 227 kg batches of a broiler grower diet (Table I-3) with or without xylanase through pellet mill dies differing in L:D. Experimental procedures were as in Experiment 1, except as described below. The experiment was a 2 x 2 factorial arrangement utilizing two pellet mill die configurations and two xylanase inclusions. The L:D of the pellet mill dies employed in this experiment included 8:1 and 10:1 with dimensions of 4.4 x 35.2 mm and 4.4 x 44 mm, respectively. The working surface area of each die was 548 cm². Before pelleting feed through each individual pellet mill die, the dies were warmed to 82°C with 454 kg of feed. After warming the pellet mill die, 227 kg of control feed was pelleted, followed by 227 kg of feed amended with xylanase. The pellet mill die with an L:D of 8:1 was employed first, followed by the pellet mill die with an L:D of 10:1.

Xylanase Activity Assay

Xylanase activity was determined via proprietary methods developed by the xylanase supplier that utilized a colorimetric assay with commercially available substrate [30]. The samples selected for analysis were chosen based on conditioning temperature at time of sample collection and corresponded to those selected for PDI analysis.

Statistical Analysis

The feed manufacturing data of Experiment 1 and Experiment 2 were analyzed as 3 x 2 factorial designs with three percentages or sources of MAF, respectively, and two xylanase inclusions. Experiment 3 feed manufacturing data was analyzed as a 2 x 2 factorial design

with two pellet mill die configurations and two xylanase inclusions. Feed manufacturing data were analyzed utilizing the Fit Model platform of JMP 13.0 [31] for ANOVA. Means were considered statistically significant at $P \leq 0.05$ unless otherwise noted. Significant differences were separated utilizing Tukey's HSD, except for those found in Experiment 3 which required the use of Student's t-test.

Relative xylanase recoveries were also analyzed utilizing the Fit Model platform of JMP 13.0. The percentage MAF, degree of MAF saturation, and pellet mill die L:D were considered continuous factors for these analyses. Linear and quadratic models were generated for Experiment 1 and Experiment 2, while only a linear model was generated for Experiment 3. Differences were considered statistically significant at $P \leq 0.05$ unless otherwise noted.

The relationship between dependent continuous factors generated during the pelleting process (PDI, ΔT , and P MEC) and relative xylanase recoveries were modeled utilizing the Fit Y x X platform of JMP 13.0. Differences were considered statistically significant at $P \leq 0.05$ unless otherwise noted. An overall model was generated utilizing the Fit Model platform of JMP 13.0 that used the independent and dependent factors of the experiment explained previously to correlate xylanase recoveries with different factors related to thermal processing to determine if enzyme recoveries might be able to be predicted utilizing a multiple regression model. Differences were considered statistically significant at $P \leq 0.05$ unless otherwise noted.

RESULTS AND DISCUSSIONS

Experiment 1

The percentage MAF significantly affected PDI, ΔT , and PMEC ($P < 0.01$; Table I-4). As the percentage MAF increased, PDI, ΔT , and PMEC decreased. These data were in agreement with other reports [9, 20, 32]. These effects most likely occurred because MAF acted as a lubricant between the feed and the pellet mill die-wall, which reduced the pressure within the pellet mill die-hole [17]. It has been reported that starch gelatinization was inhibited or delayed by the presence of fat, which may have contributed to the negative impact that MAF imposed upon PDI [33]. Another report suggested ingredients that inherently reduced electrical energy consumption also reduced the amount of heat transferred to feed as it transitioned the pellet mill die-wall [34]. Removing MAF increased the coefficient of friction of the feed, which resulted in increased ΔT and PMEC.

The percentage MAF significantly affected xylanase recovery in pellets relative to conditioned mash ($P < 0.01$; Table I-7). The diet containing the greatest percentage MAF exhibited the least loss of xylanase activity as feed passed through the pellet mill die. This could potentially be explained by the same factors that affected PDI, ΔT , and PMEC. Greater percentage MAF was associated with decreased heat transfer and pressure within the pellet mill die, both of which could mitigate enzyme activity loss since these less harsh conditions were more favorable to conserving native proteins [17, 34]. This was in agreement with Gehring *et al.* [20] who suggested that increased percentage MAF might have had an enzyme sparing effect. Despite the xylanase activity-conserving effect that greater percentage MAF extended within the pellet mill die, the overall xylanase activity in pellets relative to unconditioned mash was unaffected by percentage MAF.

Experiment 2

The source of MAF significantly affected PDI ($P < 0.05$), but did not affect ΔT or PMEC (Table I-5). The diets containing canola oil produced the most durable pellets, the diets with poultry fat produced the least durable pellets, and the coconut oil diets were intermediate. Despite statistically significant differences, the range in PDI between the most and least durable pellets was only 2.59 points, which would be unlikely to produce significant differences in broiler live performance.

Xylanase activity in pellets relative to conditioned mash was significantly affected by fat source ($P < 0.01$; Table I-8). The diets containing coconut oil and poultry fat exhibited improved xylanase activity in pellets relative to conditioned mash when compared to the diet containing canola oil, indicating that MAF source impacted xylanase activity loss as feed transitioned the pellet mill die. Despite the xylanase activity-conserving effect that certain sources of MAF extended within the pellet mill die, the xylanase activity in pellets relative to unconditioned mash was unaffected by MAF source.

Experiment 3

Pellet mill die L:D impacted PDI, ΔT , and PMEC ($P < 0.01$; Table I-6). The thicker pellet mill die (L:D 10:1) produced more durable pellets, but increased ΔT and PMEC when compared to the thinner pellet mill die (L:D 8:1). This was in agreement with other data, which demonstrated that an increase in the L:D of a pellet mill die resulted in an increased PDI, but negatively impacted PMEC [19]. As the L:D of the pellet mill die increased, the feed experienced more shear and remained in a high heat and pressure environment for a longer period of time. The increased residence time within the high heat and pressure environment of the thicker pellet mill die, along with an increase in friction and

backpressure, most likely promoted greater particle binding, which improved PDI, but increased ΔT and negatively impacted P MEC.

Pellet mill die L:D impacted the activity of xylanase in pellets relative to unconditioned and conditioned mash ($P < 0.01$; Table I-9). Producing feed with the thicker pellet mill die decreased xylanase activity in pellets relative to unconditioned and conditioned mash when compared to the thinner pellet mill die. This was likely the result of increased pressure and friction, the same mechanisms by which thicker pellet mill dies have improved PDI [19]. An increased residence time in a high heat and pressure environment was likely the cause of increased xylanase activity loss, which reduced the final xylanase activity in pellets.

Dependent Variables Affecting Xylanase Activity

The xylanase activity in conditioned mash relative to unconditioned mash was not well described by PDI, ΔT , or P MEC (Figure I-1; $R^2=0.08, 0.08, \text{ and } 0.06$, respectively). This indicated that the factors generally accepted to impact the aforementioned parameters monitored in feed manufacturing, such as percentage MAF, did not accurately describe xylanase destruction in the conditioner. This suggests that xylanase manufacturers would be responsible for reduced loss of xylanase activity in the conditioner since parameters monitored in a typical feed mill did not independently explain the fate of the enzyme in the conditioner very well.

However, the activity of xylanase in pellets relative to conditioned mash was well described by PDI, ΔT , and P MEC ($P < 0.01$; Figure I-2; $R^2=0.73, 0.67, \text{ and } 0.71$, respectively). An inverse relationship between each parameter and xylanase activity in pellets relative to conditioned mash existed, which indicated that efforts to improve PDI, which

were often accompanied by an increase in ΔT and P MEC, might result in decreased xylanase activity. Similarly, the activity of xylanase in pellets relative to unconditioned mash was well described by PDI, ΔT , and P MEC ($P < 0.01$; Figure I-3; $R^2=0.77, 0.59$, and 0.64 , respectively).

Predicting Relative Enzyme Activity

The xylanase activity in pellets relative to conditioned mash and unconditioned mash was highly predictable when the controlled factors and dependent variables generated were combined to make a prediction on the fraction of xylanase that would remain active after pelleting ($P < 0.01$; Figure I-4; $R^2=0.89$ and 0.84 , respectively). This indicated that predictive modeling could be used to anticipate the stability of an enzyme when these factors, such as percentage MAF, MAF source, pellet mill die L:D, PDI, ΔT , and P MEC were available. These commonly collected parameters in feed manufacturing could be used in conjunction with in-feed enzyme assays to generate a model that accurately predicted feed additive enzyme stability. This could be used as a method to predict enzyme stability field results, which could be used to more accurately predict the benefit of an exogenous enzyme when feeding broilers.

CONCLUSIONS AND APPLICATIONS

1. Greater percentage MAF and a thinner pellet mill die mitigated the loss of xylanase activity in pellets relative to conditioned mash.
2. An increased PDI, which was often accompanied by increased ΔT and P MEC, resulted in decreased xylanase recovery in pellets relative to unconditioned and conditioned mash.
3. Measures taken to improve PDI, such as removing MAF or increasing pellet mill die thickness, reduced xylanase recovery.

4. A multiple regression model was successfully created to describe the relationship between xylanase activity and thermal processing factors.

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26. Pellet mill, model PM1112-2, California Pellet Mill, Crawfordsville, IN.
27. Steam conditioner, model C18LL4/F6, California Pellet Mill, Crawfordsville, IN.
28. Pellet durability tester, model NHP100, Tekpro, Norfolk, United Kingdom.

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Table I-1. Composition of broiler grower diets differing in percentage mixer-added fat (MAF) and xylanase (XYL) inclusion in Experiment 1.

Ingredients	1.0% MAF		3.0% MAF		5.0% MAF	
	CON ¹	XYL ²	CON	XYL	CON	XYL
	(%)					
Corn	60.50	60.50	58.50	58.50	56.50	56.50
SBM (48% CP)	34.77	34.77	34.77	34.77	34.77	34.77
Poultry fat	1.00	1.00	3.00	3.00	5.00	5.00
Limestone	0.68	0.68	0.68	0.68	0.68	0.68
Dicalcium phosphate (18.5% P)	1.73	1.73	1.73	1.73	1.73	1.73
Salt	0.50	0.50	0.50	0.50	0.50	0.50
DL-Methionine	0.19	0.19	0.19	0.19	0.19	0.19
L-Lysine	0.04	0.04	0.04	0.04	0.04	0.04
L-Threonine	0.08	0.08	0.08	0.08	0.08	0.08
Trace mineral premix ³	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin premix ⁴	0.05	0.05	0.05	0.05	0.05	0.05
Choline chloride (60%)	0.20	0.20	0.20	0.20	0.20	0.20
Selenium ⁵ (0.06%)	0.05	0.05	0.05	0.05	0.05	0.05
Xylanase ⁶	0.00	0.01	0.00	0.01	0.00	0.01
Calculated Nutrients						
Protein	21.50	21.50	21.65	21.65	21.81	21.81
Calcium	0.80	0.80	0.80	0.80	0.80	0.80
Available phosphorous	0.40	0.40	0.40	0.40	0.40	0.40
Total lysine	1.20	1.20	1.20	1.20	1.21	1.21
Total methionine + cysteine	0.86	0.86	0.87	0.87	0.87	0.87
ME, kcal/g	2.87	2.95	2.97	3.05	3.07	3.15

¹ Control diet (CON) without xylanase.

² Xylanase diet (XYL) amended with 0.01% xylanase.

³ Trace mineral premix provided the following per kg of diet: Mn, 120 mg; Zn, 120 mg; Fe, 80 mg; Cu, 10 mg; I, 2.5 mg; Co, 1 mg.

⁴ Vitamin premix provided the following per kg of diet: vitamin A, 6600 IU; vitamin D3, 1980 IU; vitamin E, 33 IU; vitamin B12, 0.02 mg; biotin, 0.13 mg; menadione, 2 mg; thiamine, 2 mg; riboflavin, 6.6 mg; pantothenic acid, 11 mg; vitamin B6, 4 mg; niacin, 55 mg; folic acid, 1.1 mg.

⁵ Selenium premix provided Se at 0.3 mg/kg of feed.

⁶ Provided 12.5 xylanase units/g of feed in amended diets.

Table I-2. Composition of broiler grower diets differing in source of mixer-added fat and xylanase (XYL) inclusion in Experiment 2.

Ingredients	Coconut Oil		Poultry Fat		Canola Oil	
	CON ¹	XYL ²	CON	XYL	CON	XYL
	(%)					
Corn	64.12	64.12	64.12	64.12	64.12	64.12
SBM (48% CP)	30.59	30.59	30.59	30.59	30.59	30.59
Coconut oil	1.60	1.60	0.00	0.00	0.00	0.00
Poultry fat	0.00	0.00	1.60	1.60	0.00	0.00
Canola oil	0.00	0.00	0.00	0.00	1.60	1.60
Limestone	0.70	0.70	0.70	0.70	0.70	0.70
Dicalcium phosphate (18.5% P)	1.75	1.75	1.75	1.75	1.75	1.75
Salt	0.50	0.50	0.50	0.50	0.50	0.50
DL-Methionine	0.15	0.15	0.15	0.15	0.15	0.15
L-Lysine	0.05	0.05	0.05	0.05	0.05	0.05
L-Threonine	0.03	0.03	0.03	0.03	0.03	0.03
Trace mineral premix ³	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin premix ⁴	0.05	0.05	0.05	0.05	0.05	0.05
Choline chloride (60%)	0.20	0.20	0.20	0.20	0.20	0.20
Selenium ⁵ (0.06%)	0.05	0.05	0.05	0.05	0.05	0.05
Xylanase ⁶	0.00	0.01	0.00	0.01	0.00	0.01
Calculated Nutrients						
Protein	20.00	20.00	20.00	20.00	20.00	20.00
Calcium	0.80	0.80	0.80	0.80	0.80	0.80
Available phosphorous	0.40	0.40	0.40	0.40	0.40	0.40
Total lysine	1.10	1.10	1.10	1.10	1.10	1.10
Total methionine + cysteine	0.78	0.78	0.78	0.78	0.78	0.78
ME, kcal/g	2.95	3.03	2.94	3.02	2.96	3.04

¹ Control diet (CON) without xylanase.

² Xylanase diet (XYL) amended with 0.01% xylanase.

³ Trace mineral premix provided the following per kg of diet: Mn, 120 mg; Zn, 120 mg; Fe, 80 mg; Cu, 10 mg; I, 2.5 mg; Co, 1 mg.

⁴ Vitamin premix provided the following per kg of diet: vitamin A, 6600 IU; vitamin D3, 1980 IU; vitamin E, 33 IU; vitamin B12, 0.02 mg; biotin, 0.13 mg, menadione, 2 mg; thiamine, 2 mg; riboflavin, 6.6 mg; pantothenic acid, 11 mg; vitamin B6, 4 mg; niacin, 55 mg; folic acid, 1.1 mg.

⁵ Selenium premix provided Se at 0.3 mg/kg of feed.

⁶ Provided 12.5 xylanase units/g of feed in amended diets.

Table I-3. Composition of broiler grower diets differing in xylanase (XYL) inclusion to be pelleted through pellet mill dies with different length-to-diameter ratios in Experiment 3.

Ingredients	CON ¹	XYL ²
	(%)	
Corn	64.12	64.12
SBM (48% CP)	30.59	30.59
Poultry fat	1.60	1.60
Limestone	0.70	0.70
Dicalcium phosphate (18.5% P)	1.75	1.75
Salt	0.50	0.50
DL-Methionine	0.15	0.15
L-Lysine	0.05	0.05
L-Threonine	0.03	0.03
Trace mineral premix ³	0.20	0.20
Vitamin premix ⁴	0.05	0.05
Choline chloride (60%)	0.20	0.20
Selenium ⁵ (0.06%)	0.05	0.05
Xylanase ⁶	0.00	0.01
Calculated Nutrients		
Protein	20.00	20.00
Calcium	0.80	0.80
Available phosphorous	0.40	0.40
Total lysine	1.10	1.10
Total methionine + cysteine	0.78	0.78
ME, kcal/g	2.94	3.02

¹ Control diet (CON) without xylanase.

² Xylanase diet (XYL) amended with 0.01% xylanase.

³ Trace mineral premix provided the following per kg of diet: Mn, 120 mg; Zn, 120 mg; Fe, 80 mg; Cu, 10 mg; I, 2.5 mg; Co, 1 mg.

⁴ Vitamin premix provided the following per kg of diet: vitamin A, 6600 IU; vitamin D3, 1980 IU; vitamin E, 33 IU; vitamin B12, 0.02 mg; biotin, 0.13 mg, menadione, 2 mg; thiamine, 2 mg; riboflavin, 6.6 mg; pantothenic acid, 11 mg; vitamin B6, 4 mg; niacin, 55 mg; folic acid, 1.1 mg.

⁵ Selenium premix provided Se at 0.3 mg/kg of feed.

⁶ Provided 12.5 xylanase units/g of feed in amended diets.

Table I-4. Main and interaction effects of percentage mixer-added fat (MAF) and enzyme inclusion on pellet durability (PDI) as determined by the Holmen method for 30 s of testing, difference in temperature between hot pellets and conditioned mash (ΔT), and pellet mill energy consumption (PMEC) in Experiment 1.

MAF (%)	Enzyme ¹	n	PDI (%)	ΔT (°C)	PMEC (kWh/Ton)
Main Effects					
1.00		6	88.00 ^A	6.13 ^A	7.42 ^A
3.00		6	75.58 ^B	2.69 ^B	6.87 ^A
5.00		6	45.17 ^C	-0.48 ^C	5.85 ^B
<i>P</i> -value			0.001	0.001	0.001
SEM ²			1.043	0.396	0.198
	CON	9	70.33	2.41	6.74
	XYL	9	68.83	3.16	6.68
	<i>P</i> -value		0.237	0.128	0.809
	SEM ²		0.852	0.323	0.162
Interaction Effects					
1.00	CON	3	89.83	5.68	7.36
1.00	XYL	3	86.17	6.57	7.48
3.00	CON	3	75.83	2.21	6.82
3.00	XYL	3	75.33	3.18	6.92
5.00	CON	3	45.33	-0.67	6.04
5.00	XYL	3	45.00	-0.29	5.65
<i>P</i> -value			0.468	0.843	0.597
SEM ²			1.475	0.600	0.280

^{A, B, C} Means within each column that possess different superscripts differ significantly ($P \leq 0.01$).

¹ Xylanase (XYL) was added at 0.01% or withheld to form a control (CON).

² Standard error of mean (SEM) for n=6 samples for each main effect of MAF concentration, n=9 samples for each main effect of enzyme, and n=3 samples for each interaction of MAF concentration and enzyme.

Table I-5. Main and interaction effects of mixer-added fat (MAF) source and enzyme inclusion on pellet durability (PDI) as determined by the Holmen method for 30 s of testing, difference in temperature between hot pellets and conditioned mash (ΔT), and pellet mill energy consumption (PMEC) in Experiment 2.

MAF Source	Enzyme ¹	n	PDI (%)	ΔT (°C)	PMEC (kWh/Ton)
Main Effects					
Coconut oil		6	90.67 ^{ab}	4.24	7.83
Poultry fat		6	89.83 ^b	4.50	7.92
Canola oil		6	92.42 ^a	5.83	8.65
<i>P</i> -value			0.015	0.219	0.114
SEM ²			0.536	0.651	0.279
	CON	9	91.06	4.61	7.88
	XYL	9	90.89	5.11	8.38
	<i>P</i> -value		0.792	0.513	0.150
	SEM ²		0.437	0.531	0.228
Interaction Effects					
Coconut oil	CON	3	90.67	4.15	7.48
Coconut oil	XYL	3	90.67	4.33	8.17
Poultry fat	CON	3	89.00	4.26	8.10
Poultry fat	XYL	3	90.67	4.74	7.74
Canola oil	CON	3	93.50	5.41	8.08
Canola oil	XYL	3	91.33	6.26	9.22
<i>P</i> -value			0.076	0.937	0.189
SEM ²			0.758	0.920	0.394

^{a, b} Means within each column that possess different superscripts differ significantly ($P \leq 0.05$).

¹ Xylanase (XYL) was added at 0.01% or withheld to form a control (CON).

² Standard error of mean (SEM) for n=6 samples for each main effect of MAF source, n=9 samples for each main effect of enzyme, and n=3 samples for each interaction of MAF source and enzyme.

Table I-6. Main and interaction effects of pellet mill die length-to-diameter ratio (L:D) and enzyme inclusion on pellet durability (PDI) as determined by the Holmen method for 30 s of testing, difference in temperature between hot pellets and conditioned mash (ΔT), and pellet mill energy consumption (PMEC) in Experiment 3.

L:D	Enzyme ¹	n	PDI (%)	ΔT (°C)	PMEC (kWh/Ton)
Main Effects					
8:1		6	89.83 ^B	4.50 ^B	7.92 ^B
10:1		6	95.42 ^A	6.73 ^A	11.26 ^A
<i>P</i> -value			0.001	0.001	0.001
	CON	6	92.08	5.74	10.00 ^a
	XYL	6	93.17	5.50	9.18 ^b
	<i>P</i> -value		0.301	0.690	0.042
	SEM ²		0.467	0.417	0.241
Interaction Effects					
8:1	CON	3	89.00	4.26	8.10
8:1	XYL	3	90.67	4.74	7.74
10:1	CON	3	95.17	7.22	11.91
10:1	XYL	3	95.67	6.26	10.61
<i>P</i> -value			0.404	0.250	0.208
SEM ²			0.661	0.582	0.341

^{a, b} Means within each column that possess different superscripts differ significantly ($P \leq 0.05$).

^{A, B} Means within each column that possess different superscripts differ significantly ($P \leq 0.01$).

¹ Xylanase (XYL) was added at 0.01% or withheld to form a control (CON).

² Standard error of mean (SEM) for n=6 samples for each main effect of pellet mill die L:D, n=6 samples for each main effect of enzyme, and n=3 samples for each interaction of pellet mill die L:D and enzyme.

Table I-7. Effect of percentage mixer-added fat (MAF) on the relative activity of xylanase in conditioned mash compared to unconditioned mash (CM:UCM), pellets compared to conditioned mash (P:CM), and pellets compared to unconditioned mash (P:UCM) in Experiment 1.

MAF	n	Relative Xylanase Activity		
		CM:UCM	P:CM	P:UCM
(%)			(%)	
1.00	3	36.7	66.4	24.0
3.00	3	42.6	60.6	25.7
5.00	3	28.1	111.6	30.4
Linear <i>P</i> -value		0.337	0.027	0.302
Quadratic <i>P</i> -value		0.291	0.006	0.593
SEM ¹		5.8	6.6	4.1

¹ Standard error of mean (SEM) for n=3 samples for each percentage of MAF.

Table I-8. Effect of mixer-added fat (MAF) source on the relative activity of xylanase in conditioned mash compared to unconditioned mash (CM:UCM), pellets compared to conditioned mash (P:CM), and pellets compared to unconditioned mash (P:UCM) in Experiment 2.

MAF Source ¹	n	Relative Xylanase Activity		
		CM:UCM	P:CM (%)	P:UCM
Coconut Oil	3	20.2	61.1	12.4
Poultry Fat	3	23.3	60.1	13.9
Canola Oil	3	22.3	38.6	8.9
Linear <i>P</i> -value		0.564	0.043	0.433
Quadratic <i>P</i> -value		0.801	0.007	0.231
SEM ²		3.4	3.6	1.9

¹Analyzed as a continuous factor based on the degree of saturation of each fat with coconut oil, poultry fat, and canola oil being 91.9, 31.2, and 7.4% saturated, respectively.

²Standard error of mean (SEM) for n=3 samples for each MAF source.

Table I-9. Effect of pellet mill die length-to-diameter ratio (L:D) on the relative activity of xylanase in conditioned mash compared to unconditioned mash (CM:UCM), pellets compared to conditioned mash (P:CM), and pellets compared to unconditioned mash (P:UCM) in Experiment 3.

L:D	n	Relative Xylanase Activity		
		CM:UCM	P:CM (%)	P:UCM
8:1	3	23.3	60.1 ^A	13.9 ^A
10:1	3	26.5	22.9 ^B	6.0 ^B
Linear <i>P</i> -value		0.451	0.003	0.003
SEM ¹		2.7	3.9	0.8

^{A, B} Means within each column that possess different superscripts differ significantly ($P \leq 0.01$).

¹ Standard error of mean (SEM) for n=3 samples for each pellet mill die L:D.

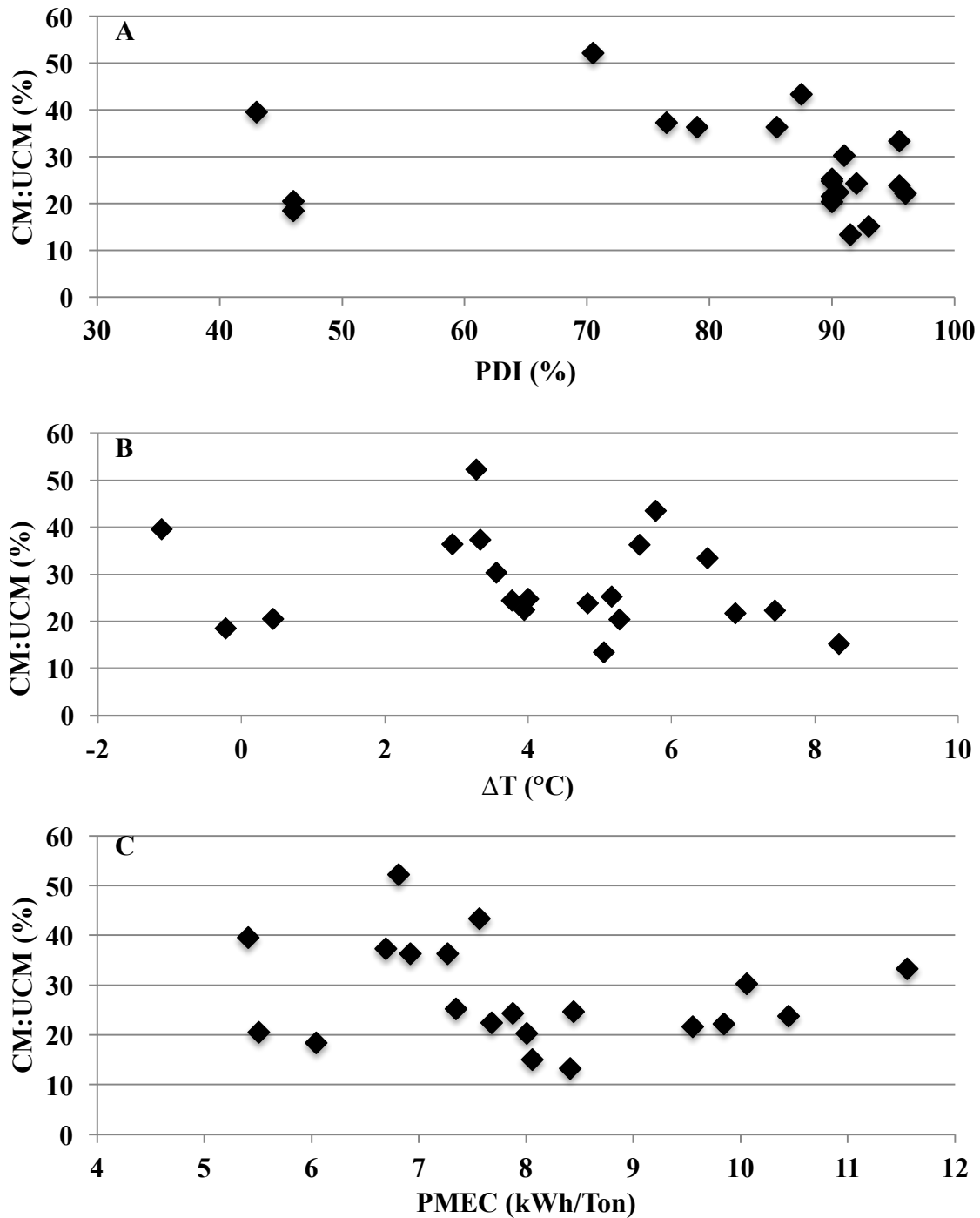


Figure I-1. Xylanase activity in conditioned mash relative to unconditioned mash (CM:UCM) vs. A) pellet durability index (PDI) ($P=0.235$; $R^2=0.08$), B) change in temperature between hot pellets and conditioned mash (ΔT) ($P=0.211$; $R^2=0.08$), and C) pellet mill energy consumption (PMEC) ($P=0.289$; $R^2=0.06$).

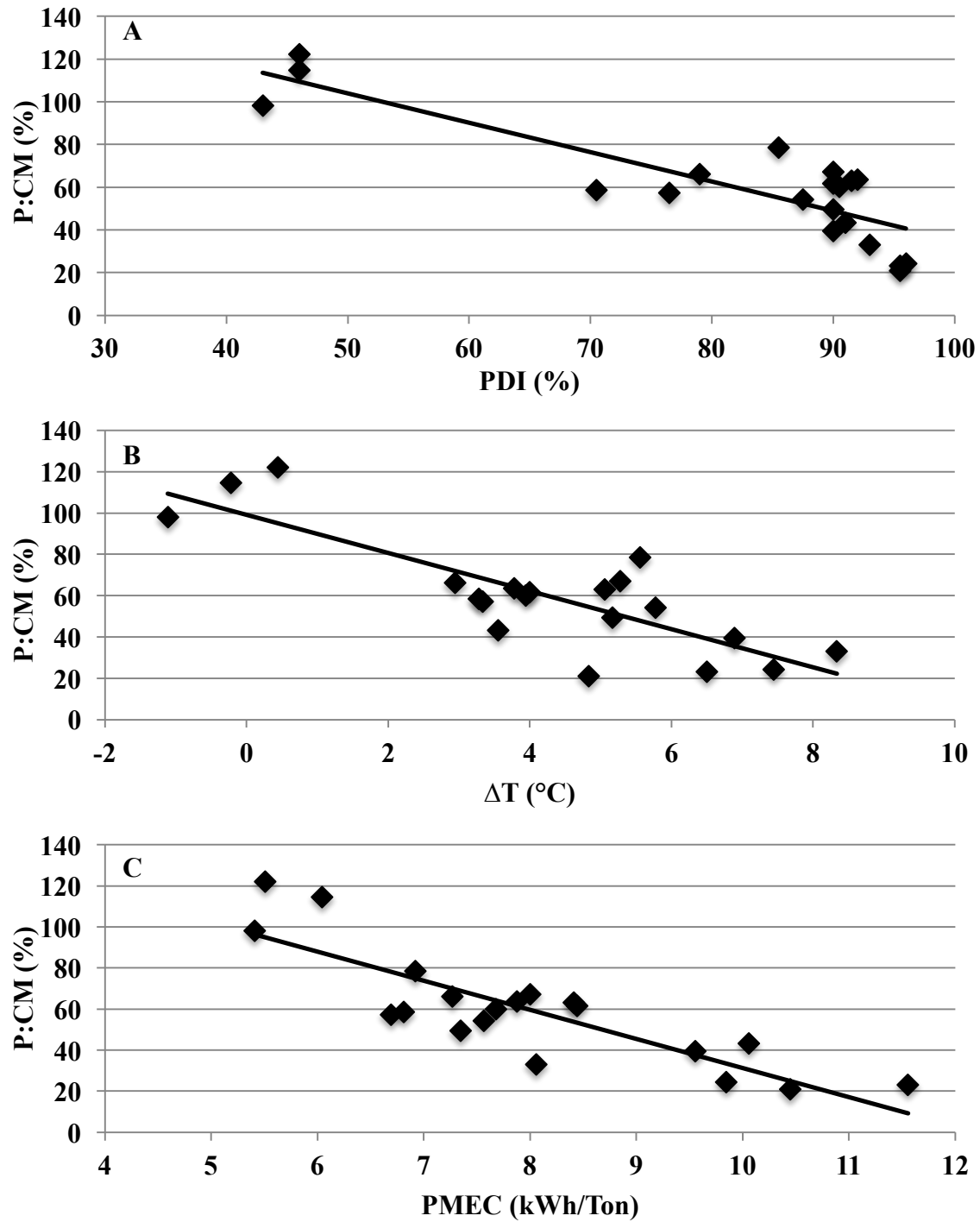


Figure I-2. Xylanase activity in pellets relative to conditioned mash (P:CM) vs. A) pellet durability index (PDI) ($P=0.001$; $R^2=0.73$; $y=172.7-1.4*PDI$), B) change in temperature between hot pellets and conditioned mash (ΔT) ($P=0.001$, $R^2=0.67$; $y=99.1-5.1*\Delta T$), and C) pellet mill energy consumption (PMEC) ($P=0.001$; $R^2=0.71$; $y=173.1-14.2*PMEC$).

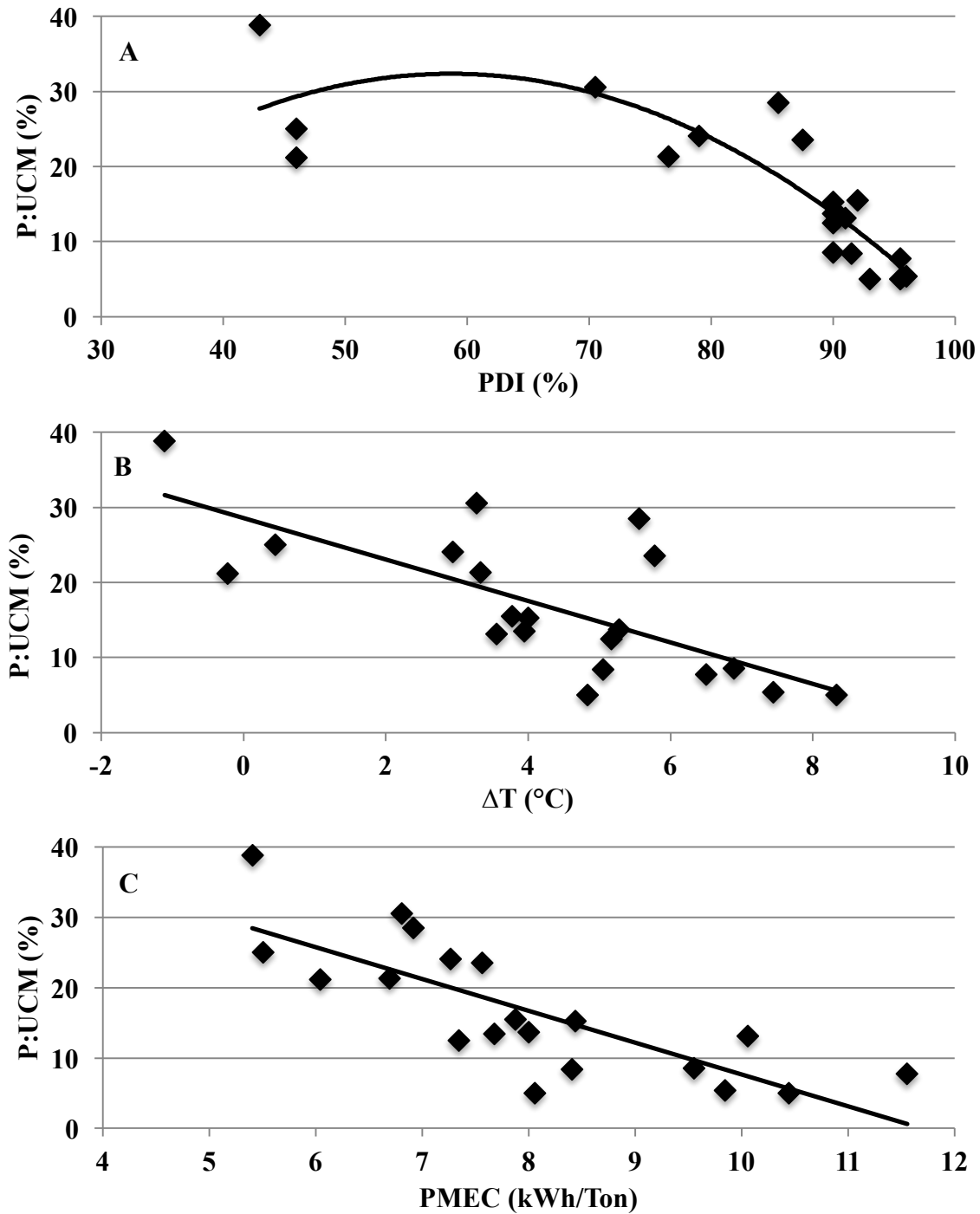


Figure I-3. Xylanase activity in pellets relative to unconditioned mash (P:UCM) vs. A) pellet durability index (PDI) ($P=0.001$; $R^2=0.77$; $y=94.3-0.8*PDI-0.01*PDI^2$), B) change in temperature between hot pellets and conditioned mash (ΔT) ($P=0.001$; $R^2=0.59$; $y=30.1-1.7*\Delta T$), and C) pellet mill energy consumption (PMEC) ($P=0.001$; $R^2=0.64$; $y=55.2-4.8*PMEC$).

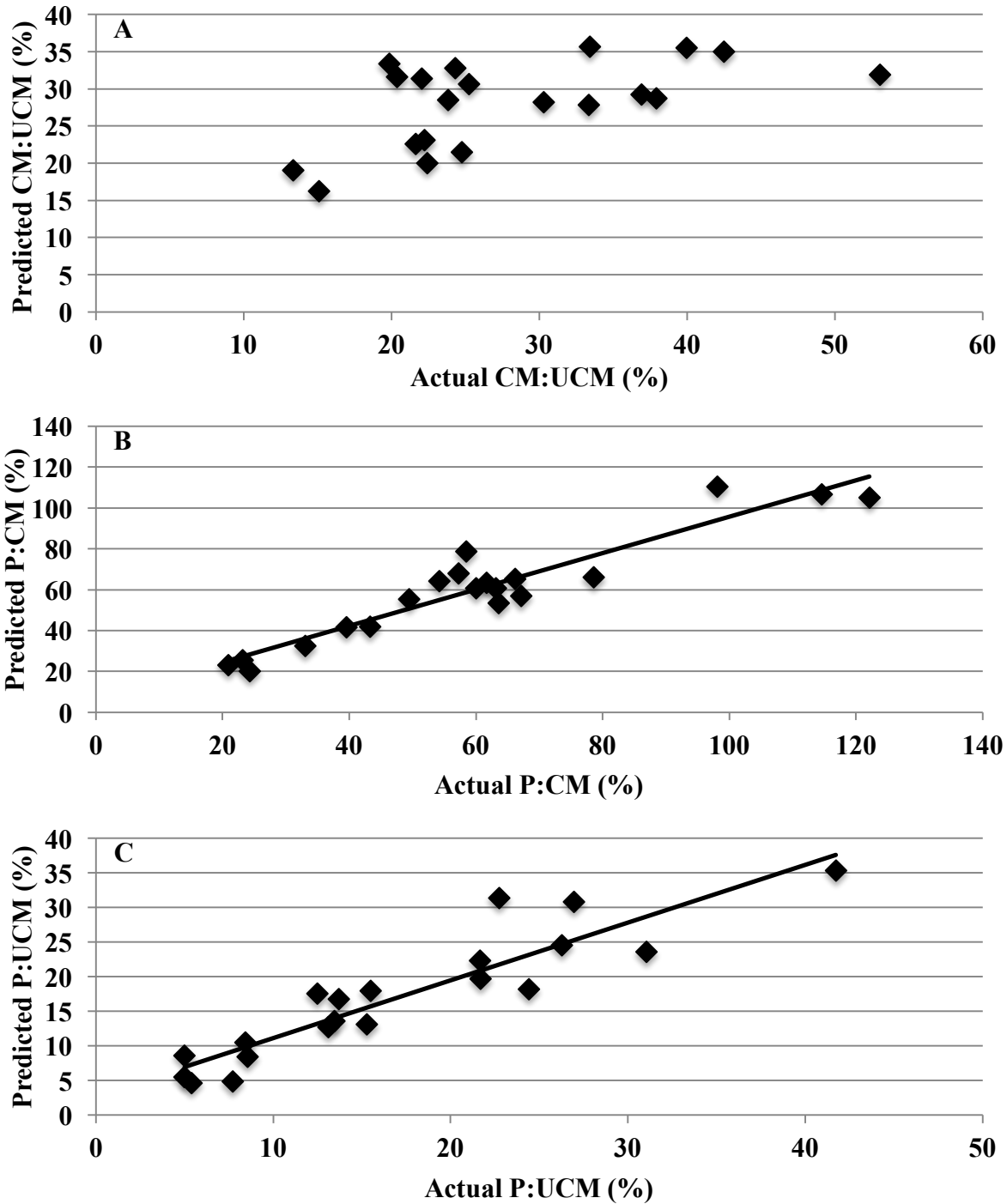


Figure I-4. Predicted xylanase activity in conditioned mash relative to unconditioned mash (CM:UCM), pellets relative to conditioned mash (P:CM), and pellets relative to unconditioned mash (P:UCM) vs. A) actual CM:UCM ($P=0.563$; $R^2=0.33$), B) actual P:CM ($P=0.001$; $R^2=0.89$), and C) actual P:UCM ($P=0.001$; $R^2=0.84$), respectively.

CHAPTER II

The effect of the level of mixer-added water and mash conditioning temperature on parameters monitored during pelleting and phytase and xylanase thermostability

SUMMARY

Exogenous enzymes have been used in broiler and swine rations to improve nutrient utilization and reduce feed costs, both of which improved the efficiencies of live production. Feed manufacturers have also utilized practices, such as high mash conditioning temperatures and the addition of moisture at the mixer or mash conditioning chamber, to maintain adequate physical feed quality. While the impact of conditioning practices on enzyme thermostability had been well described, the impact of mixer-added water (MAW) on enzyme thermostability was scarcely investigated in previous literature. The present experiment intended to investigate the interaction of three mash conditioning temperatures and three levels of MAW on the thermostability of a phytase and xylanase exogenous enzyme within a swine gestation diet in a 3 x 3 factorial randomized complete block design. The mash conditioning temperatures included 80, 86, and 92°C and the levels of MAW included 0, 1, and 2%. Parameters monitored during the pelleting process included pellet durability index (PDI), pellet mill energy consumption (PMEC), and the change in temperature between hot pellets and conditioned mash (ΔT). Conditioning mash at 92°C resulted in a 70.1 percentage-point reduction in phytase recovery when compared to diets pelleted at 80°C ($P < 0.01$). The xylanase, however, was not affected by mash conditioning temperature. Phytase and xylanase recovery in pellets was not affected by the level of MAW. As PDI and PMEC increased, phytase recovery in pellets relative to unconditioned mash decreased ($P < 0.01$). As ΔT increased, phytase recovery in pellets relative to unconditioned mash increased ($P < 0.01$). Xylanase denaturation was poorly described by the parameters monitored during pelleting, likely because it was minimally denatured during pelleting. A robust multiple regression model was generated to describe phytase activity in pellets relative

to unconditioned mash ($R^2=0.95$; $P < 0.01$) when the controlled factors and monitored parameters were correlated. In conclusion, the different enzymes exhibited different heat tolerances and MAW was not detrimental to the activity of phytase or xylanase in pelleted feed.

DESCRIPTION OF PROBLEM

Mixer-added exogenous enzymes had been used extensively in nonruminant feed rations since their commercial inception to reduce feed costs and improve nutrient extraction from feedstuffs [1, 2, 3, 4]. The majority of nonruminant feeds in the United States had been pelleted, which exposed mixer-added exogenous enzymes to heat, moisture, and pressure [2]. The aforementioned factors were necessary for feed particle adhesion into pellets [5], but negatively affected the stability and efficacy of mixer-added exogenous enzymes [6]. Although enzyme technologies had evolved to include the post-pelleting application of liquid enzymes to avoid the stressors of pelleting, the capital investment in the application systems and variation in metering minute volumes of liquid made post-pellet application of enzymes a poor candidate for many feed manufacturers [2].

The benefits of producing feeds with improved pellet quality had been well documented in previous reports [7, 8]. Feed manufactures had improved pellet quality through the manipulation of several factors, with increasing mash conditioning temperature and retention time being well established methods to improve physical feed quality [9]. Additional reports suggested that pellet quality could be improved via moisture addition at the mixer by facilitating further starch gelatinization when compared to diets that did not receive additional moisture [10]. During periods of low grain moisture, applying adequate water vapor to provide the moisture necessary to facilitate particle adhesion may have made

the rations more challenging and costly to pellet. This had led several feed manufactures to add additional moisture to rations via mixer or mash conditioning chamber added liquid water. While the practice of moisture addition via liquid water to mash prior to pelleting had been a proven method to improve pellet quality, its impact on mixer-added exogenous enzyme thermostability was scarcely investigated in previous literature other than an *in vitro* assay report which suggested that additional moisture may have exacerbated exogenous enzyme denaturation [11].

Additionally, it had been hypothesized that experimental coolers employed for pilot pellet mill enzyme thermostability trials may have been a causative factor for typically poorer enzyme recoveries in experimental conditions when compared to commercial conditions. It had been reasoned that experimental coolers adequately removed heat from pellets, but not moisture, which may have resulted in continued enzyme denaturation post-pelleting. Thus, it was necessary to determine the effect of mixer-added water (MAW), and its interaction with mash conditioning temperature, on the stability of mixer-added exogenous enzymes with varying heat tolerances (phytase and xylanase) in an experimental setting with an experimental batch pellet cooling device.

MATERIALS AND METHODS

Feed Manufacturing

The experiment was conducted by pelleting nine 909 kg batches of a swine gestation diet (Table II-1) containing phytase [12] and xylanase [13] to determine the effect of mash conditioning temperature and MAW on phytase and xylanase recovery post-pelleting. The mash conditioning temperatures included 80, 86, and 92°C and the levels of MAW included

0, 1, and 2%. The experiment was arranged as a 3 x 3 factorial randomized complete block design.

Individual batches were sourced from a common basal to reduce ingredient composition variation between batches. The basal and individual diets were blended utilizing a twin shaft counterpoise ribbon mixer [14] for 180 s of dry mixing time. The basal was devoid of fat, water, phytase, and xylanase. The individual batches were amended with phytase, xylanase, fat, and water when appropriate. For batches amended with water, the water was added after 180 s of dry mixing with enzymes, but before 90 s of wet mixing with fat. Upon mixer discharge, a composite mixer sample was collected.

The batches were conditioned for 30 s at 80, 86, and 92°C in a single pass conditioner [15] and then pelleted utilizing a 30 HP CPM pellet mill [16] equipped with a 4.4 x 35.2 mm pellet mill die with 548 cm² of working surface area at a production rate of 908 kg/hr. The steam pressure was 207 kPa. The pellet mill die was warmed with 455 kg of feed before proceeding with the experimental batches. After obtaining the appropriate mash conditioning temperature, five hot conditioned mash, cooled conditioned mash, and cooled pellet samples were collected over a five-minute period of pelleting and were blended into composite samples. The hot conditioned mash samples were collected as feed transitioned between the conditioner and the pellet mill die in sample bags that were sealed upon sample collection to determine moisture content [17]. The cooled conditioned mash samples were cooled to ambient temperature in custom manufactured 30 x 30 cm trays over open grates with circulating air and were analyzed for phytase [18] and xylanase [19] activity. The pellet samples were cooled in custom manufactured 30 x 30 cm trays, which were placed in a custom manufactured pellet cooler resembling a counterflow cooler, and were analyzed for

moisture content and phytase and xylanase activity. After collecting samples at 80°C, the steam valve on the mash conditioning chamber was further opened to increase the conditioning temperature and the sample collection process was repeated at 86 and 92°C.

Parameters monitored during the pelleting process included the change in temperature between hot pellets and conditioned mash (ΔT), pellet durability index (PDI), and pellet mill energy consumption (PMEC). Pellet durability was determined by placing 100 g of pellets, screened with a #5 US sieve, into a pellet durability tester [20] for 30 s. Pellets were removed directly from the testing chamber and weighed to represent a proportion of the initial mass added to the testing chamber, with each sample being tested in duplicate. The PMEC data were collected by the feed mill automation system [21] as a continuous input variable at the programmable logic controller.

Statistical Analysis

The moisture content of feed forms, parameters monitored during feed manufacturing, and phytase and xylanase recoveries were analyzed as a 3 x 3 factorial randomized complete block design. The Fit Model platform of JMP 13 [22] for ANOVA was employed and the means were separated utilizing the LSMeans procedure of JMP. Means were considered statistically significant at $P \leq 0.05$. Significant differences were separated utilizing Tukey's HSD test.

The relationship between parameters monitored during feed manufacturing, moisture of hot conditioned mash and pellets, and xylanase and phytase recoveries were regressed utilizing the Fit Y x X platform of JMP 13. Differences were considered statistically significant at $P \leq 0.05$. The multiple regression models to describe phytase and xylanase recoveries were generated with the Fit Model platform of JMP 13 considering the controlled

factors and monitored parameters of the experiment as input variables. The models were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSIONS

The moisture content of unconditioned mash increased in rations that were amended with 1 or 2% MAW when compared to rations that were not amended with water ($P < 0.01$; Table II-2). The water added to rations before pelleting was not adequately removed from pellets within the experimental batch cooler, as evidenced by the increased pellet moisture in rations amended with 1 and 2% water when compared to rations that were not amended with water ($P < 0.01$; Table II-2). While rations pelleted at 86 and 92°C contained greater levels of moisture in hot conditioned mash when compared to rations pelleted at 80°C ($P < 0.01$; Table II-2), the additional moisture from steam was adequately removed from pellets within the experimental batch cooler as evidenced by similar pellet moistures as affected by mash conditioning temperature ($P < 0.01$; Table II-2). These data indicated that the moisture added to rations via steam was more likely to volatilize and escape the pellet matrix within an experimental batch cooler when compared to moisture added to rations via water at the mixer. For commercial feed manufacturers adding water to rations at the mixer, it may be necessary to implement additional measures, such as cooler baffle adjustments or increased pellet cooling duration, to ensure that the moisture content of finished feed is reduced to an adequate level. There were no interactions between mash conditioning temperature and level of MAW on moisture content of the various feed forms.

Mash conditioning temperature significantly affected PDI, P MEC, and ΔT ($P < 0.01$; Table II-3). The PDI of rations conditioned and pelleted at 86 and 92°C were significantly greater than those pelleted at 80°C. These data were in agreement with other reports that

demonstrated the positive benefit of increasing mash conditioning temperatures on pellet durability [23, 24]. This likely occurred due to increased heat and moisture penetration of feed particles, which likely improved surface binding properties via physicochemical changes in native starch and protein structures. Rations conditioned and pelleted at 92°C required significantly greater amounts of energy to pellet when compared to rations conditioned and pelleted at 80 and 86°C. Winowiski [25] described the effect of applying excessive heat or moisture to rations during conditioning on the pelleting process. If conditioning practices resulted in levels of heat or moisture beyond those necessary for particle adhesion, pellet mill production capacity was impaired, which resulted in decreased pelleting efficiency. As the mash conditioning temperature increased, there was a stepwise reduction in the frictional heat transferred to the pellets, as evidence by ΔT ($P < 0.01$; Table II-3). Skoch *et al.* [26] determined that rations pelleted with steam resulted in reduced frictional heat transfer during pelleting when compared to rations pelleted without steam, which indicated that water added via steam acted as a lubricant. Data from the current trial supported this conclusion, and it was apparent that an increased conditioning temperature likely reduced the frictional heat transferred to pellets as they traversed the pellet mill die.

The level of MAW affected PDI ($P < 0.01$) and PMEC ($P < 0.05$; Table II-3). Diets receiving 2% MAW contained pellets that were significantly more durable than pellets from diets that did not receive MAW. Moisture was previously determined to affect the gelatinization, denaturation, and solubilization processes necessary to alter and prepare feed particles for binding within the pellet mill die [5]. Although inferior to moisture added via condensed steam with respect to pellet durability [27], the additional moisture in diets containing 2% MAW likely facilitated improved particle binding, and thus the manufacture

of more durable pellets. Diets that received 2% MAW also required significantly greater amounts of energy to pellet, which was in agreement with the report from Winowski [25] previously mentioned. There were no interaction effects between mash conditioning temperature and the level of MAW on parameters monitored during the pelleting process.

Mash conditioning temperature also significantly affected phytase recovery in conditioned mash relative to unconditioned mash ($P < 0.01$; Table II-4). Phytase recovery in conditioned mash relative to unconditioned mash was reduced when mash was conditioned at 92°C compared to mash conditioned at 80 and 86°C. These data were in agreement with other reports that demonstrated or discussed the negative impact of high conditioning temperatures on enzyme thermostability [2, 28, 29, 30].

Increasing the mash conditioning temperature resulted in a stepwise decrease in phytase activity in pellets relative to unconditioned mash and conditioned mash ($P < 0.01$; Table II-4). The reduction in phytase activity in conditioned mash relative to unconditioned mash (CM:UCM), or the quantification of phytase activity loss within the conditioning chamber, when rations were pelleted at 92°C compared to 80°C was 22.5 percentage points (Table II-4). The reduction in phytase activity in pellets relative to conditioned mash (P:UCM), or the quantification of phytase activity loss within the pellet mill die, when rations were pelleted at 92°C compared to 80°C was 70.1 percentage points. These data indicated that the greatest contributor to phytase denaturation was not simply exposure to steam within the conditioning chamber, but rather the accumulation of forces necessary to bind particles within the pellet mill die such as moisture, heat, and pressure. The majority of previous literature pertaining to enzyme thermostability had indicated that enzyme exposure to steam within the conditioning chamber was the determinant factor of the extent of enzyme

denaturation [28, 30]. The current data indicated that the pellet mill die should also be a focal point when characterizing enzyme thermostability.

When compared to the effect of mash conditioning temperature on phytase denaturation, the level of MAW added to rations minimally affected phytase thermostability. Phytase recovery in CM:UCM was greatest in rations that were amended with 2% MAW when compared to those amended with 1% MAW ($P < 0.05$; Table II-4), but poorest in P:CM when compared to 1% MAW ($P < 0.05$; Table II-4). Overall, there were no significant differences in phytase recoveries in P:UCM attributed to the level of MAW, which indicated that the addition of water to rations was not detrimental to the stability of the phytase utilized (Table II-4). This was not in agreement with other reports [11] which suggested that high moisture conditions facilitated heat conductivity and transfer to enzyme particles. However, the previous report came to this conclusion based on an *in vitro* assay with an unprocessed enzyme, which did not seem to directly mimic pelleting systems. These data also indicated that the reduction in phytase activity in P:UCM as a result of increased mash conditioning temperatures may not have been primarily the result of increased moisture from steam since MAW did not affect phytase recovery in pellets relative to unconditioned mash. Xylanase recovery in all feed forms was unaffected by mash conditioning temperature and level of MAW (Table II-3). No significant interactions between mash conditioning temperature and MAW were apparent with respect to phytase and xylanase thermostability.

Phytase activity in CM:UCM mash was accurately characterized by ΔT ($P < 0.01$; Figure II-1), but not PDI or PMEC (Figure 1). As ΔT increased, the amount of phytase recovered in conditioned mash relative to unconditioned mash increased, which was likely due to the fact that higher ΔT values corresponded with lower conditioning temperatures.

Phytase activity in P:UCM was accurately characterized by PDI, PMEC, and ΔT ($P < 0.01$; Figure II-2). A negative correlation between PDI and PMEC and phytase recovery in pellets relative to unconditioned mash existed, whereas a positive correlation existed for ΔT . These data indicated that the parameters monitored during the pelleting process were better indicators of phytase denaturation within the pellet mill die as compared to denaturation within the mash conditioning chamber.

While the relationships between parameters monitored during pelleting and enzyme thermostability had been previously reported [31], opposite relationships existed for ΔT and phytase thermostability in the current report. When factors were varied that did not affect exposure to heat and moisture within the mash conditioning chamber, such as the level of mixer-added fat or pellet mill die configurations, an inverse relationship between ΔT and enzyme thermostability was observed. When factors were varied that affected exposure to heat and moisture within the mash conditioning chamber, such as conditioning temperature and level of MAW, a positive relationship between ΔT and phytase thermostability was observed (Figure II-2). These differences indicated that the interaction of enzymes and the pellet mill die hole wall with respect to enzyme thermostability were not subject to the same outcomes, but rather dependent upon several factors that may affect ΔT such as mash conditioning temperature, diet ingredient composition, and pellet mill die configuration.

The moisture content of conditioned mash and phytase activity in P:UCM were negatively correlated ($P < 0.05$; Figure II-3). However, there was no significant correlation between phytase recovery in P:UCM and cooled pellet moisture (Figure II-3), which indicated that the use of an experimental cooler was not further denaturing the phytase with respect to the inadequate removal of moisture within pellets when compared to a commercial

counterflow cooler. Since there was minimal xylanase denaturation, there were no significant associations between parameters monitored during feed manufacturing (Figure 4 and 5) or moisture content of feed forms (Figure II-6) and xylanase thermostability.

Multiple regression modeling was successfully employed to describe phytase thermostability outcomes ($P < 0.01$; Figure II-7). The successful generation of models had been previously demonstrated [31] to predict enzyme recovery in pellets relative to unconditioned mash in experiments that utilized parameters monitored during pelleting as well as parameters of feed formulation and pellet mill die specifications with an $R^2=0.84$. In the current report, phytase recovery in P:UCM was correlated with an $R^2=0.95$ when mash conditioning temperature factors were applied. Despite the models having been generated with different enzymes that utilized different assays, these data indicated that adding a mash conditioning temperature variable to a multiple regression model would likely improve the robustness of an enzyme thermostability model. A significant model was not successfully generated to predict xylanase thermostability in pellets relative to unconditioned mash (Figure II-7), likely because there was minimal denaturation of the xylanase utilized in the current report. These data indicated that completely heat tolerant enzymes would not be candidates for thermostability modeling. Conversely, enzymes prone to denaturation would be better candidates for thermostability modeling so that enzyme recovery and efficacy within animals could be better predicted based on the input factors of the model.

CONCLUSIONS AND APPLICATIONS

1. Amending diets with MAW did not exacerbate overall enzyme denaturation.
2. Greater levels of phytase denaturation occurred within the pellet mill die when compared to the mash conditioning chamber.

3. Thermosensitive enzyme recoveries can be accurately described with multiple regression modeling, especially when mash conditioning temperatures are utilized as an input variable.

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Table II-1. Ingredient composition of swine gestation diets containing phytase and xylanase amended with three levels of mixer-added water (MAW).

Ingredients	MAW		
	0.0%	1.0%	2.0%
MAW	0.00	1.00	2.00
Corn	83.29	83.29	83.29
SBM (48%)	13.64	13.64	13.64
Poultry fat	1.00	1.00	1.00
Limestone	0.95	0.95	0.95
Monocalcium phosphate	0.40	0.40	0.40
Salt	0.50	0.50	0.50
Trace mineral premix ¹	0.15	0.15	0.15
Vitamin premix ²	0.04	0.04	0.04
Phytase ³	0.02	0.02	0.02
Xylanase ⁴	0.01	0.01	0.01
Calculated nutrients			
Protein	13.40	13.40	13.40
Calcium	0.62	0.62	0.62
Available phosphorous	0.33	0.33	0.33
Total lysine	0.61	0.61	0.61
ME, kcal/g	3.35	3.35	3.35

¹ Trace mineral premix contained the following concentrations of minerals: Fe, 7.3%; Zn, 7.3%, Mn, 2.2%, Cu, 1.1%, I, 198 ppm, Se, 198 ppm.

² Vitamin premix contained the following quantities of vitamins per kg of premix: vitamin A, 20568783 IU; vitamin D3, 2932090 IU; vitamin E, 117504 IU; vitamin B12, 73 mg; biotin, 589 mg, menadione, 9700 mg; riboflavin, 14690 mg; d pantothenic acid, 58790 mg; niacin, 88183 mg; folic acid, 4409 mg.

³ Quantum Blue 5G was added to provide 1000 FTU/kg of feed.

⁴ Econase XT was added to provide 16,000 BXU/kg of feed.

Table II-2. Effect of mash conditioning temperature (CT) and level of mixer-added water (MAW) on moisture content¹ of unconditioned mash (UCM), hot conditioned mash (HCM), cooled conditioned mash (CCM) and pellets (P).

CT (°C)	MAW (%)	n	Moisture Content ¹			
			UCM	HCM	CCM	P
			(%)			
Main Effects						
80		9	-	18.7 ^B	16.2	15.5
86		9	-	19.3 ^A	16.5	15.8
92		9	-	19.6 ^A	16.5	15.6
<i>P</i> -value			-	0.001	0.604	0.301
	0.0	9	13.7 ^B	18.2 ^B	15.5 ^C	14.8 ^B
	1.0	9	15.1 ^A	19.4 ^A	16.5 ^B	15.9 ^A
	2.0	9	15.7 ^A	19.9 ^A	17.2 ^A	16.2 ^A
	<i>P</i> -value		0.002	0.001	0.001	0.001
	SEM ²		0.233	0.137	0.173	0.122

^{A,B} Means within each column that possess different superscripts differ significantly ($P \leq 0.01$).

¹ Moisture content was measured in accordance with AACC 44-19.01.

² Standard error of mean (SEM) for n=9 samples for each CT and n=9 samples for each level of MAW.

Table II-3. Effect of mash conditioning temperature (CT) and level of mixer-added water (MAW) on pellet durability index (PDI) as determined by the Holmen method for 30 s of testing, pellet mill energy consumption (PMEC), and the change in temperature between hot pellets and conditioned mash (ΔT).

CT	MAW	n	PDI	PMEC	ΔT
(°C)	(%)		(%)	(kWh/T)	(°C)
Main Effects					
80		9	84.3 ^B	8.4 ^B	6.1 ^A
86		9	89.1 ^A	9.0 ^B	3.8 ^B
92		9	91.0 ^A	10.3 ^A	1.5 ^C
<i>P</i> -value			0.001	0.001	0.001
	0.0	9	86.4 ^B	8.9 ^b	3.9
	1.0	9	88.0 ^{AB}	8.9 ^b	3.7
	2.0	9	89.9 ^A	10.0 ^a	3.8
	<i>P</i> -value		0.004	0.019	0.834
	SEM ¹		0.6	0.3	0.3

^{A, B} Means within each column that possess different superscripts differ significantly ($P \leq 0.01$).

¹ Standard error of mean (SEM) for n=9 samples for each CT and n=9 samples for each level of MAW.

Table II-4. Effect of mash conditioning temperature (CT) and level of mixer-added water (MAW) on the relative activity of phytase and xylanase in conditioned mash compared to unconditioned mash (CM:UCM), pellets compared to conditioned mash (P:CM), and pellets compared to unconditioned mash (P:UCM).

CT (°C)	MAW (%)	n	Relative Phytase Recovery ¹			Relative Xylanase Recovery ²		
			CM:UCM	P:CM	P:UCM	CM:UCM	P:CM	P:UCM
Main Effects								
80		9	104.4 ^A	76.4 ^A	77.1 ^A	87.2	108.8	94.7
86		9	100.1 ^A	35.8 ^B	33.8 ^B	91.0	112.1	101.3
92		9	81.9 ^B	6.3 ^C	5.2 ^C	90.1	107.6	96.7
<i>P</i> -value			0.006	0.001	0.001	0.430	0.654	0.091
	0.0	9	95.8 ^{ab}	42.0 ^{ab}	40.8	90.9	108.8	98.2
	1.0	9	85.1 ^b	44.9 ^a	40.3	91.3	109.4	99.7
	2.0	9	105.6 ^a	31.7 ^b	35.1	86.2	110.4	94.8
	<i>P</i> -value		0.018	0.042	0.480	0.185	0.946	0.240
	SEM ³		4.4	3.5	3.6	2.0	3.5	2.1

^{a, b} Means within each column that possess different superscripts differ significantly ($P \leq 0.05$).

^{A, B} Means within each column that possess different superscripts differ significantly ($P \leq 0.01$).

¹ Quantum Blue 5G. Testing method was in accordance with ELISA specific for Quantum Blue, ESC Standard Analytical Method, SAM099; AB Vista, Marlborough, UK.

² Econase XT. Testing method was in accordance with ELISA specific for Econase XT, ESC Standard Analytical Method, SAM115; AB Vista, Marlborough, UK.

³ Standard error of mean (SEM) for n=9 samples for each CT and n=9 samples for each level of MAW.

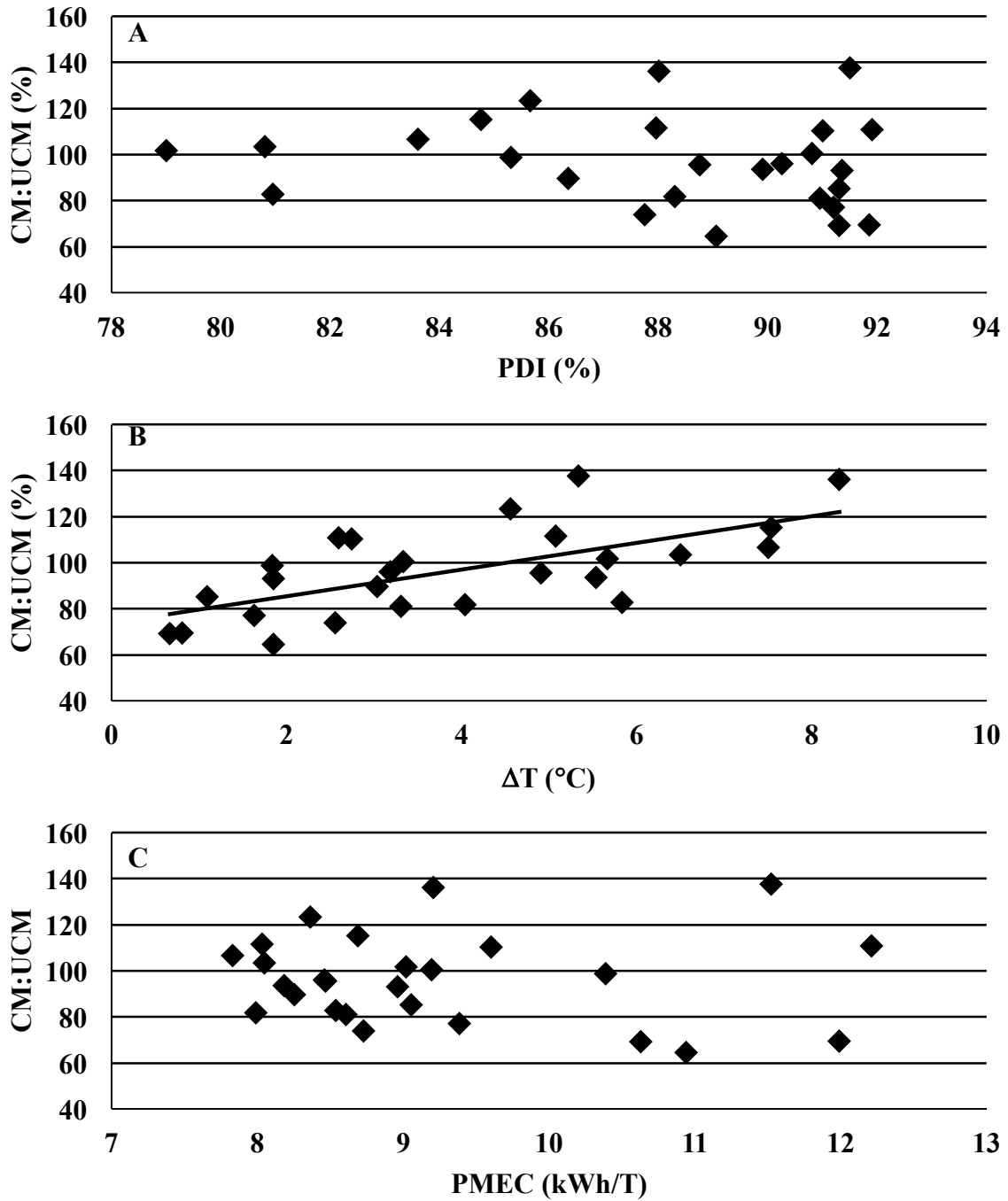


Figure II-1. Phytase activity in conditioned mash relative to unconditioned mash (CM:UCM) vs. A) pellet durability index (PDI) ($P=0.419$; $R^2=0.03$), B) change in temperature between hot pellets and conditioned mash (ΔT) ($P=0.001$; $R^2=0.43$; $y=74+5.8*\Delta T$), and C) pellet mill energy consumption (PMEC) ($P=0.754$; $R^2=0.00$).

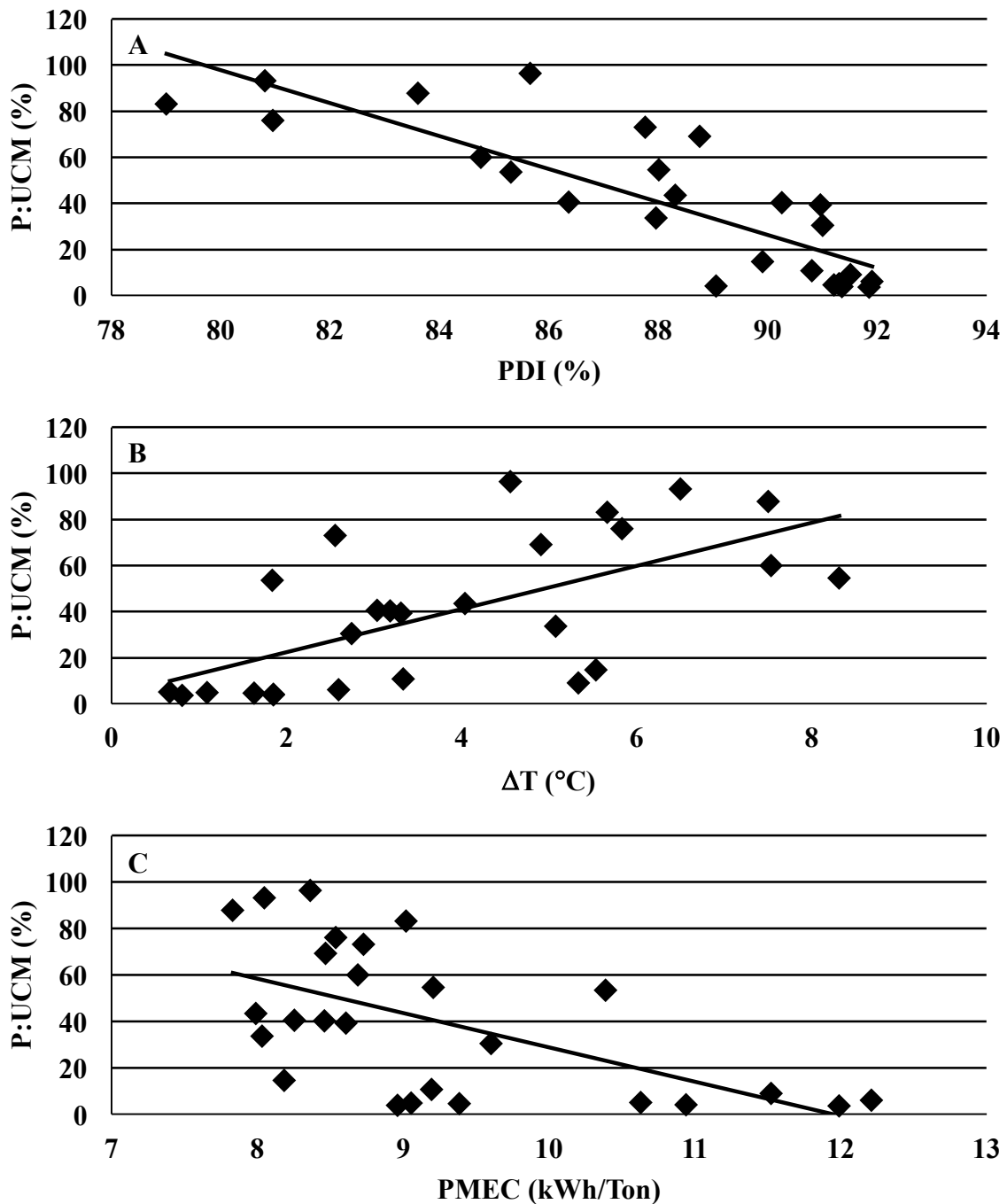


Figure II-2. Phytase activity in pellets relative to unconditioned mash (P:UCM) vs. A) pellet durability index (PDI) ($P=0.001$; $R^2=0.70$; $y=670-7.2*PDI$), B) change in temperature between hot pellets and conditioned mash (ΔT) ($P=0.001$; $R^2=0.41$; $y=3.6+9.4*\Delta T$), and C) pellet mill energy consumption (PMEC) ($P=0.002$; $R^2=0.34$; $y=176.9-14.8*PMEC$).

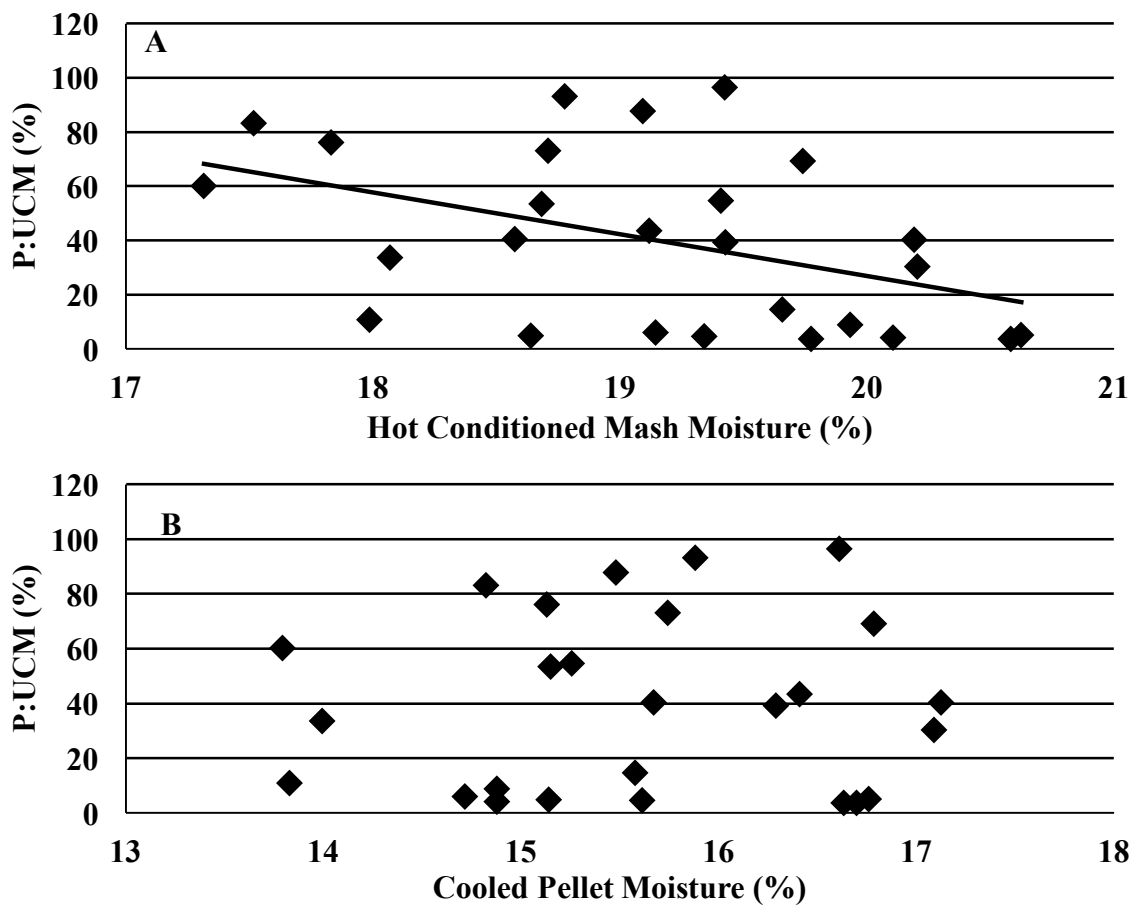


Figure II-3. Phytase activity in pellets relative to unconditioned mash (P:UCM) vs. A) hot conditioned mash moisture (HCMM)($P=0.026$; $R^2=0.19$; $y=334.5-15.4 \cdot \text{HCMM}$) and B) cooled pellet moisture ($P=0.829$; $R^2=0.00$).

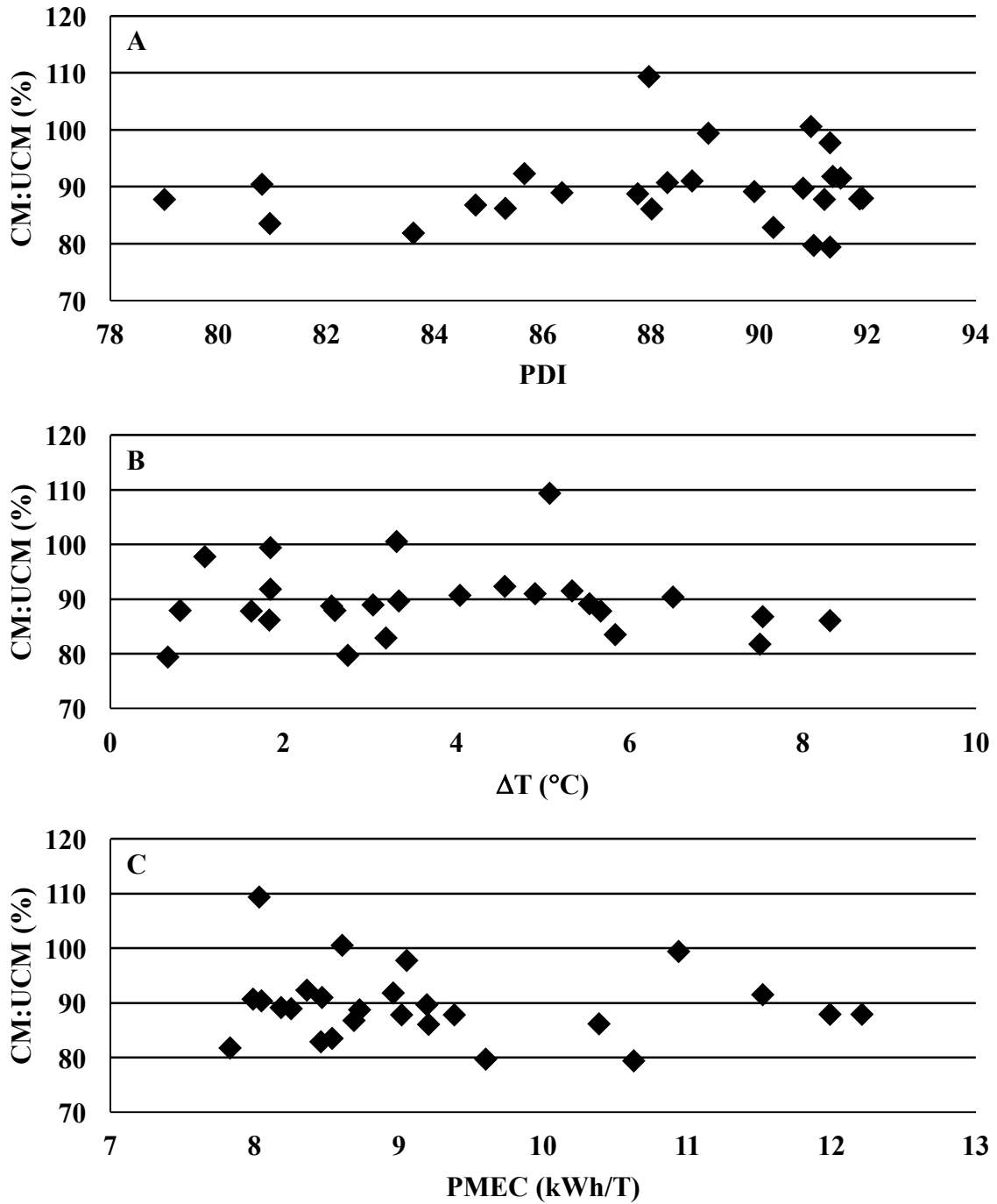
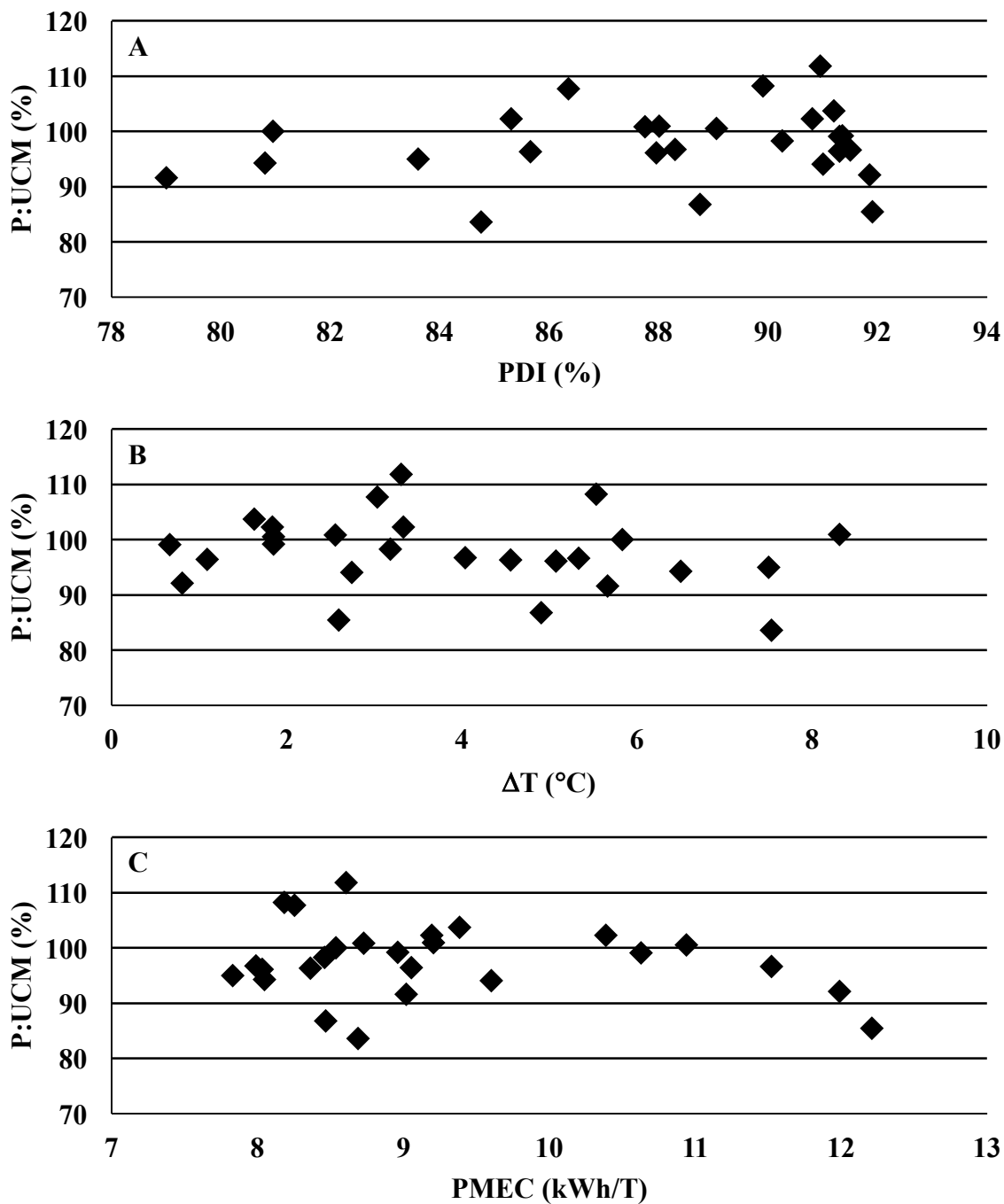


Figure II-4. Xylanase activity in conditioned mash relative to unconditioned mash (CM:UCM) vs. A) pellet durability index (PDI) ($P=0.492$; $R^2=0.02$), B) change in temperature between hot pellets and conditioned mash (ΔT) ($P=0.763$; $R^2=0.00$), and C) pellet mill energy consumption (PMEC) ($P=0.558$; $R^2=0.01$).



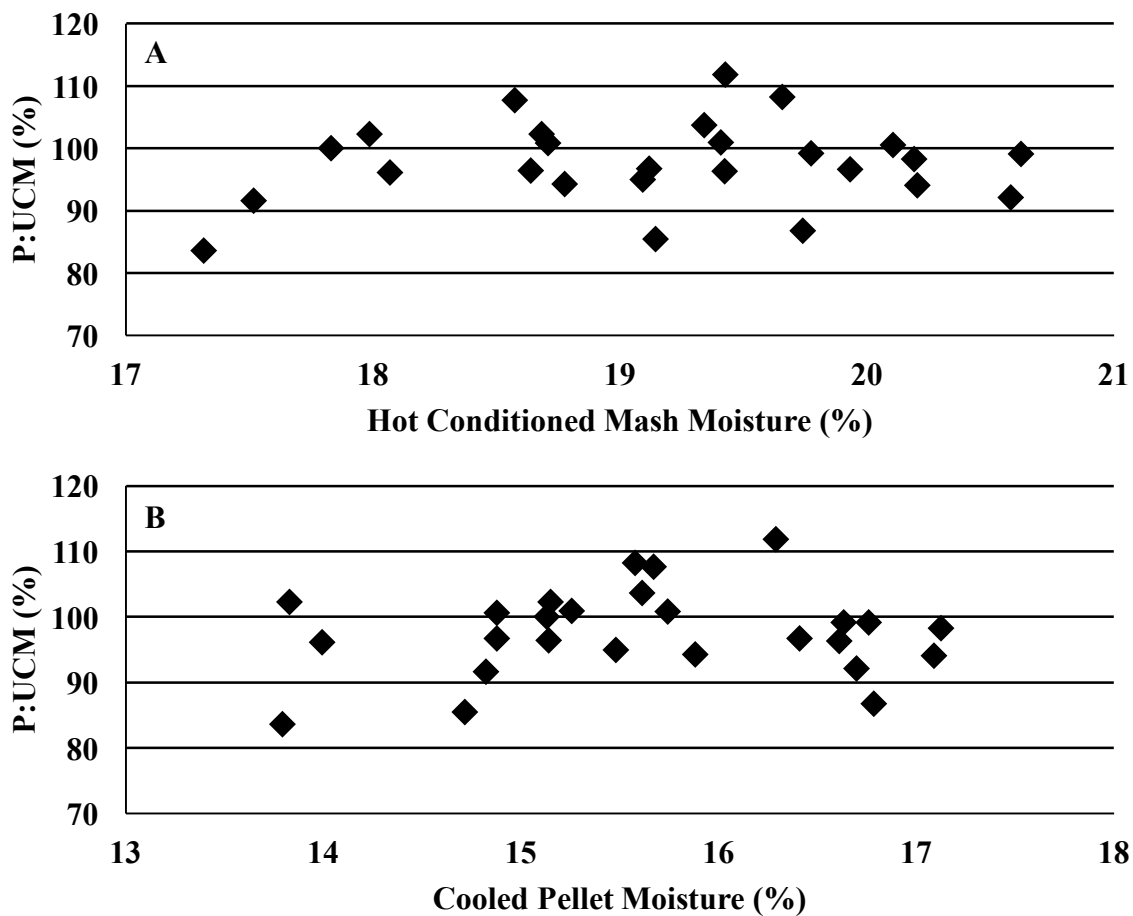


Figure II-6. Xylanase activity in pellets relative to unconditioned mash (P:UCM) vs. A) hot conditioned mash moisture ($P=0.514$; $R^2=0.02$) and B) cooled pellet moisture ($P=0.593$; $R^2=0.01$).

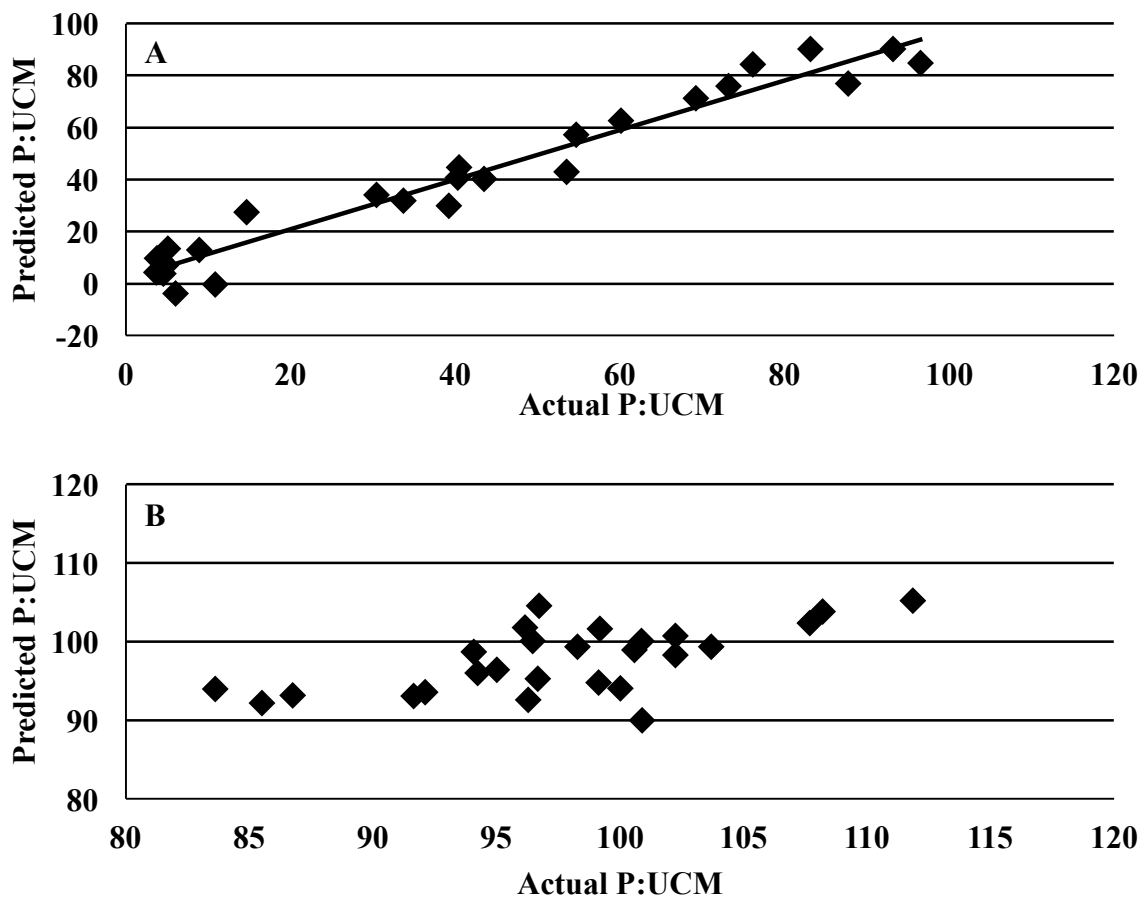


Figure II-7. Actual phytase (A) or xylanase (B) activity in pellets relative to unconditioned mash (P:UCM) vs. A) predicted phytase activity in P:UCM ($P=0.001$; $R^2=0.95$; $y=825.8+6.5*\text{mixer-added water}-4.4*\text{conditioning temperature}-4.1*\text{pellet durability index}-3.8*\text{change in temperature between hot pellets and conditioned mash}-4.4*\text{pellet mill energy consumption}$) and B) predicted xylanase activity in P:UCM ($P=0.162$; $R^2=0.40$).

CHAPTER III

The effect of pellet mill throughput and mash conditioning temperature on parameters monitored during pelleting and phytase and xylanase thermostability

SUMMARY

The use of enzymes in broilers feeds had become ubiquitous because of the benefits afforded concerning nutrient digestibility and dietary costs. However, mixer-added exogenous enzymes were susceptible to denaturation. Additionally, pilot scale pellet mills were considered to denature enzymes at a greater rate than commercial pellet mills. When pellet mill die specifications were coupled with pellet mill throughputs in research pellet mills and they were compared to commercial pellet mills, it was determined that feed was retained within research pellet mills for a greater duration. Therefore, the current investigation intended to determine the impact of pellet mill throughput, or pellet mill die feed retention, on mixer-added exogenous enzyme denaturation during the pelleting process. The present experiment was arranged as a 4 x 2 factorial where broiler starter rations amended with phytase and xylanase were subjected to four pellet mill throughput rates (PMT; 227, 454, 908, 1816 kg/hr) utilizing two mash conditioning temperatures (CT; 75 and 86°C). Parameters monitored during the pelleting process included pellet durability index (PDI) and the change in temperature between hot pellets and conditioned mash (ΔT). Although the xylanase was heat stable and experienced minimal activity loss, the phytase was subjected to denaturation and inactivation. When the PMT was 227 kg/hr, pellets that were conditioned at 75°C resulted in an 8 percentage-point reduction in phytase activity, while those conditioned at 86°C resulted in an 86.5 percentage-point reduction in phytase activity. However, when the PMT was increased to 1816 kg/hr, the phytase activities were statistically similar for diets pelleted at both 75 and 86°C. There was also a negative linear correlation between PDI and phytase recovery in pellets, especially for those diets pelleted at 86°C. Therefore, it was concluded that reducing the duration of feed retention within the

pellet mill die through increased PMT reduced the duration of exposure to heat and pressure within the pellet mill die, which decreased the extent of phytase denaturation in finished feeds. Future enzyme thermostability trials conducted utilizing pilot scale pellet mills should consider the working surface area and thickness of the pellet mill die, and then couple the pellet mill die specifications with a PMT that resembles feed retention within commercial scale pellet mills to make the interpretation of results more directly applicable to commercial scale operations.

DESCRIPTION OF PROBLEM

Enzymes have been regularly utilized in broiler diets to improve nutrient digestibility, reduce variation in ingredient quality, and to reduce ingredient costs [1, 2]. However, enzymes were determined to be susceptible to denaturation during the pelleting process [3-6]. Therefore, enzyme thermostability was routinely evaluated in product comparison studies and to characterize the thermotolerance of novel enzymes to ensure they could withstand the rigors of the pelleting process. Enzyme product evaluations frequently occurred at university operated feed mills that operated on a pilot scale, especially for new product evaluations due to ease of sampling, minimal raw material use, and batch consistency.

One example of a smaller-scale pellet mill utilized at research feed mills was the CPM 1112-2 or Master Model [7-12], which provided 85 in² of pellet mill die working surface area to pellet feeds. A typical production rate for these machines was one short ton/hr, thus providing 85 in² of pellet mill die working surface area per short ton/hr of feed pelleted. A commonly utilized pellet mill for commercial broiler feed production was the CPM 7932-12 [13], which provided 1251 in² of pellet mill die working surface area to pellet feeds. Feed manufacturers commonly operated the CPM 7932-12 at 60 short ton/hr, thus

providing 20.85 in² of pellet mill die working surface area per short ton/hr of feed pelleted. Therefore, when a research pellet mill was compared to a commercial pellet mill, the relationship between pellet mill die working surface area and pellet mill throughput (PMT) was determined to be non-linear. If the pellet mill die length-to-diameter ratio were held constant between the two machines, the feed pelleted through the research pellet mill at one short ton/hr would be retained within the pellet mill die nearly four times longer than feed pelleted through a standard commercial pellet mill die at 60 short ton/hr

Enzyme recoveries in research settings were often noted to be lower than those observed in commercial operations [14]. One of the reasons for this observation may have been the duration of feed retention within the pellet mill die when research pellet mills were utilized for enzyme thermostability evaluations. The experiment was conducted to determine whether pellet production rate, or feed retention within the pellet mill die, was a contributing factor to enzyme denaturation at different mash conditioning temperatures utilizing a partially thermostable phytase and a thermostable xylanase.

MATERIALS AND METHODS

Feed Manufacturing

The experiment was conducted by pelleting three 1910 kg batches of a broiler starter diet (Table III-1) that contained mixer-added phytase [15] and xylanase [16] to determine the effect of mash conditioning temperature (CT) and PMT on phytase and xylanase recovery post pelleting. The PMTs included 227, 454, 909, and 1818 kg/hr and the rations were conditioned and then pelleted at 75 or 86°C in a 4 x 2 factorial arrangement.

The batches were blended utilizing a twin shaft counterpoise ribbon mixer [17] for 180 s of dry mixing time and then 90 s of wet mixing time. The batches were then

conditioned for 30 s at 75 or 86°C in a single pass conditioner [18] and then pelleted utilizing a 30 HP CPM pellet mill [19] equipped with a 4.4 x 35.2 mm die with 548 cm² of working surface area. The steam pressure was 207 kPa. The pellet mill die was warmed with 455 kg of feed before proceeding with the experimental pelleting runs. After obtaining the appropriate mash CT, three conditioned mash and pellet samples were collected over a three-minute period of pelleting and were blended into composite samples. The conditioned mash samples were cooled to ambient temperature in custom manufactured 30 x 30 cm trays over open grates with circulating air and were analyzed for phytase [20] and xylanase [21] activity. The pellet samples were cooled in custom manufactured 30 x 30 cm trays, which were placed in a custom manufactured pellet cooler resembling a counterflow cooler and were analyzed for phytase and xylanase activity. The pellet mill feeder screw and mash conditioning chamber steam valve were adjusted to obtain samples at the desired temperatures and production rates. Samples were collected at a mash CT of 75°C and a production rate of 224 kg/hr first. After sample collection, the pellet mill feeder screw rate was incrementally increased to collect samples at the greater production rates. Once the samples had been collected at 75°C, the pellet mill feeder screw speed was reduced to a production rate of 224 kg/hr and the mash conditioning chamber steam valve was adjusted to condition mash at 86°C and then the sampling procedures were repeated for each of the production rates.

Parameters monitored during the pelleting process included the change in temperature between hot pellets and conditioned mash (ΔT) and pellet durability index (PDI). Pellet durability was determined by placing 100 g of pellets, screened with a #5 US sieve, into a pellet durability tester [22] for 30 s. Pellets were removed directly from the testing chamber

and weighed to represent a proportion of the initial mass added to the testing chamber, with each sample being tested in duplicate.

Statistical Analysis

The parameters monitored during feed manufacturing as well as the enzyme recoveries were analyzed as a 4 x 2 factorial arrangement. The Fit Model platform of JMP 13 [23] for ANOVA was employed and the means were separated utilizing the LSMeans procedure of JMP. Means were considered statistically significant at $P \leq 0.05$ and significant differences were separated utilizing Tukey's HSD test for the PMT factor and Student's t-test for the CT factor. The Fit Y x X platform of JMP 13 was employed to regress the association between the parameters monitored during feed manufacturing and enzyme recoveries. The regression models were considered statistically significant at $P \leq 0.05$.

RESULTS AND DISCUSSIONS

An interaction effect between mash CT and PMT was observed with regards to PDI (Table III-3; $P \leq 0.05$). When diets were pelleted at the slowest PMT (227 kg/hr), the PDI of diets pelleted at 75°C were statistically similar to those pelleted at 86°C. However, when PMT was increased to the fastest rate (1816 kg/hr), diets pelleted at 86°C were significantly more durable than those pelleted at 75°C. Previous research indicated that increased PMT resulted in decreased PDI [24] when mash CT was held constant. Additionally, as mash CT increased, PDI like increased due to improved agglomeration of ingredients [7]. Thomas *et al.* [25] described the impact of lengthening the duration of feed contact with the interior of the pellet mill die. When material remained in the compacted state for a longer duration, the materials further plasticized and the elastic components became more relaxed to improve PDI [26]. While these individual principles of mash CT and PMT were upheld, the current data

suggested that pellet durability was dependent not only upon mash CT, but also the duration of exposure to heat and pressure within the pellet mill die. As evidence by an increased in ΔT , diets pelleted at 75°C gained more frictional heat while transitioning through the pellet mill die when compared to diets pelleted at 86°C, likely due to decreased lubrication at the pellet mill die as a result of decreased levels of steam [27] (Table III-2; $P \leq 0.01$).

Xylanase recoveries were not affected by mash CT or PMT, which indicated that the xylanase product was heat stable (Table III-4). However, the recovery of phytase in pellets relative to unconditioned mash (P:UCM) was subjected to an interaction effect between mash CT and PMT (Table III-4; $P \leq 0.05$). The poorest phytase recoveries in P:UCM were observed when diets were conditioned at 86°C at the slowest PMT (227 kg/hr). However, when diets were conditioned at 86°C at the fastest PMT (1816 kg/hr), the phytase recoveries in P:UCM were statistically similar to all the diets pelleted at 75°C. The range in phytase recoveries in P:UCM for all PMTs when rations were pelleted at 75°C was 46 percentage-points, while the recoveries in diets pelleted at 86°C ranged 97.5 percentage-points.

These data indicated that enzyme denaturation during the pelleting process was both temperature and time dependent. The effect of enzyme exposure to heat for varying durations was well described in previous reports. Inbarr and Bedford [3] investigated the impact of mash CT and time on β -glucanase thermostability and determined that enzyme denaturation was exacerbated by exposure to heat, especially for greater durations of time. In the current study, mash conditioning time was held constant, but the retention within the pellet mill die was varied. Diets pelleted at 227 kg/hr were retained within the pellet mill die eight times longer than diets pelleted at 1816 kg/hr. While enzyme denaturation was previously determined to increase as the duration of exposure to steam within the conditioning chamber

increased, these data suggested that denaturation may also be exacerbated by increased retention within the pellet mill die when high pelleting temperatures were utilized. When feed was retained within the pellet mill die for a greater duration, the enzyme likely experienced greater levels of shear force, pressure, and heat, all of which negatively impacted the native structure of proteins [28]. Feed manufacturers should be cautious of methods to improve pellet quality that include slow PMTs in combination with high mash CTs due to their compounded impact on heat labile enzymes and possibly other nutrients, such as lysine and vitamins.

The recovery of phytase in P:UCM was correlated with PDI (Figure III-1; $P=0.001$; $R^2=0.56$) and ΔT (Figure III-1; $P=0.001$; $R^2=0.63$). A negative correlation existed between PDI and phytase recovery in P:UCM, which indicated that the methods utilized herein that resulted in improved PDI negatively impacted phytase stability. The forces and conditions necessary for particle adhesion to achieve physical feed quality, such as heat, moisture, and pressure, likely denatured the heat labile phytase. A positive correlation was observed for phytase recovery in P:UCM and ΔT . This most likely occurred since the greatest levels of frictional heat were generated at the coolest pelleting temperature (75°C), which was not warm enough to inactivate or denature the phytase. However, if the mash CT were held constant, factors that increase frictional heat transfer into pellets would likely further denature the enzyme, especially if the mash CT were beyond the threshold of thermotolerance [29].

Regression plots for phytase recovery in P:UCM correlated with PDI separated between 75 and 86°C provided additional information on the relationship between physical and nutritional feed quality. When diets were pelleted at 75°C , there was no significant

correlation between PDI and phytase recovery in P:UCM (Figure III-2; $P=0.10$; $R^2=0.27$). However, when diets were pelleted at 86°C, there was a strong linear correlation between PDI and phytase recovery in P:UCM (Figure III-2; $P=0.001$; $R^2=0.96$). These data indicated that feed manufactures should approach solutions to improve physical feed quality judiciously, and perhaps without the use of additional steam during the pelleting process. Similar PDI values were obtained when diets were pelleted at 75 and 86°C, although significant phytase denaturation occurred primarily at 86°C, which indicated that methods utilized to improve physical feed quality independent of steam, such as reduced levels of mixer-added fat [10, 30, 31] or the inclusion of wheat [32], may be superior for nutrient preservation when compared to the use of additional heat or moisture during the pelleting process, although the impact of these factors on enzyme thermostability were not investigated in the current report.

The data reported herein also provided information for future enzyme thermostability trials. When feed was retained within the pellet mill die for a greater duration, enzyme recovery was reduced. However, the denaturation was alleviated through increased PMT. If all the feeds were pelleted at 908 kg/hr, different conclusions concerning the thermostability of the phytase utilized would have been drawn. To better correlate with industry standard conditions, future enzyme thermostability trials conducted within a research setting should consider reporting mash conditioning temperature and time, steam pressure, pellet mill die hole length and diameter, pellet mill die working surface area, and pellet mill throughput to calculate feed retention within the pellet mill die in order for researchers to make reasonable interpretations of their observations.

CONCLUSIONS AND APPLICATIONS

1. Increased PMT was associated with improved phytase recovery likely due to decreased feed retention time within the pellet mill die.
2. Increased PMT negatively affected PDI.
3. As PDI increased, phytase recovery in P:UCM decreased, especially when diets were pelleted at 86°C.
4. Future enzyme thermostability trials should consider a PMT and pellet mill die working surface area and length-to-diameter ratio that emulates industry relevant feed retention within the pellet mill die to better characterize the thermostability of an enzyme in practical scenarios.

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Table III-1. Ingredient composition of broiler starter diets containing phytase and xylanase.

Ingredients	(%)
Corn	58.12
SBM (48%)	34.77
Poultry by-product Meal	2.26
Poultry fat	2.00
Limestone	0.84
Salt	0.50
Titanium dioxide	0.50
DL-Methionine	0.24
Choline chloride (60%)	0.20
Trace mineral premix ¹	0.20
Dicalcium phosphate (18.5% P)	0.14
L-Lysine	0.08
Vitamin premix ²	0.05
Selenium premix ³ (0.06%)	0.05
Phytase ⁴	0.02
L-Threonine	0.02
Xylanase ⁵	0.01
Calculated nutrients	
Protein	22.95
Calcium	0.85
Available phosphorous	0.43
Total lysine	1.29
ME, kcal/g	3.00

¹ Trace mineral premix provided the following per kg of diet: Mn, 120 mg; Zn, 120 mg; Fe, 80 mg; Cu, 10 mg; I, 2.5 mg; Co, 1 mg.

² Vitamin premix provided the following per kg of diet: vitamin A, 6600 IU; vitamin D3, 1980 IU; vitamin E, 33 IU; vitamin B12, 0.02 mg; biotin, 0.13 mg; menadione, 2 mg; thiamine, 2 mg; riboflavin, 6.6 mg; pantothenic acid, 11 mg; vitamin B6, 4 mg; niacin, 55 mg; folic acid, 1.1 mg.

³ Selenium premix provided Se at 0.3 mg/kg of feed.

⁴ Quantum Blue 5G was added to provide 1000 FTU/kg of feed.

⁵ Econase XT was added to provide 16,000 BXU/kg of feed.

Table III-2. Main effects of mash conditioning temperature (CT) and pellet mill throughput (PMT) on pellet durability index (PDI) as determined by the Holmen method for 30 s of testing and the change in temperature between hot pellets and conditioned mash (ΔT).

CT (°C)	PMT (kg/hr)	n	PDI (%)	ΔT (°C)
Main Effects				
75		12	82.7 ^B	6.7 ^A
86		12	89.5 ^A	2.5 ^B
<i>P</i> -value			0.001	0.001
SEM ¹			0.6	0.3
	227	6	94.9 ^A	2.7 ^B
	454	6	89.6 ^B	4.7 ^A
	908	6	84.5 ^C	5.6 ^A
	1816	6	75.6 ^D	5.3 ^A
	<i>P</i> -value		0.001	0.001
	SEM ¹		0.7	0.3

^{A, B, C, D} Means within each column that possess different superscripts differ significantly ($P \leq 0.01$).

¹ Standard error of mean (SEM) for n=12 samples for each CT and n=6 samples for each level of PMT.

Table III-3. Interaction effects of mash conditioning temperature (CT) and pellet mill throughput (PMT) on pellet durability index (PDI) as determined by the Holmen method for 30 s of testing and the change in temperature between hot pellets and conditioned mash (ΔT).

CT (°C)	PMT (kg/hr)	n	PDI (%)	ΔT (°C)
Interaction Effects				
75	227	3	93.6 ^a	4.5
75	454	3	86.3 ^b	7.3
75	908	3	81.0 ^c	7.5
75	1816	3	70.1 ^d	7.3
86	227	3	96.3 ^a	0.8
86	454	3	92.9 ^a	2.1
86	908	3	87.9 ^b	3.7
86	1816	3	81.0 ^c	3.3
	<i>P</i> -value		0.020	0.556
	SEM ¹		1.1	0.5

^{a, b, c, d} Means within each column that possess different superscripts differ significantly ($P \leq 0.05$).

¹ Standard error of mean (SEM) for n=3 samples for each interaction of CT and PMT.

Table III-4. Main effects of mash conditioning temperature (CT) and pellet mill throughput (PMT) on the relative activity of phytase and xylanase in conditioned mash compared to unconditioned mash (CM:UCM), pellets compared to conditioned mash (P:CM), and pellets compared to unconditioned mash (P:UCM).

CT (°C)	PMT (kg/hr)	n	Relative Phytase Recovery ¹			Relative Xylanase Recovery ²		
			CM:UCM	P:CM	P:UCM	CM:UCM	P:CM	P:UCM
Main Effects								
75		12	124.0	95.4 ^A	114.8 ^A	81.0	98.6	79.2
86		12	100.7	55.8 ^B	60.7 ^B	86.8	94.7	81.9
<i>P</i> -value			0.069	0.001	0.001	0.134	0.461	0.469
SEM ³			8.4	6.5	5.0	2.6	3.6	2.5
	227	6	87.7	58.3	52.8 ^C	77.7	96.1	74.0
	454	6	105.0	76.9	83.4 ^B	86.7	95.1	81.4
	908	6	127.1	70.8	90.3 ^B	86.5	95.3	82.0
	1816	6	129.4	96.5	124.5 ^A	84.8	100.1	84.7
<i>P</i> -value			0.079	0.067	0.001	0.300	0.885	0.223
SEM ³			11.8	9.2	7.1	3.7	5.0	3.6

^{A, B, C} Means within each column that possess different superscripts differ significantly ($P \leq 0.01$).

¹ Quantum Blue 5G. Testing method was in accordance with ELISA specific for Quantum Blue, ESC Standard Analytical Method, SAM099; AB Vista, Marlborough, UK.

² Econase XT. Testing method was in accordance with ELISA specific for Econase XT, ESC Standard Analytical Method, SAM115; AB Vista, Marlborough, UK.

³ Standard error of mean (SEM) for n=12 samples for each CT and n=6 samples for each PMT.

Table III-5. Interaction effects of mash conditioning temperature (CT) and pellet mill throughput (PMT) on the relative activity of phytase and xylanase in conditioned mash compared to unconditioned mash (CM:UCM), pellets compared to conditioned mash (P:CM), and pellets compared to unconditioned mash (P:UCM).

CT (°C)	PMT (kg/hr)	n	Relative Phytase Recovery ¹			Relative Xylanase Recovery ²		
			CM:UCM	P:CM	P:UCM	CM:UCM	P:CM	P:UCM
Interaction Effects								
75	227	3	115.9	88.9	92.0 ^{bc}	71.0	97.7	68.3
75	454	3	116.3	106.2	122.0 ^{ab}	80.1	100.2	79.3
75	908	3	132.1	80.9	107.0 ^b	81.8	97.8	79.5
75	1816	3	131.5	105.8	138.0 ^a	91.1	98.5	89.7
86	227	3	59.6	27.7	13.5 ^e	84.4	94.5	79.8
86	454	3	93.8	47.6	44.7 ^d	93.2	89.9	83.5
86	908	3	122.2	60.6	73.7 ^{cd}	91.2	92.9	84.4
86	1816	3	127.4	87.1	111.0 ^{ab}	78.4	101.6	79.7
	<i>P</i> -value		0.431	0.232	0.035	0.071	0.824	0.229
	SEM ³		16.7	13.0	10.1	5.2	7.1	5.0

a, b, c, d, e Means within each column that possess different superscripts differ significantly ($P \leq 0.05$).

¹ Quantum Blue 5G. Testing method was in accordance with ELISA specific for Quantum Blue, ESC Standard Analytical Method, SAM099; AB Vista, Marlborough, UK.

² Econase XT. Testing method was in accordance with ELISA specific for Econase XT, ESC Standard Analytical Method, SAM115; AB Vista, Marlborough, UK.

³ Standard error of mean (SEM) for n=3 samples for each interaction of CT and PMT.

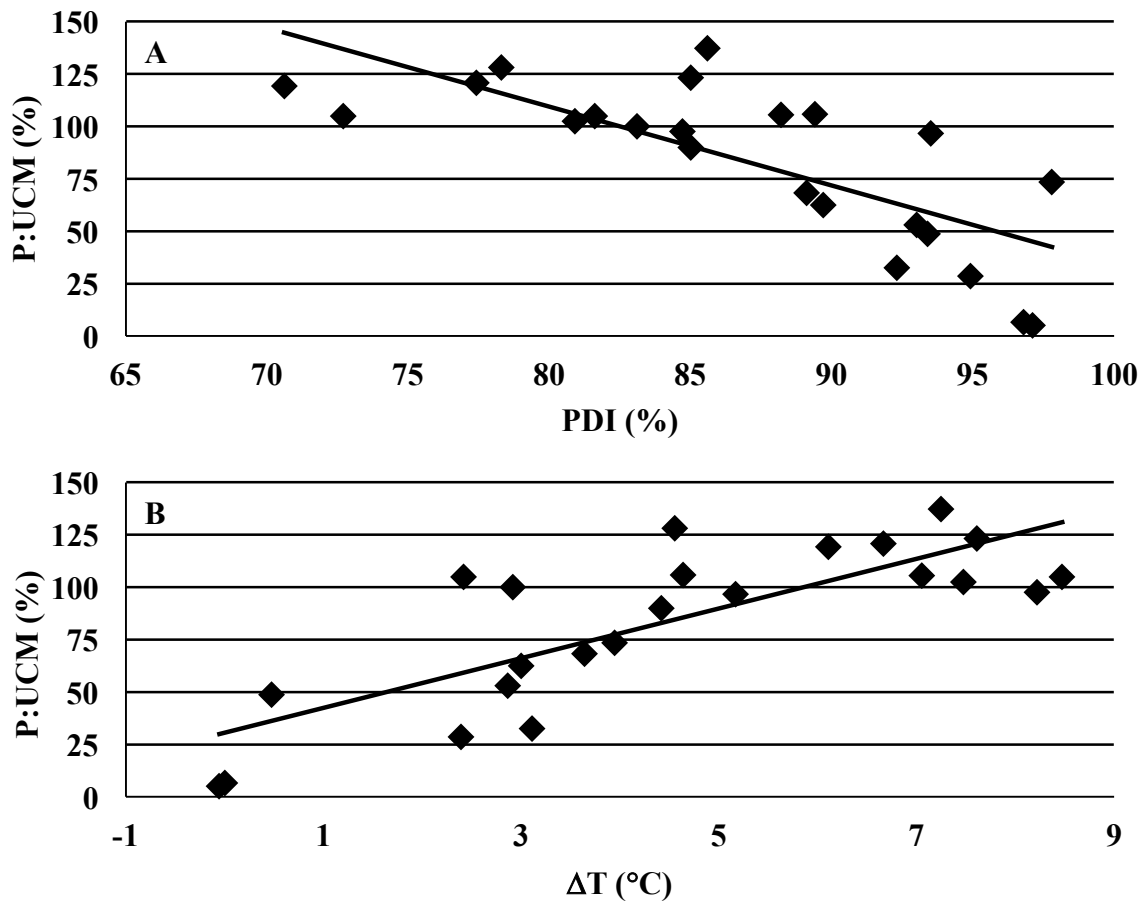


Figure III-1. Phytase activity in pellets relative to unconditioned mash (P:UCM) vs. A) pellet durability index (PDI) ($P=0.001$; $R^2=0.56$; $y=410.0-3.8*PDI$) and B) change in temperature between hot pellets and conditioned mash (ΔT) ($P=0.001$; $R^2=0.63$; $y=30.7+11.8*\Delta T$).

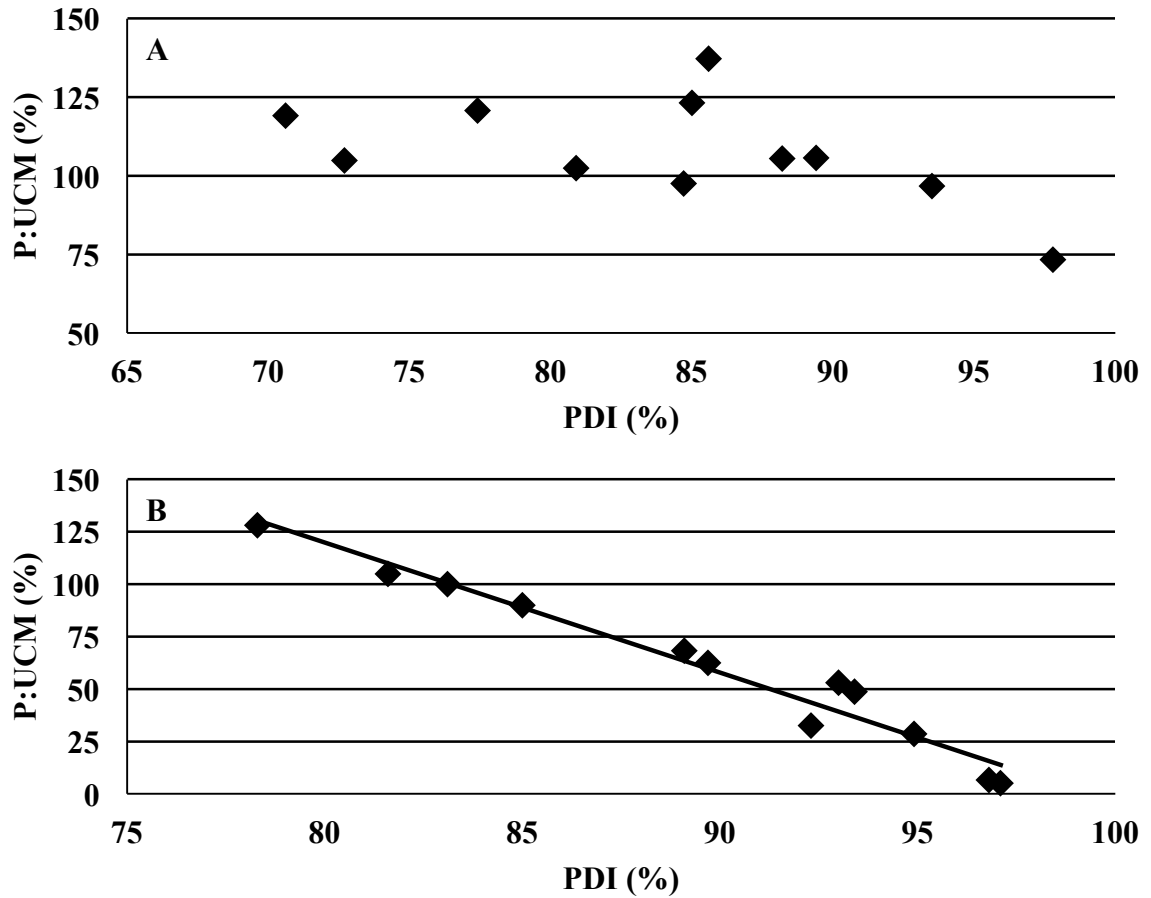


Figure III-2. Phytase activity in pellets relative to unconditioned mash (P:UCM) vs. pellet durability index (PDI) of diets pelleted at A) 75°C ($P=0.100$; $R^2=0.27$) and B) 86°C ($P=0.001$; $R^2=0.96$; $y=615.3-6.2*PDI$).

CHAPTER IV

The effect of fat application site and pellet mill die L:D on phytase thermostability and subsequent broiler growth and bone characteristics

SUMMARY

Modern broiler rations were amended with phytase to improve nutrient utilization and reduce dietary cost, but phytases were often subjected to denaturation during the pelleting process. The thermostability of phytases was well reported in regard to their ability to withstand heat added during the mash conditioning process through increased levels of steam. Increased levels of steam during the conditioning process were associated with improved physical feed quality, which improved broiler live performance parameters. However, feed manufacturers also utilized other methods to improve physical feed quality, such as decreased levels of mixer-added fat (MAF) and increased pellet mill die length-to-diameter ratios (L:D), without fully understanding their impact on enzyme thermostability. Therefore, a trial was conducted to determine the impact of fat application site (FAS) and pellet mill die L:D on phytase thermostability and subsequent broiler growth performance and bone characteristics. A positive control (PC) diet was formulated to meet the nutritional requirements of broilers. A negative control (NC) diet was formulated to be inadequate in calcium (Ca) and available phosphorous (AvP) and then was amended with 1000 FTU/kg phytase (NC+PHY). The NC + PHY diets were subjected to a 2 x 2 factorial arrangement of FAS and pellet mill die L:D. The FASs included 3.5% MAF or 0.5% MAF with the remaining 3.0% being applied via post-pellet liquid application (PPLA). The pellet mill die L:Ds included 8:1 and 10:1 and all the diets were pelleted at 86°C and then crumbled to remove the confounding effect of feed form on broiler growth performance. The PC, NC, and NC+PHY diets were fed to broilers from 0-35 d of age as a single-phase diet to allow for one-time manufacturing and to remove the confounding effects of ambient conditions on the pelleting process and subsequent enzyme thermostability. A 96-pen broiler house was

utilized with 20 male broilers in each pen, which provided 16 replicate pens per diet. All the feeds were tested for phytase recovery and broiler BW and feed intake were collected weekly starting at 7 d and FCR was calculated. At 14 and 28 d, the left tibia of two broilers per pen were excised and tibia bone breaking strength (BBS) and bone ash was determined. Diets pelleted with PPLA fat retained 523 FTU/kg of phytase activity, while those pelleted with MAF retained 935 FTU/kg of phytase activity ($P \leq 0.01$). The pellet mill die did not affect phytase recovery. At 35 d, broilers offered the NC+PHY diets exhibited improved BW and feed intake ($P \leq 0.01$) when compared to the NC, although there were no significant differences at 35 d in broiler FCR. At 14 d, broilers consuming NC+PHY diets pelleted with MAF mineralized bones to the same extent as the PC, while those consuming NC+PHY diets pelleted with PPLA fat were not able to mineralize bone to the extent of the PC ($P \leq 0.01$). At 28 d of age, the tibia BBS of broilers consuming NC+PHY diets pelleted with MAF was greater than tibias from NC fed broilers, while the BBS of those consuming NC+PHY diets pelleted with PPLA fat was not ($P \leq 0.01$). It was concluded that the level of MAF utilized during the pelleting process may impact phytase recovery and may differentially impact the ability of mixer-added phytase to release calcium and phosphorous for utilization in broiler bones as evidenced by tibia BBS and bone ash.

DESCRIPTION OF PROBLEM

Mixer-added exogenous enzymes were utilized in broiler rations to improve nutrient digestibility and reduce feed costs [1]. One of the most commonly utilized enzymes was phytase, which hydrolyzed phytic acid to improve phosphorous, calcium, amino acid, and energy digestibility while reducing the levels of inorganic phosphates supplemented in broiler feeds [2-6]. During the pelleting process, enzymes were subjected to heat and

pressure, which was determined to alter the structure and functionality of some mixer-added enzymes, rendering them unable to perform their catalytic activities [7-10]. While alternative methods to include enzymes in feed were available, such as post-pellet liquid application (PPLA) systems, their relatively poor precision and cost of operation and maintenance made mixer-added exogenous enzymes the preferred choice for feed manufacturers [9, 11].

Feed manufactures were also pressured to maintain good physical feed quality because of the inherent benefits provided to broilers when improved feed forms were offered, such as improved energy utilization and the subsequent BW gain and FCR improvements associated with the more efficient prehension of rations [12]. There were several factors that affected physical feed quality which included feed formulation, conditioning temperature, grain particle size, pellet mill die specifications, and pellet cooling practices [13]. The majority of enzyme thermostability trials were conducted utilizing a range of mash conditioning temperatures to determine the thermostability of enzymes [7-10]. Utilizing this method, enzyme suppliers were able to determine the threshold of thermotolerance for specific enzymes and subsequently make recommendations on the maximum mash conditioning temperatures for enzyme products. Feed manufacturers often utilized greater levels of steam in feeds to improve physical feed quality as well as feed hygiene [14]. However, mash conditioning temperatures were not the only determining factors that explained the level of heat and pressure that an enzyme may have experienced during the pelleting process.

Other factors that influenced the level of heat and pressure generated during the pelleting process included feed formulation and pellet mill die specifications [15, 16]. Liquid fats were often included in broiler rations to improve dietary energy density, but negatively

impacted physical feed quality [17, 18]. Thus, PPLA fat systems were implemented as a solution to improve dietary energy density without adversely affecting physical feed quality. Removing liquid fats from rations during pelleting was also associated with increased levels of frictional heat and increased pellet mill die backpressure [18], which may have had negative consequences for enzyme retention during pelleting. Gehring *et al.* [19] alluded to this theory in a previous report and suggested that greater levels of mixer-added fat (MAF) during the pelleting process may have improved exogenous enzyme thermostability.

The pellet mill die may have also negatively impacted mixer-added enzyme retention due to the heat and pressure associated with feed passing through the pellet mill die. A thicker pellet mill die, or a pellet mill die with a greater length-to-diameter ratio (L:D), contained greater pellet mill die hole wall surface area and retained feed for a greater duration, which may have prolonged and extended the level of heat and pressure an enzyme was subjected to during the pelleting process [15]. However, thicker pellet mill dies were associated with improved physical feed quality, and thus were utilized to maintain feed form.

The impact of mash conditioning temperature on enzyme thermostability [7-10] and physical feed quality [17, 20] was well described in previous reports. However, increasing mash conditioning temperatures was not the only method implemented to improve physical feed quality. Feed manufacturers often utilized PPLA fat systems and thicker pellet mill dies to improve physical feed form, but minimal data existed to determine the impact of methods utilized to improve physical feed quality, other than mash conditioning temperatures on nutritional feed quality. Thus, an experiment was conducted to determine how the level of MAF, pellet mill die L:D, and the interaction thereof impacted the thermostability of a phytase when mash conditioning temperatures were held constant. The feeds were analyzed

for *in vitro* phytase recoveries and then fed to broilers from 0 to 35 d of age to determine the impact of the feed manufacturing techniques on subsequent phytase performance through broiler growth and bone characteristic assays.

MATERIALS AND METHODS

Feed Manufacturing

The experiment utilized two formulations that represented a negative control (NC) that contained inadequate levels of available phosphorous (AvP) and calcium (Ca) and a positive control (PC) that contained adequate levels of AvP and Ca (Table IV-1) fed as one phase from 0-35 d. An additional four diets were generated by amending the NC with 1000 FTU/kg of phytase [21] to achieve the AvP and Ca levels of the PC and then subjecting them to a 2 x 2 factorial arrangement of MAF and pellet mill die L:D during feed manufacture to determine whether feed manufacturing techniques affected phytase thermostability and subsequent broiler growth performance and bone characteristics. The fat application sites (FAS) included 3.5 or 0.5% MAF with the remaining 3.0% being applied via post-pellet liquid application (PPLA). The pellet mill die L:Ds included 8:1 (35.2 x 4.4 mm) and 10:1 (44.0 x 4.4 mm) and both pellet mill dies contained 548 cm² of working surface area. The diets were fed as one phase from 0-35 d to minimize the potential confounding effects of ambient conditions on the pelleting process and subsequent phytase recoveries while retaining the ability to replicate the feed manufacturing process. The NC diets containing phytase subjected to the 2 x 2 factorial were replicated four times with each replicate batch assigned to four replicate pens within the grow-out facility. All diets were manufactured from basals to reduce ingredient variation between each batch. The basals were blended with a twin shaft counterpoise ribbon mixer [22] for 180 s of dry mixing time. The individual

batches were amended with ingredients and then mixed for an additional 180 s of dry mixing time and 90 s of wet mixing time.

The pelleting process occurred over a period of two days. A 773 kg batch of the PC was conditioned for 30 s at 86°C in a single pass conditioner [23] and then pelleted at a production rate of 908 kg/hr utilizing a 30 HP CPM pellet mill [24] equipped with the L:D 8:1 pellet mill die. The steam pressure was 207 kPa. The pellet line was flushed and then four 455 kg batches, two containing 3.5% MAF and two containing 0.5% MAF, were pelleted in alternating order under the same set of conditions used for pelleting the PC. After all diets were manufactured on the L:D 8:1 pellet mill die, the pellet mill die was removed and replaced with the L:D 10:1 pellet mill die. A 773 kg batch of the NC was pelleted first, and then four 455 kg batches, two containing 3.5% MAF and two containing 0.5% MAF, were pelleted in alternating order under the same set of conditions. Any feed that did not meet the criteria of $\pm 1^\circ\text{C}$ of 86°C was discarded. The diets were cooled in a counterflow cooler [25] and were crumbled [26] to remove the confounding effect of feed form on broiler performance. After being cooled, the diets that were pelleted with 0.5% MAF were amended with 3.0% PPLA fat utilizing a mixer [22]. The process was repeated on the second day of feed manufacture.

During the pelleting process, three conditioned mash samples were collected over a ten-minute period of pelleting and were blended into composite samples. The conditioned mash samples were cooled in custom manufactured 30 x 30 cm trays over open grates with circulating air and were analyzed for phytase activity [27]. Crumbles were sampled during bagging and were analyzed for phytase activity.

Broiler Management

All broiler rearing and handling procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee. The rearing of broilers occurred in a curtain sided house with PVC and wire partitions on a concrete floor covered with new pine shavings. A total of 96 1.2 x 3.8 m pens were used, which allotted 16 replicate pens per dietary treatment. For each replicate batch of the NC amended with 1000 FTU/kg of phytase that was subjected to various feed manufacturing techniques, there were 4 replicate pens within the grow-out facility. Each pen contained one bell-type drinker and one tube feeder.

A total of 1920 Ross 708 [28] males were allocated to one of 6 dietary treatments, which provided 20 chicks in each pen. Eggs were sourced from a commercial breeder flock and were incubated at the grow-out facility hatchery. The chicks were fed a single-phase diet from 0-35 d. The lighting schedule was 23 h of light to 7 d, 22 h to 14 d, 21 h to 21 d, and 14 h thereafter. The litter brooding temperatures were 36°C at placement and then incrementally decreased to 24°C beyond 21 d.

Data Collection

Parameters monitored during the pelleting process included the change in temperature between hot pellets and conditioned mash (ΔT) and pellet durability index (PDI). The PDI of individual samples was determined by placing 100 g of pellets, screened with a #5 US sieve, into a pellet durability tester [29] for 30 s. Pellets were removed directly from the testing chamber and were weighed to represent a proportion of the initial mass added to the testing chamber, with each sample being tested in duplicate.

Live performance data collection included group BW at placement, 7, 21, and 35 d and individual BW at 14 and 28 d. Feed weighbacks were also completed at the time of BW collection to determine feed intake (FI) and adjusted feed conversion ratio (FCR). The FCR was calculated based on BW gain from hatch and was adjusted by adding the BW of any mortality from each pen into the FCR equation.

At 14 and 28 d, two birds from each pen that represented the pen median BW were euthanized for left tibia collection. The flesh of the tibiae was removed before being broken in the center utilizing a texture analyzer [30]. The tibiae were centered on a fulcrum with a width of 3.0 and 4.5 cm for tibiae collected at 14 and 28 d, respectively. A probe was attached to a 250 kg load cell with a crosshead speed of 3 mm/s. After breaking the tibiae, they were dried for 24 h at 100°C, placed in ethyl ether for 48 h, dried for an additional 24 h at 100°C, and then ashed utilizing a muffle furnace at 600°C for 24 h.

Statistical Analysis

The parameters monitored during feed manufacturing as well as the enzyme recoveries were analyzed as a 2 x 2 factorial design. The Fit Model platform of JMP 13 [31] for ANOVA was employed and the means were separated utilizing the LSMeans procedure of JMP. Means were considered statistically significant at $P \leq 0.05$ and significant differences were separated utilizing Student's t-test. The live performance and bone characteristics data were analyzed as a one-way ANOVA utilizing the Fit Model Platform of JMP 13 and the means were separated utilizing the LSMeans procedure of JMP. Means were considered statistically significant at $P \leq 0.05$ and significant differences were separated utilizing Tukey's HSD test.

RESULTS AND DISCUSSIONS

Feed Manufacturing and Phytase Recovery

Both PDI ($P \leq 0.05$) and ΔT ($P \leq 0.01$) were significantly affected by FAS and pellet mill die L:D (Table IV-2). The PDI of diets pelleted with PPLA fat or through an L:D 10:1 pellet mill die was 11.8 and 12.5 percentage-points greater than diets pelleted with MAF or through an L:D 8:1 pellet mill die, respectively. Likewise, the ΔT of diets pelleted with PPLA fat or through an L:D 10:1 pellet mill die was 2.5 and 4.1°C greater than diets pelleted with MAF or through an L:D 8:1 pellet mill die, respectively. Increased levels of fat during the pelleting process had been previously demonstrated to reduce PDI and heat transfer into pellets as they were formed within the pellet mill die [17-19]. Fat was understood to act as a lubricant during the pelleting process which reduced frictional heat transfer, but also inhibited the inherent binding properties of proteins and starches [16]. The duration of pellet contact with the pellet mill die hole wall was also demonstrated to impact physical feed quality and frictional heat transfer, such as when the pellet mill die L:D was increased [15]. As the surface area for feed contact with the pellet mill die hole wall increased, PDI and ΔT also increased. This likely occurred due to increased shear and friction within the pellet mill die as well as increased backpressure which further plasticized elastic nutrients to maximize their binding potential.

There were no significant differences in phytase recovery in conditioned mash relative to unconditioned mash (Table IV-3), which indicated that the factors applied during feed manufacturing did not impact phytase activity loss during conditioning. However, the diets that were pelleted with all the dietary fat as MAF contained 124% greater levels of phytase in crumbles relative to conditioned mash (C:CM) when compared to diets that were

pelleted and then received PPLA fat ($P \leq 0.01$). Comparing phytase recoveries in C:CM provided insight towards phytase denaturation within the pellet mill die. These data indicated that fat conferred protection to the phytase as it traversed the pellet mill die, although it did not confer protection to the phytase within the conditioning chamber. The protection afforded via increased MAF during pelleting resulted in 96% greater levels of phytase in MAF diets when compared to PPLA diets in finished feed ($P \leq 0.05$). The L:D of the pellet mill die through which the feed was extruded did not significantly affect phytase recovery (Table IV-3).

These data agreed with the findings of Gehring *et al.* [19] in which increased levels of fat during the pelleting process was proposed to improve enzyme efficacy via improved enzyme retention during pelleting as evidenced through live performance indicators, although enzyme assays were not specifically performed. Increased levels of fat during the pelleting process likely improved the thermostability of the phytase utilized through one or more mechanisms. The increased levels of fat either reduced frictional heat transfer to the extent that the phytase was spared or insulated the phytase from the pressure and heat of the pellet mill die, with the latter being more likely since similar increases in ΔT were observed for PPLA diets as well as diets pelleted through an L:D 10:1 pellet mill die even though the FAS was the only factor that affected phytase recovery. It was also possible that the phytase utilized was sensitive to pressure and that the pressure within the pellet mill die increased to the greatest extent when PPLA diets were pelleted, although pellet mill die backpressure was not directly measured. These data indicated that feed manufacturers utilizing PPLA systems should be cautious when applying the majority or all of their liquid fats subsequent to

pelleting since fats were able to spare enzyme recovery in this trial and may spare other heat sensitive nutrients or ingredients such as lysine and vitamins.

Broiler Live Performance

Broiler feed intake was significantly affected at all ages recorded by the treatments applied (Table IV-4). Broilers fed NC + PHY diets pelleted through an L:D 10:1 pellet mill die with PPLA fat consumed greater amounts of feed when compared to the PC at 7 d ($P \leq 0.05$), the PC and NC at 14 and 21 d ($P \leq 0.01$), and then compared to the NC at 28 and 35 d ($P \leq 0.01$). By 35 d, broilers that consumed NC + PHY or PC diets, regardless of the pelleting techniques, consumed greater amounts of feed when compared to the NC. Feeding diets formulated to inadequate levels of AvP often resulted in decreased feed intake in broilers [9, 32, 33]. The decreased feed intake associated with reduced levels of AvP in broiler feeds was corrected by increasing the level of AvP in rations with inorganic phosphate sources or phytase, which agreed with the data reported herein.

Broiler BW was also affected by the treatments applied (Table IV-5). The BW of broilers fed the NC diet was significantly less than broilers fed the NC + PHY diets, regardless of feed manufacturing technique, at 21, 28, and 35 d of age ($P \leq 0.01$). These data agreed with previous reports which indicated that alleviating AvP deficiencies with phytase would improve overall broiler BW [9, 32, 33]. The BW of broilers fed the NC when compared to the NC + PHY diets was directly related to feed intake. The broilers fed the NC diets consumed less feed when compared to the NC + PHY diets at 21, 28, and 35 d, and thus a decrease in BW ensued.

The FCR of broilers was affected only at 14 d of age (Table IV-6; $P \leq 0.01$). While the NC and PC converted feed to body mass at a similar rate, there were significant

differences attributed to FAS and pellet mill die L:D for broilers fed the NC + PHY rations. Broilers consuming the NC + PHY ration pelleted with MAF through a pellet mill die with an L:D of 8:1 exhibited improved FCR when compared diets pelleted with PPLA fat through an L:D 10:1 pellet mill die. These data may be directly related to the phytase recoveries previously mentioned. Only FAS significantly affected phytase recoveries, but a numerical trend was observed that may explain, in part, the effect of feed manufacturing technique on broiler FCR at 14 d when fed NC + PHY rations. The diets pelleted with PPLA fat through an L:D 10:1 pellet mill die contained an average phytase level of 431 FTU/kg, while the diets pelleted with MAF through an L:D 8:1 pellet mill die contained an average phytase level of 1053 FTU/kg. It was expected that the most rigorous pelleting techniques which generated the greatest amount of pressure and frictional heat would be the most detrimental to phytase recoveries, such as when diets were pelleted with PPLA fat through an L:D 10:1 pellet mill die. These diets contained the least amount of phytase, and the broilers consuming these diets were less efficient with feed when compared to the those consuming the diets representing the least rigorous pelleting conditions for pressure and frictional heat, which were the MAF diets pelleted through an L:D 8:1 pellet mill die.

Although the FCR differences observed at 14 d were not persistent, the extra-phosphoric effects of phytase may explain, in part, these findings. Clear data existed to substantiate the extra-phosphoric effects of phytase [4, 6], which included improved energy and amino acid digestibility. However, it was also clear that the age of broilers, dietary ingredient composition, and formulated nutrient levels influenced the phosphorous, amino acid, and energy digestibility improvements associated with feeding phytase [1, 34]. The diets utilized for the current report were formulated below the published recommended

dietary protein levels for the broiler genetics utilized since the diets were fed as a single phase to 35 d of age [28]. Thus, it was possible that the improved phytase recoveries in MAF diets pelleted through an L:D 8:1 pellet mill die improved amino acid digestibility from 0-14 d of age when dietary protein was inadequate when compared to the PPLA diets pelleted through an L:D 10:1 pellet mill die, but ceased to improve amino acid digestibility once the dietary protein requirements were met beyond 14 d of age. These data would be consistent with the known diminishing returns associated with feeding enzymes when nutrient levels were adequate without enzyme supplementation [34]. Additionally, young broilers were typically more responsive to enzyme supplementation when compared to mature broilers due to an underdeveloped gastrointestinal tract and physical limitations on feed intake [2, 34].

Bone Characteristics

Tibia bone breaking strength (BBS) and bone ash were affected at 14 and 28 d of age by the factors applied (Table IV-7; $P \leq 0.01$). The tibia BBS of broilers fed the NC diets was less than those fed the NC + PHY or PC diets at 14 d of age. At 28 d of age, the tibia BBS of broilers fed NC + PHY diets pelleted with MAF was greater than the tibia BBS of broilers fed the NC. However, the tibia BBS of broilers fed the NC + PHY diets pelleted with PPLA fat was statistically similar to the tibia BBS of broilers fed the NC at 28 d of age. At 14 d of age, the tibia bone ash of broilers that consumed the NC + PHY diets pelleted with MAF was statistically similar to the PC, while the tibia of broilers fed the NC + PHY diets pelleted with PPLA fat failed to reach the same level of bone ash as the PC. At 28 d of age, the tibia bone ash of broilers that consumed the PC or NC + PHY diets was greater than the NC.

Several reports had previously demonstrated that tibia BBS and bone ash improved when diets inadequate in AvP were supplemented with inorganic phosphate or phytase [33,

35, 36]. The bone characteristic data reported herein provided evidence that non-conditioning factors during the pelleting process may have influenced phytase recoveries and mineral deposition into bone. When diets were pelleted with MAF as opposed to PPLA fat, phytase recoveries were improved. The improved phytase recoveries in MAF diets improved tibia bone ash to the level of mineralization observed in the PC and BBS beyond the durability of NC bones at 14 and 28 d of age, respectively. These data further confirmed the findings of Gehring *et al.* [19] that reported improved enzyme recoveries when utilizing increased levels of MAF during the pelleting process. Pelleting diets with or without the use of PPLA fat systems may have differentially impacted the ability of phytase to release phosphorous for utilization in broiler bones as evidenced by the current data. Therefore, feed manufacturers should routinely test for thermosensitive ingredient depletion during the pelleting process when practices had been implemented that may have improved physical feed quality but adversely impacted nutritional feed quality.

CONCLUSIONS AND APPLICATIONS

1. Pellets manufactured with PPLA fat or through an L:D 10:1 pellet mill die were more durable when compared to pellets manufactured with MAF or through an L:D 8:1 pellet mill die.
2. Phytase recoveries in diets pelleted with MAF were greater than those pelleted with PPLA fat.
3. Broilers that consumed diets pelleted with MAF and phytase mineralized bones to the extent of the PC, while broilers fed diets manufactured with PPLA fat and phytase did not at 14 d of age.

4. Broilers that consumed diets pelleted with MAF and phytase contained bones that were more durable than the NC, while those that consumed diets pelleted with PPLA fat were not different from the NC at 28 d of age.

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Table IV-1. Ingredient composition of broiler diets.

Ingredients	NC	NC + PHY (%)	PC
Corn	59.46	59.46	59.79
SBM (48%)	32.33	32.33	29.15
Poultry by-product Meal	1.74	1.74	3.87
Poultry fat	3.50	3.50	3.50
Limestone	0.86	0.86	0.78
Salt	0.50	0.50	0.50
Titanium dioxide	0.50	0.50	0.50
DL-Methionine	0.21	0.21	0.20
Choline chloride (60%)	0.20	0.20	0.20
Trace mineral premix ¹	0.20	0.20	0.20
Dicalcium phosphate (18.5% P)	0.25	0.25	1.06
L-Lysine	0.09	0.09	0.09
Vitamin premix ²	0.05	0.05	0.05
Selenium premix ³ (0.06%)	0.05	0.05	0.05
L-Threonine	0.01	0.01	0.01
Coccidiostat ⁴	0.05	0.05	0.05
Phytase ⁵	0.00	0.02	0.00
Calculated nutrients			
Protein	21.50	21.50	21.50
Calcium	0.64	0.85	0.85
Available phosphorous	0.23	0.43	0.43
Total lysine	1.20	1.20	1.20
TSAA	0.87	0.87	0.87
Total threonine	0.83	0.83	0.83
ME, kcal/g	3.10	3.10	3.10
Analyzed Nutrients			
Calcium	0.53		0.78
Total Phosphorous	0.49		0.63

¹ Trace mineral premix provided the following per kg of diet: Mn, 120 mg; Zn, 120 mg; Fe, 80 mg; Cu, 10 mg; I, 2.5 mg; Co, 1 mg.

² Vitamin premix provided the following per kg of diet: vitamin A, 6600 IU; vitamin D3, 1980 IU; vitamin E, 33 IU; vitamin B12, 0.02 mg; biotin, 0.13 mg; menadione, 2 mg; thiamine, 2 mg; riboflavin, 6.6 mg; pantothenic acid, 11 mg; vitamin B6, 4 mg; niacin, 55 mg; folic acid, 1.1 mg.

³ Selenium premix provided Se at 0.3 mg/kg of feed.

⁴ Monensin was included at 99 mg/kg of feed (Coban 90, Elanco, Greenfield, IN).

⁵ Quantum Blue 5G was added to provide 1000 FTU/kg of feed.

Table IV-2. Main and interaction effects of fat application site (FAS) and pellet mill die length-to-diameter ratio (L:D) on pellet durability index (PDI) as determined by the Holmen method for 30 s of testing and the change in temperature between hot pellets and conditioned mash (ΔT) in negative control diets amended with phytase.

FAS ¹	L:D	n	PDI (%)	ΔT (°C)
Main Effects				
MAF		8	80.9 ^b	3.1 ^B
PPLA		8	92.7 ^a	5.6 ^A
<i>P</i> -value			0.015	0.001
	8:1	8	80.6 ^b	2.3 ^B
	10:1	8	93.1 ^a	6.4 ^A
	<i>P</i> -value		0.011	0.001
	SEM ²		2.879	0.380
Interaction Effects				
MAF	8:1	4	71.6	1.2
MAF	10:1	4	90.3	5.0
PPLA	8:1	4	89.6	3.4
PPLA	10:1	4	95.9	7.8
	<i>P</i> -value		0.157	0.599
	SEM ²		4.071	0.537

^{A, B} Means within each column that possess different superscripts differ significantly ($P \leq 0.01$).

^{a, b} Means within each column that possess different superscripts differ significantly ($P \leq 0.05$).

¹ FAS included 3.5% mixer-added fat (MAF) or 0.5% MAF with the remaining 3.0% being applied via post-pellet liquid application (PPLA).

² Standard error of mean (SEM) for n=8 samples for each FAS and L:D and n=4 samples for each interaction of FAS and L:D.

Table IV-3. Main and interaction effects of fat application site (FAS) and pellet mill die length-to-diameter ratio (L:D) on the absolute phytase recovery in unconditioned mash (UCM), conditioned mash (CM), and crumbles (C) and the relative phytase recovery in CM compared to UCM, C compared to CM, and C compared to UCM in negative control diets amended with phytase¹.

FAS ²	L:D	n	Absolute Phytase Recovery			Relative Phytase Recovery		
			UCM	CM	C	CM:UCM	C:CM	C:UCM
			(FTU/kg)			(%)		
Main Effects								
MAF		8	1235	1188	935 ^A	100.0	84.1 ^A	80.5 ^a
PPLA		8	1328	1392	523 ^B	107.3	37.5 ^B	41.0 ^b
<i>P</i> -value			0.519	0.171	0.008	0.613	0.007	0.011
	8:1	8	1414	1423	834	106.4	59.5	60.1
	10:1	8	1150	1157	624	100.9	62.1	61.5
<i>P</i> -value			0.088	0.084	0.127	0.705	0.861	0.916
	SEM ³		99	99	90	9.9	10.0	9.1
Interaction Effects								
MAF	8:1	4	1537 ^a	1420	1053	98.1	75.9	70.5
MAF	10:1	4	932 ^b	955	817	101.9	92.3	90.5
PPLA	8:1	4	1290 ^{ab}	1425	614	114.7	43.1	49.6
PPLA	10:1	4	1368 ^{ab}	1359	431	99.9	31.9	32.4
<i>P</i> -value			0.034	0.181	0.836	0.522	0.350	0.178
	SEM ³		141	140	127	14.1	14.2	12.9

^{A, B} Means within each column that possess different superscripts differ significantly ($P \leq 0.01$).

^{a, b} Means within each column that possess different superscripts differ significantly ($P \leq 0.05$).

¹ Quantum Blue 5G. Testing method was in accordance with ELISA specific for Quantum Blue, ESC Standard Analytical Method, SAM099; AB Vista, Marlborough, UK.

² FAS included 3.5% mixer-added fat (MAF) or 0.5% MAF with the remaining 3.0% being applied via post-pellet liquid application (PPLA).

³ Standard error of mean (SEM) for n=8 samples for each FAS and L:D and n=4 samples for each interaction of FAS and L:D.

Table IV-4. Effect of fat application site (FAS) and pellet mill die length-to-diameter ratio (L:D) on feed intake when negative control diets were amended with phytase (NC + PHY) compared to negative control (NC) and positive control (PC) formulations.

Formulation ¹	FAS ²	L:D	n	Feed Intake				
				7 d	14 d	21 d	28 d	35 d
NC			16	159 ^{ab}	546 ^C	1202 ^C	2065 ^B	3159 ^B
NC + PHY	MAF	8:1	16	164 ^{ab}	567 ^{ABC}	1252 ^{AB}	2154 ^A	3335 ^A
NC + PHY	MAF	10:1	16	162 ^{ab}	567 ^{ABC}	1264 ^{AB}	2171 ^A	3352 ^A
NC + PHY	PPLA	8:1	16	165 ^{ab}	572 ^{AB}	1255 ^{AB}	2168 ^A	3370 ^A
NC + PHY	PPLA	10:1	16	168 ^a	583 ^A	1278 ^A	2174 ^A	3355 ^A
PC			16	157 ^b	548 ^{BC}	1227 ^{BC}	2132 ^{AB}	3296 ^A
<i>P</i> -value				0.025	0.001	0.001	0.002	0.001
SEM ³				2	5	12	20	32

^{A, B, C} Means within each column that possess different superscripts differ significantly ($P \leq 0.01$).

^{a, b} Means within each column that possess different superscripts differ significantly ($P \leq 0.05$).

¹ The NC diet was formulated to 0.23% available phosphorous (AvP) and 0.64% calcium (Ca) and the PC was formulated to 0.43% AvP and 0.85 Ca; the NC + PHY diet was the NC diet amended with 1000 FTU/kg of phytase and contained the same AvP and Ca levels as the PC diet once amended with phytase.

² FAS included 3.5% mixer-added fat (MAF) or 0.5% MAF with the remaining 3.0% being applied via post-pellet liquid application (PPLA).

³ Standard error of mean (SEM) for n=16 pens for each interaction of formulation, FAS, and L:D.

Table IV-5. Effect of fat application site (FAS) and pellet mill die length-to-diameter ratio (L:D) on body weight (BW) when negative control diets were amended with phytase (NC + PHY) compared to negative control (NC) and positive control (PC) formulations.

Formulation ¹	FAS ²	L:D	n	BW				
				7 d	14 d	21 d	28 d	35 d
NC			16	173 ^{AB}	472 ^B	973 ^C	1560 ^B	2348 ^C
NC + PHY	MAF	8:1	16	178 ^A	496 ^A	1019 ^{AB}	1635 ^A	2482 ^{AB}
NC + PHY	MAF	10:1	16	173 ^{AB}	496 ^A	1037 ^A	1659 ^A	2501 ^A
NC + PHY	PPLA	8:1	16	176 ^A	489 ^{AB}	1019 ^{AB}	1644 ^A	2491 ^{AB}
NC + PHY	PPLA	10:1	16	180 ^A	497 ^A	1030 ^{AB}	1645 ^A	2493 ^A
PC			16	167 ^B	473 ^B	990 ^{BC}	1606 ^{AB}	2398 ^{BC}
<i>P</i> -value				0.001	0.001	0.001	0.001	0.001
SEM ³				2	5	10	16	23

^{A, B, C} Means within each column that possess different superscripts differ significantly ($P \leq 0.01$).

¹ The NC diet was formulated to 0.23% available phosphorous (AvP) and 0.64% calcium (Ca) and the PC was formulated to 0.43% AvP and 0.85 Ca; the NC + PHY diet was the NC diet amended with 1000 FTU/kg of phytase and contained the same AvP and Ca levels as the PC diet once amended with phytase.

² FAS included 3.5% mixer-added fat (MAF) or 0.5% MAF with the remaining 3.0% being applied via post-pellet liquid application (PPLA).

³ Standard error of mean (SEM) for n=16 pens for each interaction of formulation, FAS, and L:D.

Table IV-6. Effect of fat application site (FAS) and pellet mill die length-to-diameter ratio (L:D) on feed conversion ratio (FCR) when negative control diets were amended with phytase (NC + PHY) compared to negative control (NC) and positive control (PC) formulations.

Formulation ²	FAS ³	L:D	n	FCR ¹				
				7 d	14 d	21 d	28 d	35 d
NC			16	1.238	1.278 ^{AB}	1.328	1.401	1.434
NC + PHY	MAF	8:1	16	1.228	1.258 ^B	1.312	1.388	1.425
NC + PHY	MAF	10:1	16	1.251	1.261 ^{AB}	1.307	1.383	1.423
NC + PHY	PPLA	8:1	16	1.253	1.288 ^{AB}	1.321	1.397	1.437
NC + PHY	PPLA	10:1	16	1.237	1.293 ^A	1.330	1.395	1.425
PC			16	1.278	1.282 ^{AB}	1.328	1.402	1.456
<i>P</i> -value				0.299	0.007	0.180	0.630	0.340
SEM ⁴				0.016	0.008	0.007	0.009	0.011

^{A, B} Means within each column that possess different superscripts differ significantly ($P \leq 0.01$).

¹ The FCR was calculated based on BW gain from hatch and was adjusted by adding the BW of any mortality from each pen into the FCR equation.

² The NC diet was formulated to contain 0.23% available phosphorous (AvP) and 0.64% calcium (Ca) and the PC was formulated to contain 0.43% AvP and 0.85 Ca; the NC + PHY diet was the NC diet amended with 1000 FTU/kg of phytase and contained the same AvP and Ca levels as the PC diet once amended with phytase.

³ FAS included 3.5% mixer-added fat (MAF) or 0.5% MAF with the remaining 3.0% being applied via post-pellet liquid application (PPLA).

⁴ Standard error of mean (SEM) for n=16 pens for each interaction of formulation, FAS, and L:D.

Table IV-7. Effect of fat application site (FAS) and pellet mill die length-to-diameter ratio (L:D) on tibia bone breaking strength (BBS) and tibia bone ash when negative control diets were amended with phytase (NC + PHY) compared to negative control (NC) and positive control (PC) formulations.

Formulation ¹	FAS ²	L:D	n	Tibia BBS ⁴		Tibia Bone Ash ⁵	
				14 d	28 d	14 d	28 d
NC			16	7.6 ^B	22.1 ^B	45.0 ^C	50.6 ^B
NC + PHY	MAF	8:1	16	10.4 ^A	26.0 ^A	48.5 ^{AB}	52.6 ^A
NC + PHY	MAF	10:1	16	10.0 ^A	26.0 ^A	49.3 ^{AB}	52.8 ^A
NC + PHY	PPLA	8:1	16	9.9 ^A	24.6 ^{AB}	48.3 ^B	52.3 ^A
NC + PHY	PPLA	10:1	16	10.2 ^A	24.1 ^{AB}	48.1 ^B	53.0 ^A
PC			16	10.3 ^A	25.4 ^A	50.3 ^A	53.3 ^A
<i>P</i> -value				0.001	0.001	0.001	0.001
SEM ³				0.3	0.7	0.5	0.3

^{A, B, C} Means within each column that possess different superscripts differ significantly ($P \leq 0.01$).

¹ The NC diet was formulated to contain 0.23% available phosphorous (AvP) and 0.64% calcium (Ca) and the PC was formulated to contain 0.43% AvP and 0.85 Ca; the NC + PHY diet was the NC diet amended with 1000 FTU/kg of phytase and contained the same AvP and Ca levels as the PC diet.

² FAS included 3.5% mixer-added fat (MAF) or 0.5% MAF with the remaining 3.0% being applied via post-pellet liquid application (PPLA).

³ Standard error of mean (SEM) for n=16 pens of two replicate birds per pen for each interaction of formulation, FAS, and L:D.

⁴ Tibiae were broken utilizing a texture analyzer equipped with a 250 kg load cell with a crosshead speed of 3 mm/s and a fulcrum width of 3.0 and 4.5 cm at 14 and 28 d, respectively.

⁵ Tibia bone ash was determined after broken bones were dried for 24 h at 100°C, placed in ethyl ether for 48 h, dried for an additional 24 h at 100°C, and then ashed in a muffle furnace at 600°C for 24 h.

OVERALL CONCLUSIONS

Since the commercial inception of enzyme technologies in animal rations, many improvements have been made with regards to enzyme thermostability. However, the majority of enzyme thermostability research focused solely on mash conditioning temperatures. While mash conditioning temperature was a logical focal point for enzyme thermostability trials, there were several other factors subjected to manipulation during feed formulation and pelleting that may have additionally impacted the ability of an enzyme to retain catalytic activity subsequent to pelleting. The goal of this research was to determine what those factors might be and how they affect the broilers fed those rations.

The data in Chapter I indicated that mash conditioning temperature was not the only factor that may impact enzyme denaturation, especially at the pellet mill die. Increased levels of mixer-added fat and a thinner pellet mill die improved xylanase recoveries even though the diets were pelleted at the same mash conditioning temperature. Furthermore, all of the parameters that were monitored during the pelleting process were correlated with xylanase recovery. When the monitored parameters were combined with the controlled factors, a robust multiple regression model was generated. The goal of such a model would be to provide feed manufacturers with instantaneous, continuous parameters to monitor that would allow for rapid feedback on nutritional feed quality.

The data in Chapter II further expanded on modeling capabilities and provided insight as to the primary location of enzyme denaturation during the pelleting process. It was determined that including a mash conditioning variable in enzyme thermostability modeling could improve the robustness of the model, as would be expected. Additionally, the data indicated that the primary site of denaturation for the phytase utilized was at the pellet mill

die, not the conditioning chamber which may have been expected since the conditioning chamber was the location of steam injection. These data indicated that the phytase utilized may have been stable to heat, but not to heat and pressure which would be experienced as the enzyme traversed the pellet mill die.

Chapter III also provided insight as to why enzyme denaturation may be occurring at the pellet mill die, especially within research settings. Research pellet mills, when operated under normal conditions, retained feed within the pellet mill die for a greater duration when compared to commercial mills. While the phytase utilized was not particularly stable during pelleting at the slowest pellet mill throughput and the greatest temperature, the thermostability of the phytase improved as the pellet mill throughput was increased. These data provided additional information for future enzyme thermostability testing and indicated that greater throughputs should be utilized when conducting such work on pilot pellet mills. Additionally, strong correlations were observed between PDI and phytase recovery, which indicated that achieving physical feed quality while maintaining nutritional feed quality may be a challenge for commercial feed producers.

The data presented in Chapter IV indicated that the level of fat in rations may have impacted the recovery of the phytase utilized, and that it may impact subsequent broiler bone characteristics. The use of PPLA systems became ubiquitous within the feed industry to provide a solution to maintain physical feed quality in rations that were formulated to include levels of fat that exceeded the threshold to maintain particle binding. While this practice may have improved physical feed quality, more data was necessary to determine how the implementation of such systems affected nutritional feed quality. Broilers that consumed diets that contained phytase pelleted and then post-coated with fat exhibited poorer bone

strength and mineralization characteristics when compared to broilers that were fed diets that contained all the formulated fat during the pelleting process.

The body of work herein provided several conclusions that should be directly applicable to commercial feed manufacturing as well as future enzyme thermostability research. Future enzyme thermostability research should consider the use of a standard set of formulations and procedures, especially when completing thermostability comparison trials of separate products. Standard reporting for future enzyme thermostability experiments should include conditioning time and temperature, steam pressure, pellet mill die hole wall length and diameter, pellet mill die working surface area, and pellet mill production rates. To ensure that dietary interactions do not affect thermostability, a standardized diet for testing enzyme thermostability should also be considered. Additionally, the correlation between parameters monitored during pelleting and enzyme recoveries may provide a platform to improve or expand upon the knowledge of physical feed quality and its relationship to nutrients retained within feed subsequent to pelleting. The modeling capabilities demonstrated herein may provide a platform for continuous process monitoring during pelleting to provide insight towards nutritional feed quality in addition to operations data. Finding the balance between nutritional and physical feed quality was a problem that feed operations experienced, but the models and data generated within this body of work may improve the understanding of how they interact and may help feed manufacturers and nutritionists make more informed decisions on their operation objectives with regards to their physical and nutritional feed quality goals.