Evaluation of functional feed additive administration in broiler chickens to 21 days

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1 **FUNCTIONAL FEED ADDITIVE ADMINISTRATION**

2 **Evaluation of functional feed additive administration in broiler chickens to 21 days**

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15 preparations, functional feed additives

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17 Primary Audience: Nutritionists, Researchers, Veterinarians

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SUMMARY

Administration of functional feed additives, including Direct-Fed Microorganisms (**DFM**), dietary prebiotics, and phytogenic preparations, has been demonstrated to improve growth performance, animal health, and microbial food safety in poultry and is thought to be a potentially important component of antibiotic-free poultry production. In this study, we investigated the administration of *Bacillus licheniformis* (**BL**) as a DFM, co-administration of BL with an additive blend of a dietary prebiotic and a phytogenic preparation (**BL+A**), and co-administration of BL with a dietary prebiotic as a synbiotic (**SYN**) in broiler chickens in comparison to an antibiotic over a 21 d growth period. Administration of BL improved FCR as compared to untreated broilers through 14 d post-hatch. Administration of BL +A and SYN increased total Lactic Acid Bacteria and decreased *Clostridium perfringens* compared to the antibiotic-treated and untreated broilers, respectively. Administration of BL was also observed to increase the villus height to crypt depth ratio as compared to the untreated control. Overall, our results suggest co-administration of *B. licheniformis* as a DFM with other functional feed additives is able to improve feed efficiency, promote positive shifts in populations of gastrointestinal microbiota, and improve measures of gastrointestinal function.

DESCRIPTION OF PROBLEM

The administration of sub-therapeutic antibiotics has been used widely to increase weight gain (Engberg et al., 2000), improve feed efficiency (Miles et al., 1984; Harms et al., 1986), and reduce poultry and human foodborne pathogens (Williams, 1985; Sims et al., 2004) in poultry production. Although they have been applied in poultry for over 50 years, the use of antibiotic growth promoters (**AGP**) has declined (Sneeringer et al., 2015) due to consumer preferences (Brewer and Rojas, 2007) and regulations (Castanon, 2007) resulting from concerns over the development of antibiotic resistance in bacteria (McEwen and Fedorka-Cray, 2002; Forgetta et al., 2012). As the demand for antibiotic-free (**ABF**) production of poultry and other livestock continues to grow, the continued development of alternatives to antibiotics will become increasingly important. Because the beneficial effects of antibiotics are attributed to their activities on the microbial community in the gastrointestinal (**GI**) tract (Visek, 1978; Gaskins et al., 2002), the GI microbiota is an important target for the development of alternatives to AGP (Askelson and Duong, 2015).

The United States Food and Drug Administration has defined direct-fed microbial products as those that "are purported to contain live microorganisms (FDA, 1995)", and the International Scientific Association for Probiotics and Prebiotics has defined a prebiotic as "a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017)". Phytogenic preparations consist of plant derived products used in animal diets to improve productivity or feed quality (Windisch et al., 2008). The use of Direct-Fed Microorganisms (**DFM**), prebiotics, and phytogenic preparations as functional additives, ingredients that may provide a benefit beyond satisfying traditional nutrient requirements (Marriott, 2000), is seen as a potentially important alternative to the use of AGP in poultry

production. When administered to poultry individually or in combination, DFM, prebiotics, and phytogenic preparations have been demonstrated to promote growth and performance (Flores et al., 2019a; Flores et al., 2019b), reduce GI colonization by human foodborne and poultry pathogens (Askelson et al., 2018; Froebel et al., 2019), and improve measures of intestinal function (Xu et al., 2003).

Because of their benefits, interest in the administration of functional additives as alternatives to the use of AGP has increased. Although the benefits of the administration of DFM, prebiotics, and phytogenic preparations have been widely reported, their application in the poultry industry is inconsistent, their overall effectiveness is mixed, and the functionalities of specific additives are not well understood. In this study, we investigated the co-administration of *Bacillus licheniformis* as a DFM with functional additive blend including dietary prebiotics and phytogenic preparations on the growth performance and GI microbiota of broiler chickens.

MATERIALS AND METHODS

Experimental Animals and Husbandry

Male broilers chicks (Cobb 500) were obtained from a commercial hatchery on day of hatch and vaccinated for *Eimeria* (Advent, Huvepharma Inc, Peachtree City, GA), weighed, wing banded, and assigned randomly to treatment pens with statistically similar starting weights at an initial stocking density of 0.096 m^2 per bird. Experimental animals were raised in floor pens on built-up litter under conditions simulating commercial poultry production and provided access to water and experimental rations *ad libitum* for the 21 d duration of the study. An industry lighting program was used in accordance with standard operating procedures of the Texas A&M University Poultry Research Center (Flores et al., 2019b) with temperature guidelines following the breeder's recommendations (Cobb-Vantress, 2013). All experimental procedures were performed as approved by the Texas A&M University Institutional Animal Care and Use Committee.

Experimental Design and Diets

The effect of functional feed additive administration on growth performance, GI microbiota, ileal histomorphometry, and serum antioxidant capacity was evaluated in comparison to an AGP. Broiler chicks (n=1960) were allocated to 5 experimental treatment groups with total of 49 pens of 40 birds arranged, due to housing constraints, as a randomized incomplete block design and fed experimental rations supplemented with functional additives using the manufacturers' recommended incorporation rates. The 5 experimental treatment groups were as follows: bacitracin methylene disalicylate (**BMD**) treated (50 g ton^{-1}) feed (10 pens); untreated (**UNT**) feed (9 pens); administered *Bacillus licheniformis* DSM 28710 (**BL**) in-feed at 1.6 x10^9 cfu kg^{-1} feed (Huvepharma, Inc., Peachtree City, GA) as a DFM (10 pens); co-administered BL

with a functional feed additive blend (**BL+A**) consisting of a multi-strain DFM culture of *Lactobacillus acidophilus* and *Enterococcus faecium* in-feed at 4.4 x10^7 cfu kg^{-1} feed, yeast cell wall extract at 113.40 g ton^{-1} feed (Phileo, Marcquen-Baroel, FR), and a phytogenic preparation of capsicum, cinnamaldehyde, and carvacrol at 45.36 g ton^{-1} (Allied Nutrition, Doringkloof, ZA) (10 pens); or administered a synbiotic (**SYN**) combination of BL and a yeast cell wall extract (Altech, Lexington, KY) at 226.79 g ton^{-1} (10 pens).

Experimental rations (**Table 1**) were fed for the duration of the study in two phases: starter (0 to 14 d post-hatch, crumble) and grower (14 to 21 d post-hatch, pellet). Diets for each phase were mixed using a 2-ton horizontal double-ribbon Scott mixer, pelleted using a 1 ton/hr California Pellet Mill at 175°F equipped with a 4.4 mm diameter die and conditioner, and crumbled using a roller mill when appropriate. Feed was manufactured as a single commercial-type corn/soybean meal basal diet with 5 % distiller's dried grains with solubles and added phytase and xylanase and divided for inclusion of dietary treatments as appropriate. Full matrix values for enzyme contribution of aP, Ca, Na, digestible AA, and ME as recommended by the manufacturer were used.

Growth Performance Measurements

Experimental animals and residual feed were weighed by pens at 0, 14, and 21 d post-hatch for determination of BW and feed consumption. Mortalities and post-mortem weight were recorded for calculation of percent mortality, ADG, ADFI, and mortality corrected FCR.

Tissue Sample Collection

At 21 d post-hatch, one representative (median weight ± 5%) experimental animal was selected from each pen, killed humanely, and dissected aseptically for the collection of tissues. Ileal sections of approximately 6 cm taken at the midpoint between the ileocecal junction and

Meckel's diverticulum were collected from each bird and divided in half with the proximal and distal segments being used for enumeration of ileal microbiota and histomorphometry, respectively. Additionally, the ceca and whole blood were collected from each bird for enumeration of cecal microbiota and determination of serum antioxidant capacity, respectively.

Bacterial Enumeration

Ileal specimens were homogenized and diluted serially using Fluid Thioglycolate Medium (**FTM**; BD, Franklin Lakes, NJ). One cecal specimen from each broiler was homogenized and diluted serially using sterile phosphate buffered saline (**PBS**, ThermoFisher Scientific, Waltham, MA), whereas the other was placed in 10 mL Bolton's Enrichment Broth (**BEB**; Hardy Diagnostics, Santa Maria, CA).

Clostridium perfringens was enumerated from the ileal specimens using Tryptose Sulfite Cycloserine-Egg Yolk agar (BD) incubated at 37 ^{o}C anaerobically (Coy Laboratory Products, Inc., Grass Lake, MI) for 48 h; *Campylobacter* spp. were enumerated using Campy Cefex agar (Hardy) incubated at 42 ^{o}C in 10% CO_2 for 48 h; and *Lactobacillus* spp. were enumerated from the ileum and cecal specimens using Rogosa Selective Lactobacilli agar (BD) supplemented with 100 μg mL^{-1} cylcoheximide (Amresco, Solon, OH). *C. perfringens* were selectively enriched from the ileum using FTM and Iron Milk Media (HiMedia; Mumbai, India), whereas *Campylobacter* spp. were selectively from the cecum using BEB and Campy Cefex Agar. Specimens for which no colonies appeared on enumeration plates but were positive by selective enrichment were assigned the limit of detection for enumeration (100 cfu g^{-1}).

Histomorphometry

Ileal specimens were flushed and fixed using sterile PBS and 10% neutral buffered formalin (ThermoFisher), respectively. Fixed ileal specimens were trimmed, embedded in

paraffin, sectioned, and prepared on slides for analysis using Alcian Blue and Periodic Acid Schiff staining. Measurements of five intact villi and crypts were recorded over three cross-sections for each broiler at 100× magnification. Villus heights and crypt depths were used to calculate the villus height to crypt depth ratio (**VH:CD**).

Serum Antioxidant Capacity

Whole blood was collected post-mortem using blood collection tubes (SST Plus, BD), incubated (room temperature, 30 min) and centrifuged (1000× g, 10 min, 4°C), and serum was collected as the resultant supernatant. Antioxidant capacity was determined as the Trolox equivalent inhibition of metmyoglobin-induced oxidation of 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) (**ABTS**) according to the manufacturer's instructions (Cayman Chemical Co, Ann Arbor, MI). Oxidation of ABTS was monitored colorimetrically using absorbance at 405 nm (Tecan Systems Inc., San Jose, CA). Serum antioxidant capacity was reported as mM Trolox equivalents.

Statistical Analysis

Univariate tests were used to verify normality and homoscedasticity of data so that all assumptions of ANOVA were fulfilled. Percent mortality was arcsine square root transformed for analysis (Gotelli and Ellison, 2004), whereas bacterial counts were $\log_{10}$ transformed for analysis. The General Linear Model was used to determine significant treatment effects, and significantly different means were separated *post-hoc* using Duncan's Multiple Range Test ($P \leq 0.05$).

RESULTS AND DISUCSSION

Although the administration of sub-therapeutic doses of antibiotics has been used to great benefit in the production of poultry and other livestock, growing consumer (Brewer and Rojas, 2007) and regulatory pressures (Brewer and Rojas, 2007) have increased the need for the development of alternatives to the use of AGP. Antibiotics have been suggested to improve growth and performance of livestock through competition for nutrients between GI microbiota and the host animal, decreased production of toxins and other growth depressing metabolites by the microbiota, and inhibition of subclinical infections (Corpet, 2000; Butaye et al., 2003). Because the growth promoting effects of antibiotics come as a result from their activities on the microbial community in the GI tract (Gaskins et al., 2002), the GI microbiota is an important target for the development of alternatives to AGP (Askelson and Duong, 2015). Because their beneficial effects on growth promotion and animal health are mediated through their activities on the GI microbiota, the administration of DFM prebiotics, and phytogenic preparations as functional additives, ingredients that may provide a benefit beyond satisfying traditional nutrient requirements (Marriott, 2000), is seen as a potentially important alternative to the use of AGP in poultry production. Although the administration of DFM is used widely in poultry production, their effects when co-administered with other functional additives is not well understood. In this study, we investigated the co-administration of DFM with other functional additives, including prebiotics and phytogenic preparations, as potential alternatives to AGP in poultry production.

Growth Performance

The effects of functional additive administration on the growth performance of broiler chickens was evaluated in comparison to antibiotic-treated and untreated control groups (**Table 2**). No significant treatment effects were observed on BW, ADG, ADFI, or mortality over the 21-

d course of the study. However, a significant treatment effect on FCR was observed over 0 to 14 d post-hatch (P = 0.049). Feed conversion ratio was highest when broilers were fed the untreated diet. Administration of BMD and direct-fed *B. licheniformis* (**BL**) decreased FCR when compared to the untreated broilers. Although FCR over 0 to 14 d was not significantly reduced when compared to the untreated broilers, co-administration of direct-fed *B. licheniformis* and the feed additive blend (**BL+A**) or the synbiotic (**SYN**) did reduce FCR to a level similar to that of BMD-treated broilers. No significant treatment effect was observed on FCR over 14 to 21 or 0 to 21 d post-hatch.

Improvements in BW, body weight gain, or feed consumption are often not observed in the absence of a disease or stress challenge (Midilli et al., 2008), whereas significant improvements to growth have been reported previously when broilers are raised under conditions of experimentally applied stress (Knap et al., 2010; Song et al., 2014; Johnson et al., 2020). Poor *Eimeria* vaccine cycling or absence of a direct microbial challenge due to low litter moisture (Edens et al., 1998) may have contributed the lack of an observable growth response in our study. Although litter moisture was not measured, low relative humidity in the outside environment lead to observably dry and dust conditions within the research barn. Administration of *B. licheniformis* has been reported previously to improve FCR during the starter phase (Midilli et al., 2008; Gong et al., 2018). *B. licheniformis* and other *Bacillus* spp. are valued as industrial microorganisms because of their production of important digestive enzymes including amylases, phytases, and proteases (Rozs et al., 2001; Tye et al., 2002; Hmidet et al., 2010; Gong et al., 2018). Increased digestive enzyme activity has been observed when administered in poultry (Gong et al., 2018) suggesting enzyme production in situ by *Bacillus* spp. may directly improve digestibility of feed and increase feed efficiency. Indeed, administration of heterologous phytase

producing recombinant *Lactobacillus* spp. has been demonstrated to improve growth of broilers a fed phosphorous-deficient diet (Askelson et al., 2014), underscoring the significance of microbial enzyme production in animal production.

Gastrointestinal Microbiota

A significant treatment effect was observed on *Clostridium perfringens* counts (P = 0.027) in the ileum (**Figure 1A**). *C. perfringens* counts were highest when broilers were fed the untreated diet and lowest when broilers were fed diets treated with BMD or SYN. Although they were not significantly different from the untreated control, administration of BL and BL+A reduced colonization of *C. perfringens* to levels similar to BMD. Bacitracin, a non-ribosomal peptide (**NRP**) antibiotic produced commercially using strains of *B. licheniformis* (Rey et al., 2004), and its derivatives, BMD and zinc-bacitracin, have been used widely as AGP and for mitigation of necrotic enteritis in poultry because of their antibacterial activity on *C. perfringens* (Sims et al., 2004; Fasina et al., 2015). Homologs of bacitracin synthetase (*bac*) genes are distributed widely among *B. licheniformis* strains, with over half of the strains screened being reported to harbor homologs of the *bac* gene cluster (Ishihara et al., 2002), suggesting that production of an antimicrobial NRP in situ in the GI tract may be important to the functionality of direct-fed *B. licheniformis* strains as potential alternatives to AGP. Production of bacitracin by *B. licheniformis* in situ the GI tract of gnotobiotic mice has been demonstrated previously to inhibit experimental *C. perfringens* infection (Ducluzeau et al., 1976). The reduction of inflammation induced by *C. perfringens* during subclinical infection has been reported to promote growth by sparing energy otherwise lost to the immune system (Stutz and Lawton, 1984; Hofshagen and Kaldhusdal, 1992) and likely contributed to the observed improvements to the FCR of *B. licheniformis*-treated broilers in our study. Indeed, administration of DFM to

broiler chickens has been reported previously to repartition energy away from a proinflammatory response in the GI tract to other host tissues (Qiu et al., 2012). Furthermore, rapid selection of bacitracin-resistant *C. perfringens* was observed when the antibiotic was administered in-feed to experimentally infected mice (Ducluzeau et al., 1976). However, the same study reported that bacitracin-resistant bacteria were not observed when mice were administered *B. licheniformis*, suggesting a potentially important advantage to the production of antibiotics in situ by DFM in the GI tract over the use of AGP. Further characterization will be required in order to determine whether *B. licheniformis* DSM 28710 produces an antimicrobial NRP and whether it is capable of doing so in situ in the GI tract.

Poultry are a commensal host for *Campylobacter* spp. (Duong and Konkel, 2009) and serve as a primary reservoir for foodborne *Campylobacter* infection in humans (Olson et al., 2008). The treatments were not observed to have a significant effect (P=0.095) on *Campylobacter* counts in the cecum of broiler chickens (**Figure 1B**). However, fewer *Campylobacter* tended to be recovered from SYN-treated broilers as compared with those administered BL+A. Although the 0.6 $\log_{10}$ cfu reduction was not observed to be significant, a quantitative risk assessment model suggested that *Campylobacter* reductions of a similar degree should result in a 30-50 % reductions in the burden of *Campylobacter*-associated foodborne illness from poultry (Rosenquist et al., 2003).

Administration of hydrolyzed yeast-cell wall extracts, composed largely of mannanoligosaccharides (**MOS**), β-glucans, and other prebiotics, has been demonstrated previously to reduce cecal *Campylobacter* counts (Baurhoo et al., 2009; Froebel et al., 2019). However, the effectiveness of various yeast-derived prebiotics in reducing *Campylobacter* colonization is mixed and their interaction with other functional additives has not been well

characterized (Corrigan et al., 2017). The difference in MOS composition or interactions between MOS and the additional functional additives in the functional additive blend administered to the BL+A-treated broilers may have contributed to this difference. The yeast-derived prebiotic administered to the SYN-treated was a more purified MOS fraction whereas the yeast-derived prebiotic administered to the BL+A broilers contained a yeast fraction rich in both MOS and β-glucans. Previous research has shown statistically insignificant 0.6 log difference in *Campylobacter* counts between different mannan-rich fractions used at manufacturer recommended inclusion levels (Corrigan et al., 2017). Although MOS has not yet been demonstrated to agglutinate *Campylobacter* (Spring et al., 2000), it has been demonstrated to inhibit *Campylobacter* adhesion to poultry epithelial cells in vitro (Froebel et al., 2020) suggesting inhibition of adhesion in the GI tract may be important to the functionality of yeast cell-wall derived prebiotics in reducing pathogen colonization.

Lactobacillus spp. and other Lactic Acid Bacteria (LAB) are recognized widely as beneficial organisms because of their beneficial effects on GI health and host immunity (Broderick and Duong, 2016; Vieco-Saiz et al., 2019). The treatments were not observed to have a significant effect ($P = 0.090$) on counts of *Lactobacillus* in the ileum (**Figure 2C**) but were observed to have a significant effect ($P = 0.036$) in the cecum (**Figure 1D**). Although the effect in the ileum was not significant, more lactobacilli tended to be recovered from broilers fed the BL+A treated diet as compared to those fed the SYN-treated diet. However, in the cecum, fewer *Lactobacillus* were recovered when broilers were fed the BMD-treated diet as compared to the remaining treatments. BMD administration has been demonstrated previously to reduce *Lactobacillus* and other LAB in the GI tract of poultry (England et al., 1996; Lu et al., 2008) due to their sensitivity to the activity of BMD against Gram-positive bacteria (Morris, 1956; Elkins

and Mullis, 2004). The *Lactobacillus acidophilus* included in the feed additive blend administered to the BL+A treatment is likely contributed to increased recovery of *Lactobacillus* as compared to other treatments in the ileum and as compared to BMD-treated broilers in the cecum (Lan et al., 2004). Administration of *B. licheniformis* DSM 28710 has been reported previously to reduce GI pH (Trela et al., 2020), which may promote energy sparing and nutrient availability through the reduction of harmful bacteria (Thanh et al., 2009). *Lactobacillus* spp. possess an array of acid tolerance factors that increase their survivability in low pH environments and could allow for their persistence in the GI tract of broilers fed *B. licheniformis* (Broderick and Duong, 2016).

Histomorphometery

The effects of the administration of functional feed additives on intestinal morphology were evaluated to serve as an indicator of GI function (**Figure 2**). A significant treatment effect was not observed on villus height ($P = 0.116$) but was observed on crypt depth ($P = 0.009$) and villus height:crypt depth (**VH:CD**) ratio ($P < 0.001$). Crypts of broilers administered BL, BL+A, or SYN were shallower as compared to BMD treated broilers. Crypts of broilers administered DFM alone or in combination with additive blend B were also shallower when compared with the untreated broilers. Greater crypt depth is indicative of increased cell turnover and is associated with higher energy expenditure due to the increased nutrient requirement for maintenance (Yason et al., 1987). Additionally, VH:CD was also greater when broilers were administered BL or SYN as compared with the untreated control, with VH:CD of SYN-treated broilers also being greater than that of BMD-broilers. Increased VH:CD (Lei et al., 2015) and decreased crypt depth (Latorre et al., 2017) have been reported previously when broilers were administered *Bacillus* spp. as DFM. Additionally, α-amylase produced in situ by *B. licheniformis*

(Divakaran et al., 2011) may contribute to improved morphology as increased energy available to the host through increased carbohydrate degradation and absorption allows for positive structural development of the small intestine (Ritz et al., 1995). Improved VH:CD through inclusion of *B. licheniformis* and functional feed additives was driven by shallower crypts. Deeper crypts drive cell turnover in the small intestine which increases maintenance requirements and decreases efficiency(Pluske et al., 1996). Although a small fraction of body weight, the gastrointestinal tract accounts for upwards of 20% of energy expenditures (Spratt et al., 1990; Cant et al., 1996). The reduction in FCR by *B. licheniformis* and functional feed additives to levels similar to BMD may be facilitated by reduced energy expenditure due to shallower crypts.

Serum Antioxidant Capacity

Oxygen and nitrogen free radicals, products of normal metabolic activity and immune function, can damage host DNA, proteins, and lipids (Surai, 2007). Antioxidant capacity measures the ability of antioxidants in the serum to quench free radicals compared against a trolox standard (Apak et al., 2013). A significant treatment effect was not detected ($P = 0.055$) for serum antioxidant capacity (**Figure 3**). However, antioxidant capacity of broilers administered BL+A or SYN tended to be greater when compared to those administered DFM alone. Neither the positive or negative control was distinguishable from the treated groups. Functional feed additives are capable of increasing total antioxidant capacity in broilers (Paraskeuas et al., 2017). Administration of phytogenic preparations and synthetic antioxidants as functional feed additives has been demonstrated previously to increase serum antioxidant capacity in addition to reducing lipid oxidation in broiler meat (Wang et al., 1997; Jang et al., 2008; Cherian et al., 2013). *Saccharomyces*-derived MOS have been reported scavenge reactive

oxidative radicals and exhibit anti-mutagenic activity *in vitro* (Križková et al., 2001). Thus, the yeast-derived MOS administered in BL+A and SYN treatments likely contributed to the increased antioxidant capacity compared to BL alone. When administered to poultry, yeast or MOS has been reported to increase the activity of antioxidative enzymes including catalase and glutathione peroxidase and other antioxidants in blood (Ognik and Krauze, 2012; Aluwong et al., 2013). An increase in antioxidant capacity may be beneficial to the immune response by mitigating inflammation in the gastrointestinal tract. Reactive oxygen species in the mucosa cause inflammation which hinders digestion and absorption of nutrients (Kruidenier et al., 2003). Additionally, oxidative stress increases lipid peroxidation which can induce metabolic disturbances (Vila et al., 2002). By increasing the capacity to mitigate influxes in reactive species, broilers are better able to tolerate disease and environmental stressors that would otherwise cause oxidative damage to lipids, proteins, or tissues (Tawfeek et al., 2014).

In this study, we investigated the effects *Bacillus licheniformis* and its co-administration with functional additive blends consisting of dietary prebiotics and phytogenic preparations in broiler chickens. Administration of direct-fed *B. licheniformis* alone and in conjunction with functional additives improves performance parameters, gastrointestinal health, and intestinal morphology. Feed conversion ratio was lower for broilers administered direct-fed *B. licheniformis* as compared to untreated broilers through 14 d post-hatch. Co-administration of DFM with functional additives decreased counts of *Clostridium perfringens* compared to the untreated control and increased counts of *Lactobacillus* spp. compared to antibiotic treated broilers. Administration of Direct-Fed *B. licheniformis* was observed to increase the villus height crypt to depth ratio (VH:CD) compared to the untreated control, whereas co-administration of DFM with functional additives increased VH:CD ratio compared to both the untreated and

antibiotic treated control. Direct-Fed *B. licheniformis* and functional feed additives are able to improve feed efficiency, promote positive shifts in populations of gastrointestinal microbiota, and improve measures of gastrointestinal function. Although their independent contributions improve performance and health metrics, there is not sufficient data to indicate a synergistic relationship between *Bacillus licheniformis* and functional additives in broiler production.

CONCLUSIONS AND APPLICATION

1. Administration of *Bacillus licheniformis* decreased FCR to levels statistically similar to an antibiotic control compared to untreated feed.
2. Functional feed additives promote a healthier GI microbiota by decreasing *Clostridium perfringens* and increasing total Lactic Acid Bacteria.
3. *Bacillus licheniformis* and functional additives improve measures of gut function (VH:CD)

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362 **FIGURE LEGENDS**

363 **Figure 1. Enumeration of gastrointestinal bacteria from broiler chickens.** (**A**) *Clostridium*
364 *perfringens* and (**B**) *Lactobacillus* spp. were enumerated from the ileum, and (**C**) *Campylobacter*
365 spp. and (**D**) *Lactobacillus* spp. were enumerated from the cecum of broiler chickens at 21 d
366 post-hatch. BMD, bacitracin methylene disalicylate; UNT, untreated; BL, direct-fed *B.*
367 *licheniformis*; BL+A, BL with additive blend; SYN, synbiotic. Counts are reported as mean ±
368 SEM $\log_{10}$ cfu g^{-1} digestive contents from 9 UNT broilers or 10 broilers for all other treatments.
369 Means not sharing common letters differ significantly ($P \leq 0.05$).
370
371 **Figure 2. Ileal histomorphometry of broiler chickens.** Ileal sections were sampled at 21d post-
372 hatch for determination of (**A**) villus height and (**B**) crypt depth and calculations of (**C**) VH:CD.
373 BMD, bacitracin methylene disalicylate; UNT, untreated; BL, direct-fed *B. licheniformis*; BL+A,
374 BL with additive blend; SYN, synbiotic. Villus height and crypt depth are reported as mean ±
375 SEM µm of 3 ileal sections from 9 UNT broilers or 10 broilers for all other treatments. Means
376 not sharing common letters differ significantly ($P \leq 0.05$).
377
378 **Figure 3. Serum antioxidant capacity of broiler chickens.** Serum was separated from whole
379 blood and collected at 21 d post-hatch. Antioxidant capacity is reported as the treatment mean ±
380 SEM mM trolox equivalents from 9 UNT broilers or 10 broilers for all other treatments. Means
381 not sharing common letters differ significantly ($P \leq 0.05$).

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Table 1. Ingredient composition and nutrient content of the basal diet

Item (%)	0 to 14 d	14 to 21d
Ingredients		
Corn	60.76	65.74
Soybean Meal	26.56	22.25
Meat and Bone Meal	5.00	4.43
Corn DDGS	5.00	5.00
Fat, A/V blend	0.66	0.77
DL-Met	0.26	0.25
Lysine HCL	0.27	0.25
Limestone	0.65	0.55
$CaH_4(PO_4)_2$	0.12	0.00
NaCl	0.37	0.27
$NaHCO_3$	0.04	0.19
Trace Minerals[1]	0.05	0.05
Vitamins[2]	0.25	0.25
Phytase[3]	0.01	0.01
Xylanase[4]	0.01	0.01
Calculated Nutrients		
Available P	0.45	0.40
dig Met	0.57	0.54
dig TSAA	0.84	0.78
dig Lys	1.18	1.04
dig Trp	0.20	0.18
dig Thr	0.69	0.62
Analyzed Nutrients[5]		
Dry Matter	88.86	89.13
Crude Protein	21.60	19.20
ME (kcal kg^1)	3014	3124
Crude Fat	4.17	4.89
Crude Fiber	4.20	3.50
Ash	4.73	4.29
Ca	0.80	0.77
Total P	0.97	0.50
Na	0.22	0.17

[1]Trace mineral premix added at this rate yields 60.0 mg manganese, 60 mg zinc, 60 mg iron, 7 mg copper, 0.4 mg iodine, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium per kg of diet. The carrier is calcium carbonate and the premix contains less than 1% mineral oil.
[2]Vitamin premix added at this rate yields 22,045 IU vitamin A, 7,716 IU vitamin D3, 91 IU vitamin E, 0.04 mg B12, 11.9 mg riboflavin, 91.8 mg niacin, 40.4 mg d-pantothenic acid, 261.1 mg choline, 2.9 mg menadione, 3.50 mg folic acid, 14.3 mg pyroxidine, 5.87 mg thiamine, 1.10 mg biotin per kg diet. The carrier is ground rice hulls.
[3]OptiPhosPF, Huvepharma. Peachtree City, GA. 748 units kg^{-1} feed
[4]Hostazym X, Huvepharma. Peachtree City, GA. 1500 units kg^{-1} feed
[5]Performed by Midwest Laboratories, Inc., Omaha, NE

Table 2. Growth performance of broiler chickens

	Treatments[1]						Pooled
Item	BMD	UNT	BL	BL+A	SYN	*P*-value	SEM
BW (kg)							
0 d	0.044	0.044	0.044	0.044	0.044	0.891	0.000
14 d	0.439	0.436	0.441	0.442	0.440	0.800	0.016
21 d	0.888	0.888	0.903	0.887	0.894	0.555	0.004
ADG (kg bird-day^{-1})							
0 to 14 d	0.031	0.031	0.031	0.032	0.031	0.697	0.000
14 to 21 d	0.071	0.072	0.074	0.071	0.072	0.417	0.001
0 to 21 d	0.042	0.042	0.043	0.043	0.042	0.492	0.000
ADFI (kg bird-day^{-1})							
0 to 14 d	0.035	0.035	0.035	0.035	0.035	0.986	0.000
14 to 21 d	0.096	0.098	0.100	0.098	0.098	0.248	0.001
0 to 21 d	0.055	0.056	0.056	0.056	0.055	0.457	0.000
Mortality corrected FCR (Feed:Gain)							
0 to 14 d	1.239^{b}	1.263^{a}	1.239^{b}	1.243^{ab}	1.248^{ab}	0.049	0.004
14 to 21 d	1.350	1.361	1.357	1.369	1.353	0.717	0.004
0 to 21 d	1.301	1.317	1.305	1.314	1.305	0.272	0.003
Mortality (%)							
0 to 14 d	1.111	0.333	0.667	0.667	1.000	0.794	0.222
14 to 21 d	0.000	0.333	0.333	0.000	0.800	0.581	0.110
0 to 21 d	1.111	0.667	0.911	0.667	1.800	0.386	0.301

[a,b] Superscripts indicate significant differences between treatments ($P \leq 0.05$)

[1]Treatments: BMD, bacitracin methylene disalicylate; UNT, untreated; BL, direct-fed *B. licheniformis*; BL+A, BL with additive blend; SYN, synbiotic

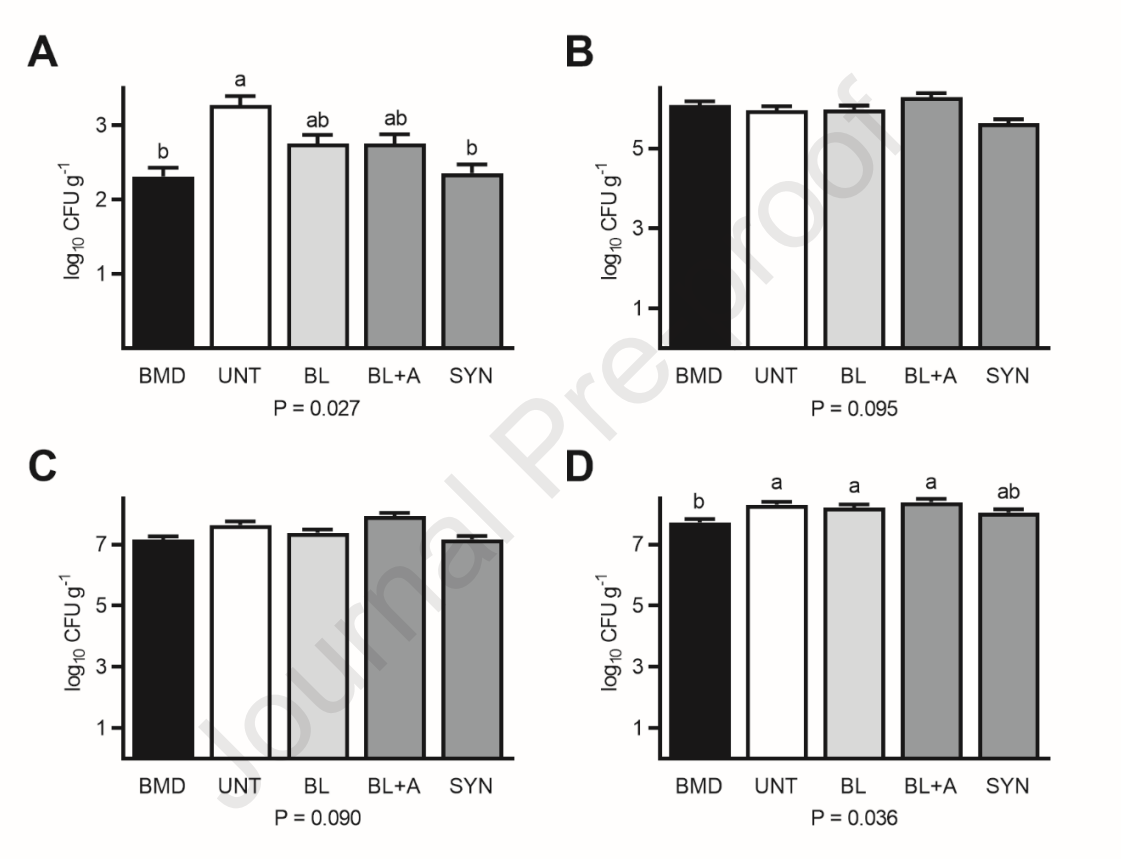


Figure 1. Enumeration of gastrointestinal bacteria from broiler chickens at 21 d post-hatch. (**A**) *Clostridium perfringens* and (**B**) *Campylobacter* spp were enumerated from the ileum and cecum, respectively; *Lactobacillus* spp. were enumerated from the (**C**) ileum and (**D**) cecum. . BMD, bacitracin methylene disalicylate; UNT, untreated; BL, direct-fed *B. licheniformis*; BL+A, BL with additive blend; SYN, synbiotic. Counts are reported as mean ± SEM $\log_{10}$ cfu g^{-1} digestive contents from 9 UNT broilers or 10 broilers for all other treatments. Means not sharing common letters differ significantly ($P \leq 0.05$).

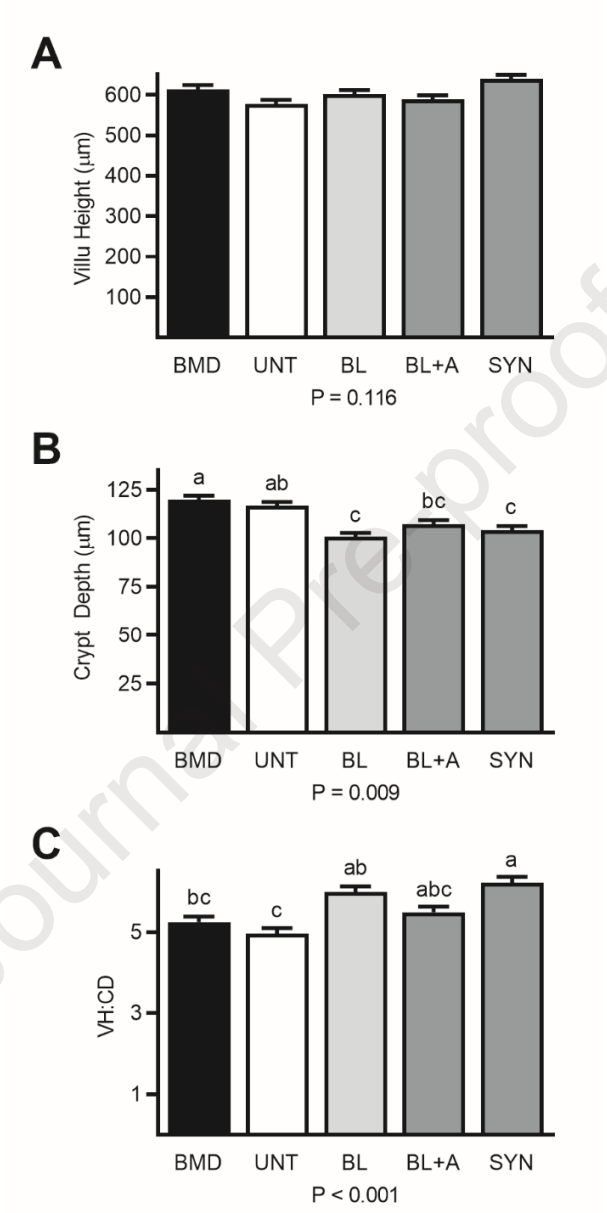


Figure 2. Ileal histomorphometry in broiler chickens. Ileal sections were sampled at 21d post-hatch for determination of (**A**) villus height and (**B**) crypt depth and calculations of (**C**) VH:CD. BMD, bacitracin methylene disalicylate; UNT, untreated; BL, direct-fed *B. licheniformis*; BL+A, BL with additive blend; SYN, synbiotic. Villus height and crypt depth are reported as mean ± SEM µm of 3 ileal sections from 9 UNT broilers or 10 broilers for all other treatments. Means not sharing common letters differ significantly ($P \leq 0.05$).

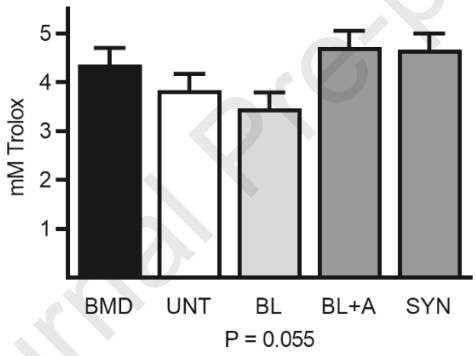
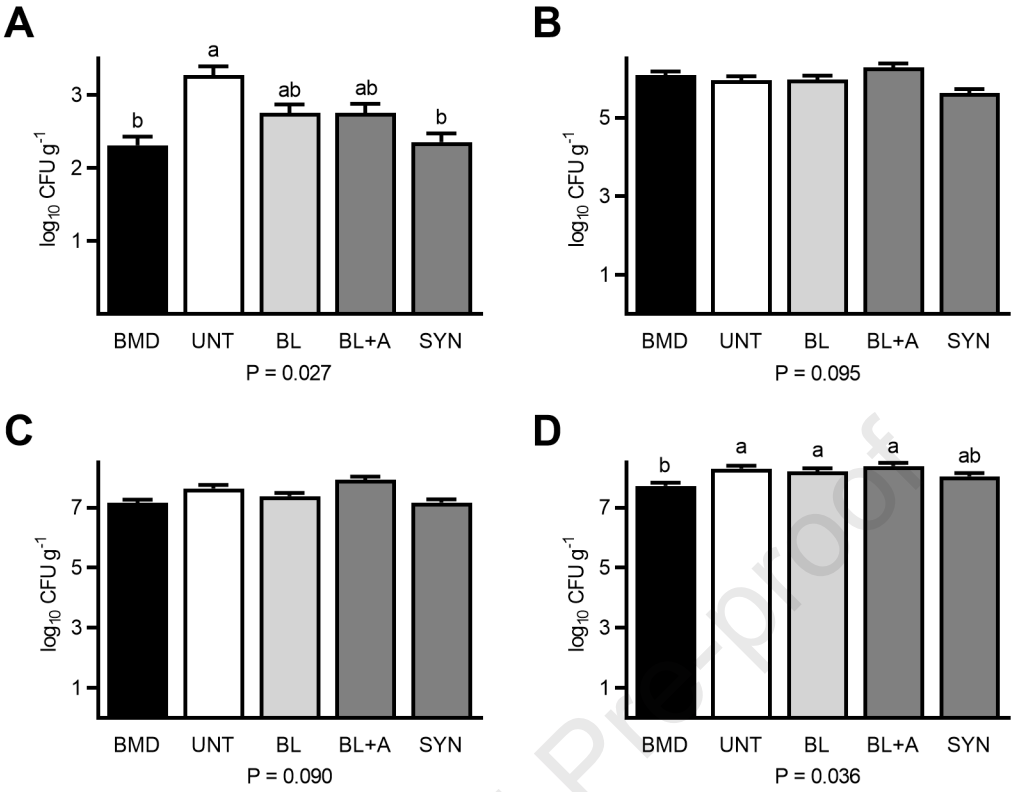
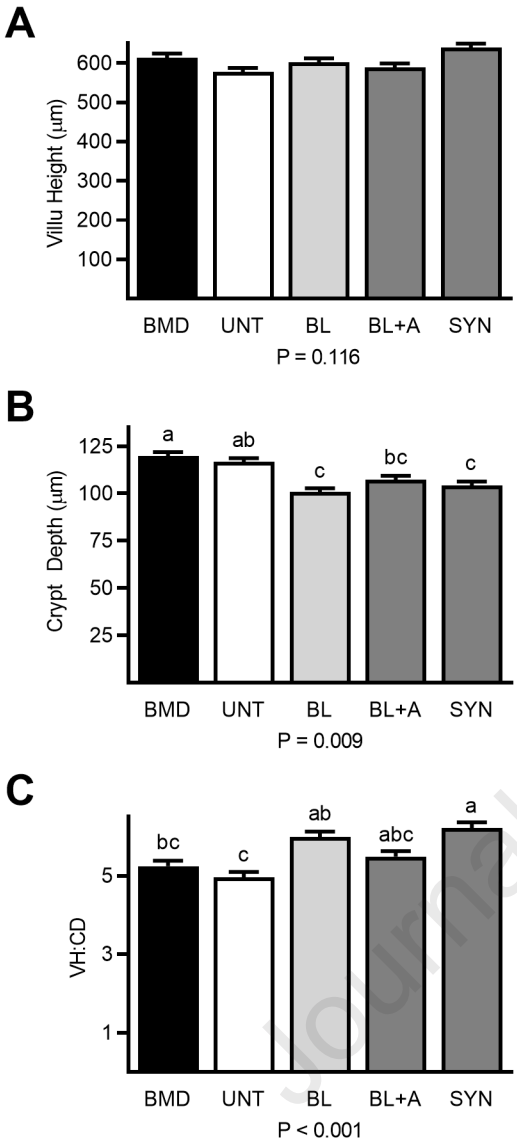
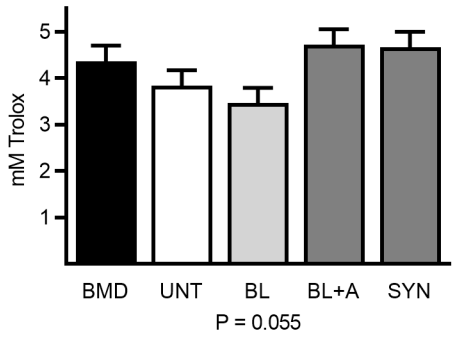


Figure 3. Serum antioxidant capacity of broiler chickens. Serum was separated from whole blood and collected at 21 d post-hatch. Antioxidant capacity is reported as the treatment mean ± SEM mM trolox equivalents from 9 UNT broilers or 10 broilers for all other treatments. Means not sharing common letters differ significantly ($P \leq 0.05$).







The authors declare no conflict of interest