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Conditioning Thermal Stability and Dose Effects of Commercially Available Phytase Preparations on Performance and Tibia Mineralization of Young Broiler Chickens

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ABSTRACT

Conditioning Thermal Stability and Dose Effects of Commercially Available Phytase Preparations on Performance and Tibia Mineralization of Young Broiler Chickens

Victoria Homan

Phytases have become a vital part of broiler diets due to their ability to decrease feed cost and potential environmental pollution caused by the use of excessive phosphorus. The objective of the first study was to compare the effects of various inclusions of a Transgenic Phytase Corn (TPC) to a Granulated Bacteria Phytase (GBP) on 21-d broiler performance and tibia mineralization. Treatments varied in non-phytate phosphorus (Negative control (NC) = 0.2, positive control (PC) = 0.4, 0.3, and 0.35), phytase product (TPC or GBP), and phytase dose (500-3000 FTU/kg). A 2x2 factorial analysis was performed with the main effects phytase activity and phytase product. The inclusion of granulated bacterial phytase resulted in increased broiler performance and tibia mineralization compared to the inclusion of transgenic phytase corn. This may be attributed to variations in dispersion of enzyme within the diet or the general efficacy of the expressed enzyme. The inclusion of a commercial phytase at 3,000 FTU/kg increased broiler performance and tibia mineralization compared to inclusion at 500 FTU/kg. These results may be due to a greater reduction of phytate, alleviating anti-nutrient effects.

The objective of the second study was to evaluate the effects three commercial 6-phytase products with a phosphorus deficient diet subjected to increased steam conditioning temperatures on broiler performance and tibia mineralization. Treatments included a negative control, and a negative control with the addition of a Buttiauxella phytase expressed in Trichoderma reesei (B Trich), an E. coli phytase expressed in Pichia pastoris (E Pichia), or an E. coli phytase expressed in Trichoderma reesei (E Trich), steam conditioned at 82, 88, or 93°C, for a total of twelve treatments. Broiler performance and tibia mineralization were analyzed using a 4 x 3 factorial with the main effects of product and conditioning temperature. The commercial phytase products demonstrated differences in supporting the phosphorus requirement in broilers. Increasing conditioning temperatures had a detrimental effect on broiler performance and tibia mineralization. The increased conditioning temperatures may have decreased nutrient availability, denatured phytase products, or both of these events may have influenced the results.

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CHAPTER 1

Literature Review

Conditioning Thermal Stability and Dose Effects of Commercially Available Phytase Preparations on Performance and Tibia Mineralization of Young Broiler Chickens

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I.   Introduction

Poultry receive the majority of their phosphorus in the form of phytate (myo-inositol hexakisphosphate, IP6). Phytate accounts for 60-70% of phosphorus in typical corn and soybean based poultry diets (NRC, 1994). Phosphorus is essential for proper bone development and for the utilization of energy. Phytate is not easily digested, as it forms insoluble complexes with other nutrients. Poultry also lack the endogenous enzymes required to effectively dephosphorylate phytate, having the ability to only digest around 10% of the phytate provided to them (Adeola and Sands, 2003; Bedford and Schulze, 1998). As a result, phosphorus is excreted in manure, which can make its way into local water sources, where it has been implicated as having a detrimental environmental effect (Biehl et al., 1998). Sharpley (1999) found that increased phosphorus content in water increases eutrophication, which restricts water use for fisheries, recreation, industry, and drinking, due to the increased growth of undesirable algae. The uncontrolled algae growth is referred to as algal blooms, which not only contaminate drinking water, but also deplete the water of oxygen, killing aquatic life. Due to the low digestibility of phytate, excessive levels of phosphorus must be included in the diet, which increases both potential environmental pollution and feed costs.

As an alternative to increasing the inclusion of phosphorus, exogenous phytase may be added to diets. Exogenous phytase is added to most commercial broiler diets in order to improve nutrient availability, decrease costs, and reduce environmental pollution (Bedford, 2000; Dilger et al 2004). Exogenous phytase is able to make otherwise inaccessible phosphorus available to poultry by hydrolyzing the phytate molecule (Ravindran et al., 1995). Microbial phytases are classified based on the site of initial hydrolysis. A 6-phytase will dephosphorylate phytate in a sequential way, beginning with the phosphate group at position 6, whereas a 3-phytase would not begin hydrolyzing phytate

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until position 3 (Greiner et al., 2000). The full sequence of hydrolysis down to myo-inositol has yet to be determined (Maenz, 2001).

Exogenous phytase supplementation has been shown to improve broiler performance, indicated by increased weight gain, nutrient utilization, and tibia ash percentages (Gurbuz et al., 2008). Sebastian et al. (1996) found that the inclusion of exogenous phytase increased utilization of phosphorus (and therefore Ca, Zn, and Cu) leading to increased bone mineralization, which can be measured by tibia ash percentage. However, many factors play a role on the effectiveness of the phytase additive. Since phytase may be obtained from different sources, it would follow that different sources would result in different enzyme characteristics and therefore different efficacies (Onyango et al., 2005). The difference in characteristics include optimum pH, thermostability, solubility, and resistance to hydrolysis. In addition, the dosage at which the exogenous phytase is included in the diet will alter its effects (Li et al., 2016; Shirley and Edwards, 2003). For a phytase additive to be optimized, it must also be thermally stable, as well as maintain phytase activity with the gastrointestinal tract of the animal (Nyannor et al., 2009). While commercially available phytase products have led to increased digestibility, tibia mineralization, and broiler performance, there is room for greater improvement and thus, more research. Determining an efficacious phytase source and dose, and identifying conditioning temperatures that may denature the enzyme, can lead to increased broiler performance and decreased feed cost.

II.   Commercial Phytase Sources

The varying characteristics and effectiveness of phytase supplements may be explained by factors such as the phytase source. There are many commercially available 6-phytase products that have shown to be effective in improving broiler performance. The inclusion of 6-phytases, such as phytase from the bacteria sources *Escherichia coli* or *Buttiauxella*, have been shown to improve broiler performance, as indicated by increased feed intake (FI), live weight gain (LWG), tibia ash percent and weight, and decreased feed conversion ratio (FCR; Cowieson et al., 2006; Walk et al., 2014). Pillai et al. (2006) found that phytase from *Escherichia coli* are more effective than fungal phytases at releasing phosphorus, leading to increased tibia ash percent and weight. Li and coauthors (2015) found that the inclusion of a phytase from *Buttiauxella* increased LWG, FI, tibia ash percent, and tibia ash weight compared to a negative control. While research seems to unanimously agree that both *Escherichia coli* and *Buttiauxella* phytases can be effective in improving broiler performance, there is a lack of data comparing the effectiveness of these two types of phytase.

Genetic modifications are being made to increase phytate-degrading activity in the plant seed itself as an alternative strategy for improving nutrient management in animal production. This concept was first introduced by Pen et al. (1993), who found that a phytase enzyme in tobacco seeds was able to maintain *in vitro* activity for an entire year after harvest. However, tobacco seeds are not a main component in poultry diets, so other options were explored. Soybeans expressing phytase were used by Denbow et al. (1998), who found the transgenic soybean was just as effective as phytase additive in improving broiler FI, LWG, and bone ash content. The disadvantage of using a transgenic soybean product is that soybeans are subjected to high thermal processing prior to their inclusion in the diet. Corn, however, is not subjected to such processing. Nyannor et al. (2009)

concluded that a transgenic corn phytase can maintain a significant level of phytase activity and effectively dephosphorylate phytate. A study by Gao et al. (2013) found that phytase expressed as corn can be as efficacious as microbial phytases in improving phytate digestibility in laying hens. The use of transgenic phytase corn (TPC) may be more economically feasible than microbial phytases, since plants already constitute a larger land mass than microbial expression systems. However, corn is a mixer-added ingredient, meaning the phytase enzyme must be thermally stable in order to survive the pelleting process. Pelleting of the feed is necessary to decrease feed segregation and has been shown to increase broiler performance as opposed to mash diets. While transgenic phytase products may be promising, more research needs to be conducted regarding their stability and efficacy. It is also important to consider consumers' perceptions. Genetically-modified organisms (GMOs) are currently a controversial subject, and there is potential that consumers would not purchase TPC regardless of its efficacy.

III. Inclusion Levels

Perhaps equally important to the source of the phytase is the dose at which it is administered. Conflicting data exists about the optimal dosage for poultry feed. The universally accepted commercial recommendation is 500 FTU/kg (Shirley and Edwards, 2003). One phytase unit (FTU) may be defined as the quantity of enzyme required to liberate 1 µmol of inorganic P per minute from an excess of 1.5 mM sodium at 37°C, with a pH of 5.5 (International Union of Biochemistry, 1979). While 500 FTU/kg is the current recommendation, research has been performed in an attempt to show that higher doses may be beneficial.

Shirley and Edwards (2003) found that super dosing (>1,000 FTU/kg) increases amino acid digestibility and retention, and results in maximum broiler performance compared to lower doses in broiler chicks from d1-16. The authors concluded that phytase levels above commercial recommendation (12,000 FTU/kg) are required in order to make almost all (95%) of the phytate P available for maximum utilization by chicks. This is in agreement with earlier work by Shirley and Edwards (2002), which found that the supplementation of phytase at 6,000 FTU/kg improved broiler performance, bone ash, and the utilization of phytate phosphorus. Walk et al. (2013) showed that feeding a diet with exogenous enzyme inclusion >1500 FTU/kg to broilers from d0-21, resulted in almost complete hydrolysis of phytate and decrease in FCR. However, Walk and others did not observe any improvements in FI, LWG, or tibia ash content when supplementing a diet with 1500 FTU/kg versus 500 FTU/kg.

In contrast, many studies have shown that phosphorus utilization plateaus at 500 FTU/kg (Simons et al., 1990; Huyghebaert et al., 1992). Research performed by Cowieson et al. (2006) showed that the amino acid digestibility of younger birds (d1-16) was maximized with the inclusion of 250-300 FTU/kg exogenous phytase. Insignificant improvement was found from adding phytase at levels above 600 FTU/kg. This is in agreement with Pieniazek et al. (2016), who found that when phytase inclusion >250 FTU/kg, there was little to no significance in increased amino acid digestibility. While the study did not observe an improvement in amino acid digestibility, phytase inclusion at 1,200 FTU/kg did result in increased FI, LWG, tibia ash percent, and tibia ash weight. In addition, Pieniazek and others found that phytase inclusion at 2,000 FTU/kg increased amino acid digestibility, FI, and LWG when birds were raised to d42. This leads to the theory that phytase supplementation at inclusions above commercial recommendations are only beneficial when provided to older birds. Nonetheless, the supplementation of phytase at levels above commercial recommendations provides

inconsistent justification to support the additional cost. More research must be performed before commercial recommendations are modified.

IV.   Conditioning Temperatures

In addition to the phytate product source and dose, the method that the supplement is incorporated into the diet, i.e. mash or pelleted, will alter its effectiveness. When feed is pelleted, it is exposed to high temperatures, pressure, and steam. Phytase enzymes may be sprayed on to the feed post-pelleting, but this method does not ensure mix uniformity throughout the diet or protect against nutrient segregation effects associated with feed attrition during auguring. Therefore, most phytase enzymes are added at the mixer. Loop et al. (2012) stated that mixer-added phytase products have the potential to be denatured during the pelleting process due to the exposure of heat, moisture, and pressure. Phytase enzymes may contain a carbohydrate-lipid coating, be genetically modified, or be selected due to heat stability, in an attempt to circumvent issues associated with denaturation during the pelleting process. Loop and others investigated heat-stable *E. coli* derived phytases, and found that as steam conditioning temperatures increased (up to 82°C), phytase activity decreases. In addition, Loar et al. (2014) found that increasing conditioning temperatures (up to 96°C) of broiler diets per se increased FCR and decreased amino acid digestibility.

There is argument that conditioning temperatures need to remain high, regardless of the effect on phytase activity. High temperatures aid in the destruction pathogens and anti-nutritive factors. High temperatures are also required for proper agglomeration of nutrients and for the attainment of high quality pellets (Thomas and van der Poel, 1996). Since the thermal stability of commercially

available phytase products may vary drastically, more research needs to be conducted to determine what conditioning temperatures are appropriate.

V.   Conclusions and Future Research

In conclusion, due to the diversity of phytase additives, it may not be feasible to create universally appropriate doses and conditioning temperatures. Much research is still required to justify the use of transgenic phytase corn over microbial phytase. In addition, 6-phytases need to be compared to each other to determine if one phytase is superior. Future research should address individual phytase products to create a range of appropriate conditioning temperatures and doses that produce maximal broiler performance and tibia mineralization.

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CHAPTER 2

The effect of varying levels of commercial and novel phytase products on broiler performance and tibia mineralization

V.B. Homan and J.S. Moritz

SUMMARY

Innovative phytase products may be cost effective for use in feeding commercial poultry. The objective of the study was to compare a Transgenic Phytase Corn (TPC) and a Granulated Bacterial Phytase (GBP) in mash diets on 21-D Hubbard x Cobb broiler chick performance, phosphorus equivalency values, and tibia mineralization. The experiment was arranged in a randomized complete block design with ten replications of nine treatments total. Treatments varied in nPP (Negative control – 0.2 (NC), Positive control – 0.4 (PC), 0.3, and 0.35), phytase product (TPC or GBP), and phytase dose (500 – 3,000 FTU/kg). Based on the multiple comparison of all treatments, the NC and PC affected performance variables as would be expected (P<0.05). A 2x2 factorial analysis was performed with the main effects of phytase activity and phytase product. The results of this analysis showed that increased phytase activity increased feed intake (P=0.0001). Additionally, phytase activity and product interacted to affect LWG (P=0.0473). The GBP LWG improvement due to increased activity was greater than the TPC LWG improvement due to increased activity. Broiler chicks provided GBP had improved FCR (P=0.0005) compared to chicks provided TPC. Phytase product and activity both affected tibia ash content (P=0.0001). Linear regression performed on LWG of diets increasing in nPP (R^2=0.66), were used to demonstrate 3,000 FTU/kg nPP equivalency of 0.07 and 0.20 for TPC and GBP respectively. Linear regression performed on tibia ash of diets increasing in nPP (R^2=0.88), were used to demonstrate 3,000 FTU/kg nPP equivalency of 0.08 and 0.15 for TPC and GBP respectively. Calculated additions of 3,000 FTU/kg were analytically similar for TPC and GBP (2,800 and 3,100 FTU/kg, respectively). Phytase product efficacy differences may be due to dispersion of enzyme within mash. The transgenic phytase corn had a larger particle size in comparison to the granulated bacterial phytase. Despite a homogenous mix, broilers would still be less like to consume the fewer, larger particles of transgenic corn as opposed to the smaller, more

frequent particles of granulated bacterial phytase. Differences may also be attributed to the general

effectiveness of the expressed enzyme. The expression system of the granulated bacteria phytase may

have been superior to that of the transgenic phytase corn.

DESCRIPTION OF PROBLEM

Poultry receive the majority of their phosphorus in the form of Phytate (myo-inositol hexakisphosphate, IP6). Phytate is not easily digested, and it forms insoluble complexes with other nutrients. As a result, phosphorus is excreted in manure and subsequently spread on fields as a fertilizer, which has been implicated as having a potentially detrimental environmental effect (Biehl et al., 1998). Due to the reduced digestibility of cereal and oil seed phosphorus, excessive levels of phosphorus must be included in the diet, which increases both the potential for environmental pollution and feed cost. Exogenous phytase is able to make otherwise inaccessible phosphorus available to poultry by hydrolyzing the phytate molecule (Ravindran et al., 1995). Therefore, exogenous phytase is added to most commercial broiler diets in order to improve nutrient availability, decrease costs, and reduce environmental pollution (Bedford, 2000; Dilger et al., 2004). Exogenous phytase supplementation has been shown to improve broiler performance, via increased weight gain, nutrient utilization, and tibia ash percentages (Gurbuz et al., 2009; Sebastian et al., 1996). In addition, as an alternative to common phytase feed additives that are commercially available, genetic modifications are being made to corn to allow for expression of phytase enzyme (Nyannor et al., 2009).

While commercially available phytase products have led to improved nutrient digestibility, tibia mineralization, and broiler performance, there are additional factors that may affect efficiency. For example, the dosage at which the phytase product is included will alter its effects (Cowieson et al., 2006). The inclusion of exogenous phytase at or above 1,000 FTU/kg has been referred to as superdosing, and has been shown to further improve nutrient absorption and broiler performance (Shirley and Edwards, 2003; Walk et al., 2013). In addition, there is little research comparing the effectiveness of genetically modified transgenic phytase corn (TPC) to commercially available

phytase enzymes. The objective of the current study was to evaluate the effect of phytase product and

inclusion level on Hubbard x Cobb 21-d broiler performance and tibia mineralization.

MATERIALS AND METHODS

Dietary Treatments

The two phytase products evaluated were a Granulated Bacterial Phytase (GBP) created by a genetically modified strain of the yeast species Pichia pastoris and an E. coli-derived phytase expressed in Transgenic Phytase Corn (TPC). Treatments used to establish effects of increasing non-phytate phosphorus (nPP) included a negative control (NC) diet containing 0.2% nPP via dicalcium phosphate (DCP), NC + 0.1% nPP via DCP, NC + 0.15% nPP via DCP, and a positive control (PC) that contained 0.4% nPP via DCP. Additional treatments containing phytase included 500, 1000, or 3000 FTU/kg GBP + NC, and 500 or 3000 FTU/kg TPC + NC. All treatments consisted of corn and soybean-based mash diets (Table 1). There were nine dietary treatments total.

Feed Manufacture

Feed manufacture was conducted at the West Virginia University pilot feed mill. All diets were batched and mixed in one day. Two master batches were manufactured. One batch contained 0.2% nPP (NC) and the other contained 0.4% nPP (PC). Dietary treatments devoid of phytase were manufactured using one master batch or a blend of each master batch. Dietary treatments containing phytase were manufactured using the NC master batch.

Phytase inclusions for treatments were based on manufacturer supplied data (GBP treatments = 5,085 FTU/g and TPC treatments = 3,345 FTU/g). Washed builders sand was used to balance phytase inclusion of the different products and levels to a 0.1% total diet inclusion (Table 1). In order to improve homogeneity of the final mix, phytase product, sand, and 5 lbs. of ground corn were mixed for ten minutes in a 4.73-liter Hobart mixer [11] prior to final treatment batching and mixing. Each dietary treatment was mixed for ten minutes in a one-ton vertical screw Easy Automation Inc. mixer

[12]. This mix time was chosen because it has been shown to produce a homogenized diet, meaning it achieves a nutrient coefficient of variation of less than 10% (Reese et al. 2016). Multiple samples of each treatment were collected and pooled prior to analysis of crude protein (Kjeldahl analysis), total calcium (AOAC method 927.02), total phosphorus (AOAC method 964.06), phytic acid (AOAC method 986.1) and phytase (AOAC method 2000.12).

Broiler Performance and Bone Mineralization

A total of 900 straight-run Hubbard x Cobb 500 broilers were fed the dietary treatments for 21 days. The lighting program followed 23L:1D from d1 to 7, and 20L:4D from d8 to 21. Feed and water were provided for ad libitum consumption. Brooding temperature was maintained to maximize bird comfort. Birds were cared for according to West Virginia University Animal Care and Use Committee Guidelines. Feed intake (FI), live weight gain (LWG), mortality corrected feed conversion ratio (FCR), and mortality were calculated for the 21-d growth period.

On d 21, all birds were euthanized via cervical dislocation. Right tibiae were excised and used to determine ash percentage. Tibiae were dried for 24 h at 105°C. Fat was removed via extraction with petroleum ether over a 16 h period. Bones were weighed and then ashed at 600°C for 18 h. Ash weight was recorded and used to determine ash percent.

Statistical Analysis

Bird performance and tibia measures were analyzed using the PROC GLM procedure of Statistical Analysis System (SAS) [14]. Variables were analyzed in a 2 x 2 factorial arrangement with the main effects of phytase product and phytase activity within a randomized complete block design. The experimental unit was a pen of ten broilers. Significance was established at $\alpha = 0.05$. Fisher's LSD multiple comparison test was used to separate means of all nine treatments. Phosphorus

equivalency values were determined using linear regression for live weight gain, feed intake, feed

conversion ratio, and tibia mineralization.

RESULTS AND DISCUSSION

Upon analysis, calculated additions of 500 and 3,000 FTU/kg were analytically similar for TPC and GBP (TPC=290 and 2,800 FTU/kg and GBP=500 and 3,100 FTU/kg, Table 1). Performance variables and tibia mineralization are presented in Table 2. Increased phytase activity increased feed intake ($P<0.0001$). This is in agreement with Pieniazek, et al (2016), who found that increasing phytase levels increases feed intake, body weight, and tibia mineralization. Phytase product had no effect on feed intake. Nyannor et al. (2008) found no difference in FI, LWG, or tibia ash percent when comparing a TPC product to the commercially available phytase Quantum (AB Vista Feed Ingredients, Marlborough, UK), both with an inclusion of 3,650 FTU/kg. Phytase activity and product interacted to affect LWG ($P=0.0473$). The inclusion of phytase at 3,000 FTU/kg resulted in an increased LWG when compared to inclusion at 500 FTU/kg; however, the GBP improvement was greater than TPC. There was no effect of phytase activity on FCR ($P=0.7166$). This is in agreement with Waldroup et al. (2000), who found that increases in LWG correspond proportionately to FI, making it uncommon to find improved FCR when the dietary P deficiency is alleviated. The GBP product resulted in a lower FCR ($P=0.0005$) than the TPC product. The inclusion of phytase at 3,000 FTU/kg resulted in increased tibia ash content ($P=0.0001$) when compared to inclusion at 500 FTU/kg. This is in agreement with Manobhavan et al (2005), who found that superdoses of phytase result in higher tibia ash content when compared to inclusion at 500 FTU/kg. Cowieson et al. (2006) found similar results, stating that phytase concentrations >1200 FTU/kg result in higher bone ash content than when provided at 250 or 500 FTU/kg. Broiler chicks provided the GBP diet resulted in higher tibia ash percent ($P=0.0001$). This is in disagreement with Nyannor and others (2008), however they used a different phytase product (Quantum), their TPC product had a higher phytase activity than Quantum (3,300 and 2,700 FTU/kg, respectively), and they did not provide feed

manufacture data. Feeding a mash versus pellet diet could cause different results. Mortality did not exceed 4% for any treatment.

Linear regression performed on LWG of diets increasing in nPP (R^2=0.66), were used to demonstrate 3,000 FTU/kg nPP equivalency of 0.07 and 0.20 for TPC and GBP respectively (Table 3). Manobhavan et al. (2015) concluded that the inclusion of phytase supplementation at 2,500 and 5,000 FTU/kg improved FI and LWG (P=0.001). Augspurger and Baker (2004) concluded that the addition of exogenous phytase at high levels can hydrolyze nearly 100% of phytate P in broiler chicks. Linear regression performed on tibia ash of diets increasing in nPP (R^2=0.88), were used to demonstrate 3,000 FTU/kg nPP equivalency of 0.08 and 0.15 for TPC and GBP respectively. Differences in expression systems of the TPC and GBP may affect their efficacy. Additionally, particle size of the TPC product may have affected enzyme dispersion.

CONCLUSIONS

1) Providing broiler chicks mash diets with GBP resulted in increased broiler performance and tibia mineralization compared to broiler chicks provided mash diets with TPC. These results may be attributed to variations in dispersion of enzyme within the diet. The transgenic phytase corn has a larger particle size than the granulated bacterial phytase. Despite a homogenous mix, broilers would still be less like to consume the fewer, larger particles of transgenic corn as opposed to the smaller, more frequent particles of granulated bacterial phytase. Differences may also be attributed to the general effectiveness of the expressed enzyme. The expression system of the granulated bacteria phytase may have been superior to that of the transgenic phytase corn.

2) The inclusion of a commercial phytase at 3,000 FTU/kg increased broiler performance and

tibia mineralization compared to inclusion at 500 FTU/kg. These results may be due to a

greater reduction of phytate, alleviating anti-nutrient effects.

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Table 1. Ingredients, calculated nutrient content, and analyzed nutrient content of diets provided to Hubbard x Cobb 500 broilers from 1-21 d of age.

Ingredient (%)	NC	PC
Corn	55.45	54.46
Soybean meal (48%)	37.97	38.22
Soybean oil	3.02	3.30
Limestone	1.68	1.06
DL-Methionine	0.48	0.48
Dicalcium phosphate	0.37	1.45
Salt	0.29	0.29
Vitamin mineral premix[1]	0.25	0.25
Sodium Bicarbonate	0.15	0.15
Lysine	0.12	0.12
Threonine	0.12	0.12
Sand	0.10	0.10
Calculated Nutrients		
ME (kcal/kg)	1,361	1,361
Crude Protein (%)	22.27	22.33
Digestible Lysine (%)	1.20	1.20
Digestible Methionine	0.54	0.54
Digestible Methionine+ Cystine	1.15	1.14
Digestible Threonine (%)	0.84	0.84
Digestible Arginine (%)	1.39	1.40
Digestible Tryptophan (%)	0.24	0.24
Calcium (%)	0.80	0.80
Available Phosphorus (%)	0.20	0.40
Sodium (%)	0.17	0.17

Analyzed Nutrients	NC = 0.2% nPP	NC + 0.1% nPP	NC + 0.15% nPP	NC + 500 FTU GBP	NC + 1000 FTU GBP	NC + 3000 FTU GBP	NC + 500 FTU TPC	NC + 3000 FTU TPC	PC = NC + 0.2% nPP
Crude Protein (%)	21.2	19.6	21.1	20.5	21.1	19.5	20.7	21.1	19.9
Calcium (%)	0.72	0.679	0.903	0.807	0.840	0.830	0.773	0.847	0.74
Non-Phytate Phosphorus[2] (%)[2]	0.20	0.29	0.35	0.24	0.19	0.20	0.21	0.21	0.40
Phytase (ftu/kg)	--	--	--	500	1000	3100	290	2800	--

[1] Supplied per kg of diet: manganese, 0.02%; zinc 0.02%; iron, 0.01%; copper, 0.0025%; iodine, 0.0003%; selenium, 0.00003%; folic acid, 0.69mg; choline, 386mg' riboflavin, 6.61mg; biotin, 0.03mg; vitamin B_6, 1.38mg; niacin, 27.56mg; pantothenic acid, 6.61mg; thiamine, 2.20mg; manadione, 0.83mg; vitamin B_{12}, 0.01mg; vitamin E, 16.53 IU; vitamin D_3, 2133 ICU; vitamin A, 7716 IU.
[2] nPP= Total Phosphorus- (phytic acid x 0.282)
R. Angel et al. 2002

Table 2: Statistical comparison of all dietary treatments as well as the factorial arrangement of treatments on 21 d Hubbard x Cobb straight-run broiler performance.

Enzyme	Activity	Percent AP due to Dicalcium P	Starting Pen Weight (kg)	Feed Intake/pen (kg)	LWG/bird[1] (kg)	FCR[2] (kg/kg)	Percent Mortality (%)	Percent Tibia Ash (%)
No	No	0.2	0.451	9.118^C	0.585^E	1.524^{AB}	4	26.434^E
No	No	0.3	0.451	10.090^{AB}	0.697^B	1.436^{BC}	2	32.1401^C
No	No	0.35	0.451	10.423^A	0.712^B	1.461^{BC}	0	33.714^B
No	No	0.4	0.448	10.562^A	0.761^A	1.382^C	1	35.0136^A
GBP	500 FTU/kg	0.2	0.453	9.396^{BC}	0.640^D	1.448^{BC}	1	29.535^D
GBP	1,000 FTU/kg	0.2	0.449	10.221^A	0.681^{BC}	1.503^B	1	31.523^C
GBP	3,000 FTU/kg	0.2	0.451	10.555^A	0.763^A	1.398^C	4	33.592^B
TPC	500 FTU/kg	0.2	0.453	8.993^C	0.584^E	1.537^{AB}	3	26.783^E
TPC	3,000 FTU/kg	0.2	0.452	10.488^A	0.649^{CD}	1.616^A	0	30.438^D
ANOVA P-value			0.2041	0.0001	0.0001	0.004	0.1186	0.0001
SEM[3]			0.0013	0.2496	0.0128	0.0362	1.2070	0.3419
Marginal Means-Product								
GBP			0.452	9.976	0.701^A	1.423^B	2.500	31.5637^A
TPC			0.452	9.740	0.616^B	1.576^A	1.500	28.6100^B
SEM			0.0011	0.2133	0.0098	0.0285	0.8660	0.3429
Marginal Means-Activity								
500 FTU/kg			0.453	9.195^B	0.612^B	1.492	2.000	28.1589^B
3,000 FTU/kg			0.451	10.521^A	0.706^A	1.507	2.000	32.0148^A
SEM			0.0011	0.2133	0.0098	0.0285	0.8660	0.3429
Main Effects and Interactions								
Product			0.7735	0.4404	0.0001	**0.0005**	0.4196	**0.0001**
Activity			0.4298	**0.0001**	0.0001	0.7166	1.000	**0.0001**
Product x Activity			0.7554	0.5806	**0.0473**	0.1212	**0.0193**	0.6819

[1] LWG= Live Weight Gain
[2] FCR= Feed Conversion Ratio, corrected for mortality
[3] SEM= Standard Error of the Mean

Table 3. Phosphorus equivalency values calculated from standard curve linear regression using Dicalcium Phosphorus.

Treatment	Linear derived Calc nPP (%) LWG[1]	LWG Phosphorus Equivalency Value	Linear derived Calc nPP (%) FI[2]	FI Phosphorus Equivalency Value	Linear derived Calc nPP (%) FCR[3]	FCR Phosphorus Equivalency Value	Linear derived Calc nPP (%) Tibia Ash Percent[4]	Tibia Ash (%) Phosphorus Equivalency Value
GBP 500 FTU/kg	0.26	0.06	0.23	0.03	0.32	0.12	0.26	0.06
GBP 1000 FTU/kg	0.30	0.10	0.34	0.14	0.23	0.03	0.31	0.11
GBP 3000 FTU/kg	0.40	0.20	0.38	0.18	0.40	0.20	0.35	0.15
TPC 500 FTU/kg	0.19	0	0.17	0	0.18	0	0.20	0
TPC 3000 FTU/kg	0.27	0.07	0.37	0.17	0.05	0	0.28	0.08

[1]Calculated nPP values derived from the linear regression of Live Weight Gain for the standard curve (LWG − 0.41981)/0.86087 = calc. nPP, R^2=0.6645

[2]Calculated nPP values derived from the linear regression of Feed Intake for the standard curve (FI− 7.71777)/7.45832 = calc. nPP, R^2=0.2536

[3]Calculated nPP values derived from the linear regression of Feed Conversion Ratio for the standard curve (FCR − 1.64676)/-0.62663 = calc. nPP, R^2=0.1373

[4]Calculated nPP values derived from the linear regression of Tibia Ash % for the standard curve (Tibia Ash % − 18.22012)/43.53670 = calc. nPP, R^2=0.8782

CHAPTER 3

The effect of diets varying in commercial phytase product subjected to increasing steam conditioning temperature on broiler performance and tibia mineralization

V.B. Homan and J.S. Moritz

SUMMARY

Phytases are commonly added to diet formulations prior to pelleting to support phosphorus requirements in broilers. Commercial phytase products vary in thermal stability, site of action, mode of action, and manufacturer suggested protocols for determining activity. The objective of this study was to evaluate three commercial 6-phytase products within a phosphorus deficient corn and soybean meal based diet subjected to increasing steam conditioning temperature on broiler performance and tibia mineralization. Commercial phytase products included a *Buttiauxella* phytase expressed in *Trichoderma reesei* (B Trich), an E. coli phytase expressed in Pichia pastoris (E Pichia), and an E. coli phytase expressed in Trichoderma reesei (E Trich). Conditioning temperatures included 82, 88, and 93°C for a duration of 10 seconds prior to extrusion through a 4.7 x 38 mm pellet die. Commercial phytase products were analyzed for activity using the AOAC 2000.12 method at two different laboratories. The lower obtained activity was utilized to include each product at 1,000 FTU/kg of the diet prior to pelleting. Post extrusion, diets were ground to eliminate any variations in feed form and stored in a dehumidified room to ensure diets were of a similar moisture level, i.e. 12 percent. Diets containing the three commercial phytase products and a negative control diet without phytase were subjected to each conditioning treatment and fed to eight replicate raised wire cages of 10 Hubbard x Cobb straight-run broiler chicks for 20 d. Treatments were blocked by cage locations within room. Broiler performance and tibia mineralization were analyzed using a 4 x 3 factorial arrangement within a randomized complete block design. The main effect product was significant for feed intake (FI), live weight gain (LWG) and feed conversion ratio (FCR). Broilers fed diets without phytase had the lowest FI and LWG ($P<0.05$). Broilers fed E Trich had the highest FI and LWG ($P<0.05$). Broilers fed B Trich and E Pichia had lower FCR

compared to broilers fed diets without phytase and E Trich fed broilers were not distinguishable from other treatments (P<0.05). The main effect conditioning temperature was significant for FI and LWG. Increased conditioning temperature sequentially decreased both FI and LWG (P<0.05). Main effect interactions were not apparent (P>0.05). Commercial products demonstrated differences in supporting the phosphorus requirement in broilers and increasing conditioning temperature was detrimental to broiler performance. Increased conditioning temperature may have decreased nutrient availability per se, denatured phytase products, or both factors contributed to performance detriment.

DESCRIPTION OF THE PROBLEM

Phytate (myo-inositol hexakisphosphate, IP6) makes up 2/3 of the phosphorus (P) provided in poultry diets (Eeckhout and DePaepe, 1994; Harland and Oberleas, 1999; Adeola and Sands, 2003). Phytate forms insoluble complexes with other nutrients, and poultry lack the endogenous enzymes necessary for the effective hydrolysis of phytate (Adeola and Sands, 2003; Bedford and Schulze, 1998). Phytase has the ability to hydrolyze phytate, making otherwise inaccessible phosphorus available to the bird (Ravindran et al., 1995). Microbial phytases are classified based on the site of initial phytate hydrolysis. A 6-phytase will dephosphorylate phytate in a sequential way, beginning by removing iP from the sixth position on the phytic acid molecule (Greiner et al., 2000).

Exogenous phytase supplementation has been shown to decrease feed cost, as well as improve broiler performance (Bedford, 2000). These improvements are indicated by increased weight gain, feed intake, Phosphorus utilization, and tibia mineralization (Gurbuz et al., 2009; Angel et al., 2002). Specifically, the addition of a 6-phytase at 500 FTU/kg has been shown to improve feed intake and bone mineralization (Pieniazek et al., 2016). Although research has shown effects based on inclusions of 500 FTU/kg, activity inclusion of 1,000 FTU/kg was chosen for this study. Considered a super-dose, 1,000 FTU/kg was selected so that even if partial inactivation of the enzyme occurred, performance results would still be demonstrated in comparison to the negative control.

Phytase enzymes may be sprayed onto the feed post-pelleting, but this method does not ensure mix uniformity throughout the diet or protect against nutrient segregation effects. Therefore, most phytase enzymes are added at the mixer. In order for mixer-added exogenous phytase products to be efficacious in pelleted feed, they must be thermally stable. Phytase

products may contain a carbohydrate-lipid coating, be genetically modified, or be specifically selected for heat stability, in an attempt to circumvent issues associated with denaturation during the pelleting process. Increasing steam conditioning temperatures results in decreased enzyme activity (Loop et al. 2012). It has also been shown that when conditioning temperature increases (from 74 to 96°C), feed conversion ratio (FCR) increases and amino acid digestibility decreases (Loar et al., 2014). Several researchers have proposed target conditioning temperatures of 80-85°C (Jones and Richardson, 2004; Veldman et al., 1995). Equipment, ambient conditioning temperature, and nutrient composition affect the ability to condition feed at higher temperatures. However, conditioning temperature of 82°C can generally be obtained. Research focusing on thermal stability and pellet quality often condition feed at 88°C. Since the objective was to examine thermal stability, an upper limit of 93°C was chosen for this study. At this conditioning temperature, reductions in performance are expected. This is in agreement with research by Cutlip et al. (2008), who found that conditioning feed at 93°C resulted in decreased feed intake. Loar and authors (2014) also found detrimental effects associated with conditioning feed over 90°C.

There is not a universally-accepted recommendation for steam conditioning temperatures, and due to the variability of heat stability among commercially available products, it is important to establish appropriate conditioning temperatures for specific phytase products. The objective of the current study was to evaluate three commercial 6-phytase products within a phosphorus deficient corn and soybean meal based diet subjected to increasing steam conditioning temperature on broiler performance and tibia mineralization.

MATERIALS AND METHODS

Birds and Husbandry

Nine hundred sixty Hubbard x Cobb 500 straight-run broilers were obtained on the day of hatch, weighed, and randomly distributed into 96 raised-wire pens. The lighting program followed 23L: 1D from d1 to 7, and 20L: 4D from d8 to 20. Feed and water were provided on an ad libitum basis. Brooding temperature was maintained to maximize bird comfort. Birds were cared for according to West Virginia University Animal Care and Use Committee Guidelines.

Dietary Treatments and Feed Manufacture

The treatments included a negative control (NC) diet containing 0.2% non-phytate phosphorus (nPP) via dicalcium phosphate (DCP), and NC diets with the addition of three different commercial phytase products. The three phytase products evaluated were a phytase enzyme sourced from a *Buttiauxella* phytase expressed in *Trichoderma reesei* (B Trich), an E.coli phytase expressed in *Pichia pastoris* (E Pichia), and an E. coli phytase expressed in *Trichoderma reesei* (E Trich). Each phytase product was included at 1000 FTU/kg. One phytase unit (FTU) may be defined as the quantity of enzyme required to liberate 1 μmol of inorganic P per minute from an excess of 1.5 mM sodium at 37°C, with a pH of 5.5 (International Union of Biochemistry, 1979). All four different diet formulations were conditioned at 82, 88, and 93°C and subsequently pelleted.

Feed manufacture was conducted at the West Virginia University pilot feed mill. Prior to diet formulation, samples of each enzyme were sent to two different labs for determination of phytase level. Upon analysis, the lower value reported for each enzyme (B Trich= 2080 FTU/g, E Pichia = 4680 FTU/g, E Trich = 5300 FTU/g) was used in order to calculate a 1000 FTU/kg

enzyme activity inclusion. Additional samples were analyzed for phytase activity following feed manufacture to determine how feed manufacture affected phytase levels.

Washed builders sand was used to standardize volume among the different phytase product inclusions to a 0.1% total inclusion (Table 1). The phytase product, sand, and 5 lbs. of ground corn were mixed for ten minutes in a 4.73-liter Hobart mixer [14] prior to final treatment batching and mixing. Each dietary treatment was mixed for ten minutes in a 1-ton vertical screw Easy Automation Inc. mixer [15]. This mix time was chosen because it has been shown to produce a homogenized diet, meaning it achieves a nutrient coefficient of variation of less than 10% [16]. Diets were steam conditioned using a short-term (10 s) conditioner and extruded through a 4.7 × 38 mm pellet die driven by a 40-horsepower California Pellet Mill [17]. To ensure conditioning temperatures were met, hot pellet temperature was monitored for each treatment. Diets were then reduced to a small particle size via the roller mill to eliminate any variations in feed form. Finished feed was placed in open bags in a dry room containing a dehumidifier to ensure diets were of similar moisture (12%).

Multiple samples of each treatment were collected and pooled for analysis of crude protein (Kjeldahl analysis), total calcium (AOAC method 927.02), total phosphorus (AOAC method 964.06), phytic acid (AOAC method 986.1) and phytase (AOAC method 2000.12). Pellet durability was determined 24 hours post-pelleting using the New Holmen Pellet Tester (NHPT) [18] (Table 2). Particle size and particle size standard deviation were determined using a 100 g representative sample passed through a RO-TAP RX-29 for a 10-minute processing period [19].

Broiler Performance and Bone Mineralization

Feed intake, live weight gain (LWG), mortality corrected feed conversion ratio (FCR), and mortality were calculated for the 20-d growth period. On d 20, all birds were euthanized by cervical dislocation. Right tibiae were excised and used to determine ash weight and percentage. Tibiae were dried for 24 h at 105°C. Fat was removed via extraction with petroleum ether in a soxhlet apparatus over a 16 h period. Bones were weighed and then ashed at 600°C for 18 h. Ash weight was recorded for determination of ash percent and ash weight per chick.

Statistical Analysis

Bird performance and tibia measures were analyzed using the PROC GLM procedure of Statistical Analysis System (SAS) [20]. Variables were analyzed in a 4 x 3 factorial arrangement with the main effects of enzyme (No, B Trich, E Pichia, E Trich) and conditioning temperature (82, 88, 93°C) within a randomized complete block design. The experimental unit was a pen of ten broilers. Significance was established at $\alpha = 0.05$. Fisher's LSD multiple comparison test was used to separate means when interactions were significant.

RESULTS AND DISCUSSION

Each diet formed one master batch that was then split and steam conditioned at the appropriate temperature. Increasing conditioning temperature resulted in decreased analyzed phytase values (Table 3). E Trich was the most thermally stable enzyme, followed by E Pichia. B Trich was the least stable enzyme used, providing insufficient levels of phytase when conditioned at 88 and 93°C.

Broiler performance is presented in Table 4. The main effect product was significant for FI, LWG, and FCR. Broilers fed E Trich had the highest feed intake and LWG (P=0.0001). Boilers fed diets without phytase had the lowest FI and LWG, while broilers fed B Trich and E Pichia were intermediate in FI and LWG. Broilers fed B Trich and E Pichia had lower FCR (P=0.0321) when compared to broilers fed diets without phytase. Broilers fed E Trich were not distinguishable from other treatments in terms of FCR. The main effect conditioning temperature was significant for FI, and LWG. Increasing conditioning temperature sequentially decreased both FI and LWG (P=0.0001). Cutlip et al. (2008) also found that feeding pellets produced by high conditioning temperatures (93.3°C) decreases broiler feed intake. However, that study also stated that increasing conditioning temperatures decreases feed conversion ratio. It is important to remember that diets in the current study were ground via a roller mill back to fines in order to observe the effect that increasing steam conditioning temperatures has on phytase activity rather than pellet quality. Increasing steam conditioning temperatures is known to increase pellet quality (Thomas and van der Poel, 1996; Table 2), and increased pellet quality may have effected bird performance and tibia mineralization differently.

Phytase enzyme and conditioning temperature interacted to affect mortality. Broiler chicks provided the NC diet steam conditioned at 93°C had higher mortality than any other treatment. Steam conditioning at high temperatures has the ability to denature enzymes as well as other dietary protein, which would result in a smaller bird that would not be able to consume as much feed, and the nutrient deficiency may result in death. This theory is supported by Loar and others (2014), who found that increasing conditioning temperatures decreases the digestibility and bioavailability of nutrients. The main effects enzyme and conditioning temperature interacted to affect tibia ash percent (P=0.0016) and tibia ash mg/chick (P=0.0001). E Trich resulted in higher tibia measures compared to the other treatments. All enzymes resulted in decreased tibia measures with increased temperature, however the decrease was most drastic when feed was conditioned at 93°C. This negative affect was not observed with broilers fed the NC. The decreased performance and tibia mineralization when exogenous phytase is included was likely associated with the denaturation of the enzyme as conditioning temperatures increase (Jongbloed and Kemme, 1999).

CONCLUSIONS

1) Commercial enzyme products demonstrated differences in supporting the phosphorus requirement in broilers.

2) Increasing conditioning temperatures had a detrimental effect on broiler performance and tibia mineralization. The increased conditioning temperatures may have decreased nutrient availability, denatured phytase products, or both of these events may have influenced the results.

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Table 1. Diet formulation and calculated nutrient values.

Ingredient	(%)
Corn	55.45
Soybean meal (48%)	37.97
Soybean oil	3.02
Limestone	1.68
DL-Methionine	0.48
Dicalcium phosphate	0.37
Salt	0.29
Vitamin mineral premix[1]	0.25
Sodium Bicarbonate	0.15
Lysine	0.12
Threonine	0.12
Sand/Phytase[2]	0.10
Calculated Nutrients	
ME (kcal/kg)	1,361
Crude Protein (%)	22.27
Digestible Lysine (%)	1.20
Digestible Methionine	0.54
Digestible Methionine+ Cystine	1.15
Digestible Threonine (%)	0.84
Digestible Arginine (%)	1.39
Digestible Tryptophan (%)	0.24
Calcium (%)	0.80
Available Phosphorus (%)	0.20
Sodium (%)	0.17

[1] Supplied per kg of diet: manganese, 0.02%; zinc 0.02%; iron, 0.01%; copper, 0.0025%; iodine, 0.0003%; selenium, 0.00003%; folic acid, 0.69mg; choline, 386mg' riboflavin, 6.61mg; biotin, 0.03mg; vitamin B_6, 1.38mg; niacin, 27.56mg; pantothenic acid, 6.61mg; thiamine, 2.20mg; manadione, 0.83mg; vitamin B_{12}, 0.01mg; vitamin E, 16.53 IU; vitamin D_3, 2133 ICU; vitamin A, 7716 IU.

[2] NC= 1.4 lbs (100%) sand, B Trich = 0.73 lbs (52%) sand and 0.67 lbs (48%) phytase, E Pichia = 1.10 lbs (79%) sand and 0.30 lbs (21%) phytase, E Trich = 1.14 lbs (81%) sand and 0.26 lbs (19%) phytase.

Table 2: Descriptive feed manufacture data of dietary treatments steam conditioned in a 0.31 x 1.3 m conditioner for 10 s and extruded through a 4.7 x 38 mm pellet die.

Enzyme	Conditioning Temperature (°C)	Formulated Available P and Total Ca	Hot Pellet Temperature[1] (°C)	Average Pellet Mill Motor Amperage	Production Rate (Tonne/hr)	Pellet Survivability due to NHPT[2] (%)	Crumble Particle Size[3] (µm)
No	82	0.20 and 0.80	85	19.55	0.814	67.82	1061.68
No	88	0.20 and 0.80	89	19.85	0.807	83.79	1062.66
No	93	0.20 and 0.80	96	20.50	0.806	93.78	1001.29
B Trich	82	0.20 and 0.80	85	19.95	0.811	74.91	986.15
B Trich	88	0.20 and 0.80	89	19.85	0.838	83.76	1036.46
B Trich	93	0.20 and 0.80	94	21.40	0.835	91.97	991.21
E Pichia	82	0.20 and 0.80	84	20.10	0.831	73.75	1036.52
E Pichia	88	0.20 and 0.80	90	19.85	0.817	81.80	1084.57
E Pichia	93	0.20 and 0.80	96	21.90	0.866	92.84	1007.77
E Trich	82	0.20 and 0.80	86	19.75	0.844	71.87	969.80
E Trich	88	0.20 and 0.80	89	19.55	0.853	80.09	1045.37
E Trich	93	0.20 and 0.80	95	20.85	0.880	91.65	1006.39

[1]Hot pellet temperature was determined on pellets directly following extrusion from the die. Pellets were collected into an insulated container and temperature was measured using a thermocouple thermometer and an 80PK-24 temperature probe.
[2]Measurements of PDI were obtained using the New Holmen Pellet Tester, where 100 g pelleted samples are subjected to air flow within a perforated chamber for 30 s.
[3]Particle size was determined with a Ro-Tap particle size analyzer model RX-29 Type 110V 60H2.
[4]B Trich = *Buttiauxella* phytase expressed in *Trichoderma reesei*
[5]E Pichia = E.coli phytase expressed in *Pichia pastoris*
[6]E Trich = E. coli phytase expressed in *Trichoderma reesei*

Table 3. Analyzed nutrient content of diets provided to Hubbard x Cobb 500 broilers from 1-20 d of age.

Treatment	Crude Protein (%)	Calcium (%)	Non-Phytate Phosphorus (%)[1]	Phytase (ftu/kg)
NC @ 82°C	23.40	0.987	0.172	--
NC @ 88°C	22.20	0.781	0.182	--
NC @93°C	21.85	0.761	0.185	--
[2]B Trich @ 82°C	22.00	0.885	0.181	580
B Trich @ 88°C	21.92	0.805	0.184	200
B Trich @ 93°C	21.40	0.955	0.179	<70
[3]E Pichia @ 82°C	21.39	0.832	0.179	880
E Pichia @ 88°C	22.20	0.845	0.178	560
E Pichia @ 93°C	21.73	0.894	0.184	140
[4]E Trich @ 82°C	22.27	0.794	0.178	1100
E Trich @ 88°C	22.17	0.843	0.184	1400
E Trich @ 93°C	21.72	0.871	0.186	250

[1]nPP = Total Phosphorus- (phytic acid x 0.282)
R. Angel et al. 2002
[2]B Trich = *Buttiauxella* phytase expressed in *Trichoderma reesei*
[3] E Pichia = E.coli phytase expressed in *Pichia pastoris*
[4]E Trich = E. coli phytase expressed in *Trichoderma reesei*

Table 4. Statistical comparison of all dietary treatments as well as the factorial arrangement of treatments on 20 d Hubbard x Cobb straight-run broiler performance.

Enzyme	Conditioning Temp. (°C)	Starting Pen Weight (kg)	Feed Intake/ bird (kg)	Live Weight Gain / bird (kg)	FCR[1] (kg/kg)	Mortality (%)	Tibia Ash (%)	Tibia Ash (mg/chick)
No	82	0.436	0.686^e	0.597^c	1.149	1.250^{bc}	42.624^{cd}	420.38^{gh}
No	88	0.434	0.663^{ef}	0.554^d	1.156	6.250^{bc}	42.138^{de}	378.76^{gh}
No	93	0.437	0.562^h	0.471^f	1.122	15.000^a	40.838^{ef}	329.85^i
B Trich[2]	82	0.437	0.749^{abc}	0.682^a	1.094	1.250^{bc}	47.311^a	672.83^{ab}
B Trich	88	0.434	0.708^{cde}	0.641^b	1.103	3.750^{bc}	44.709^b	517.77^{cd}
B Trich	93	0.434	0.583^{gh}	0.523^e	1.103	3.750^{bc}	39.587^f	375.24^h
E Pichia[3]	82	0.434	0.744^{bcd}	0.643^b	1.107	7.500^b	44.729^b	527.11^c
E Pichia	88	0.435	0.689^e	0.615^{bc}	1.119	0^c	43.963^{bc}	484.20^{de}
E Pichia	93	0.435	0.623^{fg}	0.543^{de}	1.110	6.250^{bc}	40.208^f	356.32^{hi}
E Trich[4]	82	0.437	0.799^a	0.705^a	1.134	0^c	47.571^a	706.45^a
E Trich	88	0.438	0.775^{ab}	0.680^a	1.137	1.250^{bc}	47.058^a	646.65^b
E Trich	93	0.434	0.695^{de}	0.611^c	1.110	7.500^b	43.315^{bcd}	461.75^{ef}
ANOVA P-value		0.0723	**0.0001**	**0.0001**	0.3346	**0.0004**	**0.0001**	**0.0001**
Fisher's LSD		0.0031	0.0514	0.0294	0.0511	--	1.765	41.839
SEM[5]		0.0011	0.0183	0.0104	0.0182	2.2899	0.6268	14.857
Marginal Means- Enzyme								
B Trich		0.435	0.680^b	0.615^b	1.100^b	2.917	43.869	521.95
E Pichia		0.435	0.685^b	0.600^b	1.112^b	4.583	42.967	455.87
E Trich		0.436	0.756^a	0.665^a	1.127^{ab}	2.917	45.981	604.95
None		0.436	0.637^c	0.540^c	1.142^a	7.500	41.866	376.33
SEM		0.0006	0.0107	0.0063	0.0104	1.3200	0.3657	9.4366
Marginal Means- Conditioning Temperature °C								
82°		0.436	0.745^a	0.657^a	1.121	2.500^b	45.559	581.69
88°		0.435	0.709^b	0.622^b	1.129	2.813^b	44.467	506.85
93°		0.435	0.616^c	0.537^c	1.111	8.125^a	40.989	380.80
SEM		0.0006	0.0092	0.0054	0.0090	1.1431	0.3167	8.1724
Probability Values for Main Effects and the Interaction								
Enzyme		0.2584	**0.0001**	**0.0001**	**0.0321**	0.0520	**0.0001**	**0.0001**
Conditioning Temperature		0.4132	**0.0001**	**0.0001**	0.400	**0.0009**	**0.0001**	**0.0001**
Enzyme x Conditioning Temperature		0.0672	0.5825	0.0707	0.9356	**0.0179**	**0.0016**	**0.0001**

[1]FCR= Feed Conversion Ratio, corrected for mortality

[2]B Trich = *Buttiauxella* phytase expressed in *Trichoderma reesei*

[3]E Pichia = E.coli phytase expressed in *Pichia pastoris*

[4]E Trich = E. coli phytase expressed in *Trichoderma reesei*

[5]SEM = Standard Error of the Mean