

Effect of *Bacillus*–direct-fed microbial on leaky gut, serum peptide YY concentration, bone mineralization, and ammonia excretion in neonatal female turkey poults fed with a rye-based diet

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ABSTRACT Rye is high in nonstarch polysaccharides (NSP), a complex carbohydrate which cannot be digested by poultry as they lack the endogenous enzymes to do so. Exogenous carbohydrases must therefore be supplemented to avoid the antinutritional effects associated with a high NSP diet. The objectives of the present study were to evaluate the effects of a rye-based diet with and without supplementation of a *Bacillus* direct-fed microbial (DFM) on body weight, bone mineralization, and leaky gut, as well as its role on influencing serum concentrations of peptide YY (PPY) and the ammonia concentration in turkey manure. Two independent trials were conducted. In each experiment, day-of-hatch female turkey poults were neck tagged and randomly assigned to either a control rye-based diet or a rye-based diet supplemented with the DFM ($n = 25$ birds/group). At 10 days-of-age, poults in both groups were administered with an appropriate dose of fluorescein isothiocyanate-dextran (FITC-d) by oral gavage. One hour later, all poults were euthanized. Blood was collected to evaluate serum FITC-d and PPY concentrations. Furthermore, in Trial 2 only, both tibias were removed for assessment of bone parameters, and turkey manure was collected to evaluate physicochemical analysis. In both trials, poults treated with the DFM showed a significant increase ($P < 0.05$) in body weight

and body weight gain as compared with control nontreated poults. Poults that received the DFM also had a significant reduction in serum levels of PPY and FITC-d when compared with control nontreated poults. In Trial 2, turkeys treated with the DFM had a substantial increase in tibia strength, tibia diameter, total ash, calcium, and phosphorus when compared with control nontreated turkeys. Their manure was also shown to have a significant reduction in the concentration of ammonia. This is the first report of a commercial DFM reducing the concentration of this compound in turkey manure. In summary, the results of the present study confirm that turkeys fed with a rye-based diet have a significant increase in gut permeability, a reduced body weight, and decreased bone mineralization when compared with turkeys fed with the DFM. Turkeys that received the rye-based diet supplemented with the *Bacillus*-DFM also had a significant reduction in the serum concentration of PPY when compared with control turkeys. This finding suggests a possible prebiotic effect of rye, warranting future studies to test this effect. Further studies to evaluate the microbiota diversity, as well as the concentration of ceca short-chain fatty acids, are also necessary to confirm the reliability of PPY as a potential metabolomic biomarker in poultry.

Key words: ammonia, *Bacillus*-DFM, bone mineralization, leaky gut, peptide YY

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INTRODUCTION

As monogastrics lack the endogenous enzymes needed to digest nonstarch polysaccharides (NSP), their diets must be supplemented with exogenous enzymes to avoid the antinutritional effects associated with a high NSP diet (Bedford and Classen, 1993; Choct et al., 1995;

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Bedford and Schulze, 1998; Kiarie et al., 2013; Tellez et al., 2015). Previous studies using rye as a source of energy, without enzyme supplementation, showed that a rye-based diet reduces body weight, increases digesta viscosity, bacterial translocation, and leakage of fluorescein isothiocyanate-dextran (**FITC-d**), alters the microbiota composition, and reduces bone mineralization (Tellez et al., 2014, 2015). These adverse effects can be prevented by the inclusion of a *Bacillus* direct-fed microbial (**DFM**) that was specifically selected to produce exogenous enzymes (Latorre et al., 2014, 2015).

Probiotics, prebiotics, and synbiotics (the combination of probiotics and prebiotics) are gaining importance because they seem to exert their nutritional benefits in various animal species, and their concept is more understandable. Numerous poultry studies have demonstrated the clear benefits of probiotics in the performance and health status of birds. Furthermore, probiotics serve as an improved alternative to antibiotic growth promoters as they do not cause microbial resistance.

In recent years, our laboratory has been working on developing different models to induce gut inflammation, including the use of a rye-based diet (Tellez et al., 2014; Kuttappan et al., 2015a; Menconi et al., 2015; Vicuña et al., 2015a,b), and the identification of biomarkers as indicators for gut barrier failure in poultry (Chen et al., 2015; Baxter et al., 2019). In mammals, Peptide YY (**PPY**) has been used as an indirect biomarker of metabolomics (Xu et al., 1899; Goodacre, 2007; Musso et al., 2010). Peptide YY consists of a short 36-amino acid peptide with a similar structure to neuropeptide Y and pancreatic polypeptide. It is released in the intestine after feeding to inhibit stomach acid secretion, empty the stomach, induces the secretion of pancreatic enzymes, and promotes intestinal motility. Peptide YY is also involved in energy balance and utilization as it is related to feed intake and insulin homeostasis (Gomez et al., 1999). Fermentation of dietary fiber, such as those found in rye, increases the concentration of short-chain fatty acids (**SCFA**) and induces the release of PPY, which then causes satiety (Psichas et al., 2015; Brooks et al., 2017).

The objective of the present study was to confirm the role of the DFM used in previous studies on body weight, intestinal permeability, and bone mineralization, while extending these findings to further evaluate serum PPY concentration as a new metabolomic biomarker, as well as ammonia excretion in neonatal turkey poult fed with a rye-based diet.

MATERIAL AND METHODS

Bacillus-Based Probiotic

The present study was conducted utilizing Norum (Eco-Bio/Euxxis Bioscience LLC, Fayetteville, AR), a *Bacillus* spore DFM culture, consisting of 3 isolates: 2 *Bacillus amyloliquefaciens* and 1 *Bacillus subtilis* (Latorre et al., 2016). Each strain is cultivated and sporulated individually before being added in the same ratio to the final

Table 1. Ingredient composition and nutrient content of a rye-soybean starter diet used in all experiments on as-is basis.

Item	Amount
Ingredients (%)	
Rye	37.24
Soybean meal	48.22
Poultry oil	7.95
Dicalcium phosphate	3.66
Ground limestone	1.13
Sodium chloride	0.41
DL-Methionine	0.43
Vitamin premix ¹	0.10
L-Lysine HCl	0.50
Choline chloride 60%	0.10
Mineral premix ²	0.10
Threonine	0.12
Selenium	0.02
Antioxidant ³	0.02
Calculated analysis (CA)	
ME (kcal/kg)	2,844
Crude protein (%)	28.50
Total Calcium (%)	1.49
Available Phosphorus (%)	0.81
Total Lysine (%)	1.82
Total Methionine (%)	0.79
Total Methionine + Cystine (%)	1.18
Total Threonine (%)	1.00

¹Vitamin premix supplied the following per 1,000 kg: vitamin A, 20,000,000 IU; vitamin D₃, 6,000,000 IU; vitamin E, 75,000 IU; vitamin K₃, 9 g; thiamine, 3 g; riboflavin, 8 g; pantothenic acid, 18 g; niacin, 60 g; pyridoxine, 5 g; folic acid, 2 g; biotin, 0.2 g; cyanocobalamin, 16 mg; and ascorbic acid, 200 g (Nutra Blend LLC, Neosho, MO).

²Mineral premix supplied the following per 1,000 kg: manganese, 120 g; zinc, 100 g; iron, 120 g; copper, 10 to 15 g; iodine, 0.7 g; selenium, 0.4 g; and cobalt, 0.2 g (Nutra Blend LLC, Neosho, MO).

³Ethoxyquin.

commercial product. The product contains a concentration of stable *Bacillus* spores ($\sim 3 \times 10^{11}$ spores/g).

Animal Source and Diets

To show that similar results can be achieved independently, 2 trials, without replicates, were conducted in the present study. In each experiment, 50 day-of-hatch female turkey poults were obtained from a commercial hatchery (Cargill, Gentry, AR). Turkey poults were neck tagged and randomly assigned to either a control group, which was given a rye-based diet or a treated group fed a rye-based diet supplemented with 10^6 spores per gram of feed of Norum. Both diets met the nutritional requirements recommended by the National Research Council (1994). In each group, 25 turkey poults served as experimental units and were placed in 2 isolator chambers with a controlled age-appropriate environment where they were given *ad libitum* access to feed and water for 10 D. The number of animals used was based on published studies in which similar outcomes were measured (Tellez et al., 2015). No antibiotics were added to any of the diets (Table 1). All animal handling procedures were in compliance with the Institutional Animal Care and Use Committee at the University of Arkansas, Fayetteville. Explicitly, the Institutional Animal Care and Use Committee approved this study under protocol #11047: "Evaluation of direct-fed microbials and prebiotics in poultry."

Experimental Design

Two independent trials were conducted. In trials 1 and 2, 50 day-of-hatch, turkey poults were randomly neck tagged, weighted, and assigned to 1 of 2 groups ($n = 25/\text{group}$). At 10 day-of-age, all turkey poults in both experiments were weighed and euthanized via carbon dioxide asphyxiation. Blood was collected ($n = 20$) from the femoral vein. In Trial 2, tibias ($n = 12$) were also collected to analyze bone parameters and turkey manure ($n = 8$) for physicochemical analysis, as described below.

Serum Analysis

Intestinal leakage of FITC-d (MW 3–5 kDa; Sigma-Aldrich Co., St. Louis, MO) and the measurement of its serum concentration as a marker of paracellular transport and mucosal barrier dysfunction was performed as previously described by (Baxter et al., 2017). Fluorescence measured was then compared with a standard curve with known FITC-d concentrations. Gut leakage for each bird was reported as ng of FITC-d/mL of serum (Baxter et al., 2017). Serum levels of PYY were determined using a rabbit Peptide YY ELISA kit (cat. no. ABIN1565099, Antibodies-Online Inc, Limerick, PA 19468) according to the manufacturer's protocol.

Bone Parameters

In Trial 2, bone parameters were measured according to the methods described by Zhang and Coon (1997). Tibias from each poult were cleaned of attached tissues. Bones from the left leg were subjected to conventional assays as described below, and the tibias from the right legs were used to determine breaking strength. The bones from the left tibia were dried at 100°C for 24 h and weighed again. Then the samples were ashed in a muffle furnace (Isotemp muffle furnace; Fisher Scientific, Pittsburgh, PA) at 600°C for 24 h in crucibles, cooled in a desiccator, and weighed. Finally, the content of calcium and phosphorus in the tibia was determined using standard methods (AOAC International, 2000). The right tibial diaphysis from individual birds was cleaned of adherent tissues, the periosteum was removed, and the biomechanical strength of each bone was measured using an Instron 4502 (Norwood, MA) material testing machine with a 509 kg load cell. The bones were held in identical positions, and the mid-diaphyseal diameter of the bone at the site of impact was measured using a dial caliper. The maximum load at failure was determined using a 3-point flexural bend fixture with a total distance of 30 mm between the 2 lower supporting ends. The load, defined as the force in kg per mm² of cross-sectional area (kg/mm²), represents bone strength. The rate of loading was kept constant at 20 mm/min collecting 10 data points per second using Instron's Series IX Software.

Physicochemical Analysis of the Turkey Manure

In Trial 2, 8 samples of manure from each group were collected from the trays of the isolators chambers at day 10 to obtain the physicochemical analysis of the manure. Moisture was determined by drying the turkey manure at 65°C overnight and comparing the weight before and after drying. The water potential of incubated turkey manure was measured at 23°C using a dew point potentiometer (Model WP4; Decagon Inc., Pullman, WA). The instrument measures water potentials from 0 to -80 MPa with a precision of ± 0.1 MPa. Water potential was obtained by placing 2 g of turkey manure in the potentiometer chamber and allowing it to equilibrate for 5 to 10 min. Turkey manure pH was determined using a combination electrode (Fisher Scientific, Hampton, NH) at a 5:1 deionized water to turkey manure ratio. Total N and total C were determined by combustion of the turkey manure using a Vario Max CN analyzer (Elementar Americas, Inc., Mt. Laurel, NJ). The NH₄-N content of turkey manure was determined using a 1:10 turkey manure to 1 N KCl extraction followed by colorimetric analysis on a Skalar using chemical method no.155-324 (Skalar Inc., Buford, GA). A 7Multi Mettler Toledo probe (Mettler-Toledo, LLC, Columbus, OH) was used to measure pH on the 1:10 1N KCl extractions. The NO₃-N content was also assessed after this KCl extraction using Quickchem FIA+, method #12-107-04-1-B (Lachat Instruments). The remaining total elemental composition of poultry turkey manure was determined using inductively coupled plasma-optical emission spectroscopy analysis after HNO₃ and HCl microwave digestion. Microwave digestion was performed using a Mars 5 Microwave (CEM Corp., Matthews, NC). The procedure consisted of mixing 0.5 g turkey manure with 9 mL HNO₃ and 3 mL HCl in a Teflon microwave digestion vessel. This mixture was allowed to predigest for 45 min at room temperature and then placed in the microwave. A 6.5-minute ramp time was used to achieve a digestion temperature of 175°C, which was held for 12 min. Samples were allowed to cool to room temperature and then filtered through a Whatman 42 filter before inductively coupled plasma optical emission spectrometry analysis. Total N and NH₄-N analyses were performed on wet turkey manure. Microwave digestion for analysis by inductively coupled plasma optical emission spectrometry was performed on turkey manure dried at 65°C. Organic-N was estimated by subtracting the NH₄-N and NO₃-N values from the total N value. Organic N mineralization and total N loss were determined by mass balance. All N values were adjusted for moisture content and are reported on a dry weight basis.

Statistical Analysis

Data from body weight, body weight gain, serum determination of FITC-d and PYY, bone strength and bone composition as well as physicochemical analysis of the poultry manure were subjected to one-way analysis

of variance as a completely randomized design using the General Linear Models procedure of Statistical Analysis System (SAS Institute, 2002). Duncan's multiple range test determined significant differences among the means. All data are expressed as the mean \pm standard error. A P -value of $P < 0.05$ was set as the threshold for significance.

RESULTS

The results of the evaluation of body weight, body weight gain, PYY, and FITC-d in neonatal turkey poult fed with a rye-soybean-based diet with or without dietary inclusion of DFM in trials 1 and 2 are summarized in Table 2. In both trials, poult treated with the DFM showed a significant increase ($P < 0.05$) in body weight and body weight gain as compared with control nontreated poult on day 10. The inclusion of the DFM also led to a significant reduction in serum levels of PYY and FITC-d when compared with control nontreated poult (Table 2).

Table 3 shows the results of the evaluation of bone strength and bone composition in neonatal turkey poult fed with a rye-soybean based diet with or without dietary inclusion of DFM in trial 2. Turkeys treated with the DFM had a significant increase in tibia strength, tibia diameter, total ash, calcium, and phosphorus when compared with control nontreated turkeys (Table 3).

The results of the physicochemical analysis of the poultry manure in neonatal turkey poult fed with a rye-soybean-based diet with or without dietary inclusion of a DFM in trial 2 are summarized in Table 4. The manure of the control nontreated turkeys showed a significant increase in water content and ammonia but a significant decrease in pH when compared with DFM-treated turkeys (Table 4).

DISCUSSION

Corn is a source of energy in monogastric diets; however, owing to cost or availability, alternative grains such as rye, wheat, barley, or oats are also utilized. Although those grains are a good source of energy, they are also high in NSP which are associated with adverse effects on digestibility, productivity, and animal

health (Choct et al., 1995; Hofacre, 2001; Annett et al., 2002; Timbermont et al., 2011). In our laboratory, the supplementation of a rye-based poultry diet is one of the most robust models to induce intestinal inflammation in poultry. Inflammatory responses in the gastrointestinal tract are associated with dysbacteriosis and increased permeability (Yoshikawa et al., 2008; Kuttappan et al., 2015b; Awad et al., 2017). In just 10 D following *ad libitum* administration of a rye-based diet in chickens or turkeys, birds show a significant reduction in body weight and bone mineralization, increased digesta viscosity, bacterial translocation, intestinal permeability, and dysbiosis (Tellez et al., 2014, 2015). Remarkably, these adverse effects can be prevented by the inclusion of a *Bacillus*-DFM that was specifically selected to produce exogenous enzymes (Latorre et al., 2014, 2015). Our results confirmed that the supplementation of the *Bacillus*-DFM on a rye-based diet improved body weight and bone mineralization, as well as reduced the leakage of FITC-d in turkey poult (Tables 2, 3). The significant reduction in bone strength and mineralization in control turkeys also confirmed previous studies that have shown that high NSP diets in poultry or gluten intolerance in humans are associated with malabsorption of minerals and fat-soluble vitamins (MacAuliffe and McGinnis, 1971; Campbell et al., 1983; Rennie et al., 1993; Bianchi and Bardella, 2008; Capriles et al., 2009; Kotake et al., 2009; Wideman et al., 2011).

Probiotics are a single or mixed culture of living microorganisms which when administrated in adequate numbers exert health benefits for the host by improving the host's intestinal microbial balance, enhancing colonization resistance against pathogens, and improving a host's immune response (Schrezenmeir and de Vrese, 2001). Although the mechanisms by which probiotic bacteria assert their beneficial effects on the gastrointestinal tract are not fully understood, possible mode of actions have been proposed: 1) maintaining a healthy balance of bacteria in the gut by competitive exclusion (the process by which beneficial bacteria exclude potential pathogenic bacteria through competition for attachment sites in the intestine and nutrients) and antagonism (inhibit the growth of pathogenic bacteria); 2) promoting gut maturation and integrity; 3) modulating the immune system and preventing inflammation;

Table 2. Evaluation of body weight, body weight gain, peptide YY, and FITC-d in neonatal female turkey poult fed with a rye-soybean-based diet with or without dietary inclusion of a *Bacillus* direct-fed microbial (DFM) in trials 1 and 2 at day 10 of age.

Treatment	Body weight day 1 (g)	Body weight day 10 (g)	Body weight gain (g)	Peptide YY (ng/mL)	FITC-d (ng/mL)
Trial 1					
Control	61.33 \pm 0.41 ^a	160.52 \pm 3.01 ^b	99.19 \pm 2.96 ^b	2.55 \pm 0.69 ^a	602.71 \pm 18.06 ^a
DFM	60.12 \pm 0.37 ^a	170.04 \pm 3.55 ^a	109.92 \pm 2.85 ^a	0.4 \pm 0.16 ^b	126.85 \pm 7.37 ^b
Trial 2					
Control	61.33 \pm 0.54 ^a	150.52 \pm 5.21 ^b	89.19 \pm 2.96 ^b	3.15 \pm 0.45 ^a	802.41 \pm 28.16 ^a
DFM	59.12 \pm 0.48 ^a	165.04 \pm 6.35 ^a	105.92 \pm 3.55 ^a	0.64 \pm 0.26 ^b	216.15 \pm 17.07 ^b

^{a,b}Superscripts within columns indicate significant difference at $P < 0.05$.

Abbreviation: FITC-d, fluorescein isothiocyanate-dextran.

Data are expressed as mean \pm SE. Body weight, $n = 25$ individual turkeys; Peptide YY and FITC-d, $n = 20$ serum samples.

Table 3. Evaluation of bone strength and bone composition in neonatal female turkey poults fed with a rye–soybean–based diet with or without dietary inclusion of a *Bacillus* direct-fed microbial (DFM) at day 10 of age (Trial 2).

Treatment	Tibia strength load at yield (kg/mm ²)	Tibia diameter (mm)	Total ash from tibia (%)	Calcium (% of ash)	Phosphorus (% of ash)
Control	0.36 ± 0.12 ^b	3.45 ± 0.32 ^b	37.61 ± 0.81 ^b	22.35 ± 0.07 ^b	15.35 ± 0.42 ^b
DFM	0.54 ± 0.23 ^a	5.02 ± 0.48 ^a	52.17 ± 0.75 ^a	44.31 ± 0.46 ^a	24.77 ± 0.39 ^a

^{a,b}Superscripts within columns indicate significant difference at $P < 0.05$. Data are expressed as mean ± SE, $n = 12$ tibias.

4) improving the metabolism by increasing digestive enzyme activity, decreasing bacterial enzyme activity, and ammonia production; 5) improving feed intake and digestion (as a result from the improved microbial balance in the gut); and 6) neutralizing enterotoxins and stimulating the immune system (Yurong et al., 2005; Howarth and Wang, 2013). While the benefits of probiotics have been known for some time, the concept of prebiotics is relatively new. It developed in response to the notion that nondigestible food ingredients (e.g., nondigestible oligosaccharides) are selectively fermented by bacteria known to have positive effects on gut physiology (Schrezenmeir and de Vrese, 2001). They serve as a preferential food substrate for these bacteria and give them a proliferative advantage over other bacteria. Some prebiotics have even been shown to selectively stimulate the growth of endogenous lactic acid bacteria in the gut to improve the health of the host (Pourabedin and Zhao, 2015). Some of the essential metabolites from microbial fermentation are SCFA, such as acetate, propionate, and butyrate, which play a vital role in the gastrointestinal tract homeostasis (Flint, 2004; Li et al., 2014). Together, probiotics and prebiotics create a symbiotic relationship that has a positive effect on a host. Several studies have shown that probiotics (Xu et al., 1899; Benítez-Páez et al., 2019) and prebiotics (Bednarczyk et al., 2011) increase the production of SCFA, by stimulating the expression of G protein-coupled receptors and leads to the release of hormones like glucagon-like peptide-1, glucagon-like peptide-2, and PPY (De Vadder et al., 2014; Akiba et al., 2015; Psichas et al., 2015; Brooks et al., 2017).

In the present study, turkeys that received the control rye-based diet without the inclusion of the DFM exhibited a significant increase in PPY (Table 2). Perhaps, rye worked as a prebiotic, increasing the production of SCFA by endogenous bacteria. Unfortunately, we did not evaluate the microbiota nor did we measure the ceca concentration of SCFA. However, PPY has been used as an indirect metabolomic biomarker in mammals and is an indirect way to evaluate intestinal SCFA (Xu et al., 1899; Goodacre, 2007;

Musso et al., 2010; Psichas et al., 2015). Our results suggest that the microbiota can metabolize and ferment rye-NSP reaching the large intestine of turkeys. This finding suggests that rye may have a prebiotic effect. Further studies are needed to confirm this possible effect. Interestingly, the turkeys that received the rye-based diet supplemented with the *Bacillus*-DFM had a significant reduction in the serum concentration of PPY when compared with control turkeys (Table 2). Further studies to evaluate the microbiota, as well as the concentration of ceca SCFA, are needed to confirm the reliability of PPY as a potential metabolomic biomarker in poultry.

Similar to NSP and cellulose, exogenous proteins from the diet or endogenous proteins produced by the host (mucin, enzymes, antibodies, or dead enterocytes) become valuable nutrients for the microbiota once they reach the large intestine (Meehan and Beiko, 2014). An increased level of undigested protein and amino acids in the large intestine has been associated with high levels of putrefactive bacteria, branched-chain fatty acids, and other protein fermentation products such as 3-methylindole, p-cresol, phenol, and ammonia created by the microbiota (Zhu et al., 2015).

Poultry manure contains high levels of protein and nitrogen (Kelleher et al., 2002). A significant issue with poultry manure is the loss of nitrogen, in the form of ammonia, during microbial fermentation of uric acid (Nahm, 2003). In poultry houses, ammonia is one of the most toxic and stressful gases, having severe effects on the health and performance of the birds (Moore et al., 2011). Interestingly, one of the initial criteria for selection of the DFM candidates, before they were selected for exogenous enzyme production, was their ability to utilize ammonia as a source of energy (Latorre et al., 2015). In the present study, we observed a significant reduction in the concentration of ammonia in the manure of turkeys that received the DFM as compared with control nontreated turkeys. The present study is the first report showing that another benefit of the *Bacillus*-DFM is the reduction of this toxic gas in the turkey manure.

Table 4. Physiochemical analysis of the poultry manure in neonatal female turkey poults fed with a rye–soybean–based diet with or without dietary inclusion of a *Bacillus* direct-fed microbial (DFM) at day 10 of age (Trial 2).

Treatment	Water content (%)	pH	Ammonia-N (mg/L)	Ammonia-N wet (mg/kg)	Ammonia-N dry (mg/kg)
Control	76.19 ± 0.85 ^a	6.76 ± 0.03 ^a	87.39 ± 2.37 ^a	572.65 ± 23.56 ^a	2,414.35 ± 66.47 ^a
DFM	74.34 ± 0.99 ^a	6.24 ± 0.02 ^b	50.10 ± 2.10 ^b	499.65 ± 20.92 ^b	1,955.35 ± 57.88 ^b

^{a,b}Values within columns with different lowercase superscripts differ significantly ($P < 0.05$). Data are expressed as mean ± SE, $n = 8$ samples.

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