Effects of direct-fed microorganisms and enzyme blend co-administration on intestinal bacteria in broilers fed diets with or without antibiotics

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ABSTRACT Direct-fed microorganisms (**DFM**) and exogenous enzymes have been demonstrated to improve growth performance in poultry and are potentially important alternatives to antibiotic growth promoters (**AGP**). We investigated the administration of a feed additive composed of a DFM product containing spores of 3 *Bacillus amyloliquefaciens* strains and an enzyme blend of endo-xylanase, α-amylase, and serine-protease in diets with or without sub-therapeutic antibiotics in broiler chickens over a 42-d growth period. Evaluation of growth performance determined feed efficiency of broiler chickens which were administered the feed additive was comparable to those fed a diet containing AGPs. Characterization of the gastrointestinal microbiota using culture-dependent methods determined administration of the feed additive increased counts of total Lactic Acid Bacteria (**LAB**) relative to a negative control and reduced *Clostridium perfringens* to levels similar to antibiotic administration. Additionally, greater counts of total LAB were observed to be significantly associated with reduced feed conversion ratio, whereas greater counts of *C. perfringens* were observed to be significantly associated with increased feed conversion ratio. Our results suggest the co-administration of DFMs and exogenous enzymes may be an important component of antibiotic free poultry production programs and LAB and *C. perfringens* may be important targets in the development of alternatives to AGPs in poultry production.

Key words: DFM, enzymes, AGP, microbiota, broiler

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INTRODUCTION

Sub-therapeutic doses of antibiotics have been used to promote the growth of broiler chickens in the United States for more than 50 years (Moore et al., 1946; Stokstad and Jukes, 1950; Bridges et al., 1952). Antibiotic growth promoters (**AGP**) have been demonstrated to increase weight gain (Moore et al., 1946), improve feed efficiency (Emborg et al., 2002; Gaskins et al., 2002), and reduce mortality in livestock animals (Cromwell, 2002; Callesen, 2003). However, the use of AGPs has declined (Casewell et al., 2003) because of increased concerns regarding the development of antibiotic-resistant bacteria (Dibner and Richards, 2005), and their use has been banned in the European Union (Cogliani et al., 2011) and limited in the United States by the Veterinary Feed Directive (Food and Drug Administration, 2000). Because of growing interest in low-input and antibiotic-free (**ABF**) production practices, the development of effective alternatives to the sub-therapeutic use of antibiotics is of significant interest to animal agriculture.

The growth-promoting activity of antibiotics is attributed to their effect on the gastrointestinal microbiota (Dibner and Richards, 2005) and are not observed when administered to germ-free animals (Coates et al., 1963). However, increased growth is observed when antibiotics are administered to animals with normal microbiota (Moore et al., 1946; Stokstad and Jukes, 1950; Miles et al., 2006). Additionally, growth is depressed when germ-free animals are inoculated with normal microbiota (Coates, 1980), suggesting intestinal microorganisms are competitive with growth performance of the host animal (Gaskins et al., 2002). Modification of the host microbiota by antibiotics has been suggested to improve growth performance of livestock through inhibition of subclinical infections (Barnes et al., 1978), reduced competition for nutrients between the microbiota and host animal (Monson et al., 1954; Eyssen, 1962), decreased production of growth depressing metabolites by the resident microbiota (Dang et al., 1960), and enhanced absorption of nutrients through the thinner intestinal wall of antibiotic-fed animals (Eyssen and Desomer, 1963; Boyd and Edwards, 1967).

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Administration of probiotics, sometimes called direct-fed microorganisms (**DFM**) when used in livestock animals (Sanders, 2008), has been demonstrated to improve growth performance at levels similar to AGPs (Awad et al., 2009; Mountzouris et al., 2010). Additionally, they have been demonstrated to improve pre-harvest food safety of poultry by reducing colonization of human food-borne pathogens including *Salmonella* (Pascual et al., 1999; Shivaramaiah et al., 2011) and *Campylobacter* (Fritts et al., 2000; Neal-McKinney et al., 2012) in the gastrointestinal tract; improve poultry health by reducing colonization by poultry pathogens including *Clostridium perfringens* (La Ragione and Woodward, 2003; Rahimi et al., 2011) and avian pathogenic *Escherichia coli* (La Ragione et al., 2001); and reduce inflammation induced during *C. perfringens* associated necrotic enteritis (Cao et al., 2012).

Cereal grains commonly used in livestock animal feed contain anti-nutrients including non-starch polysaccharides (**NSP**), resistant starches, and indigestible proteins that are poorly digested by monogastric animals (Bedford, 2000; Sheppy, 2001). Additionally, NSPs exert anti-nutritive effects through chelation of important metal cations including calcium, iron, and magnesium (Debon and Tester, 2001), reduce nutrient absorption by increasing ileal viscosity (Choct et al., 1999), and alter the gastrointestinal microbiota (Choct and Annison, 1992). Digestive enzymes including xylanases, amylases, and proteases are used routinely in animal feeds to improve digestibility (Zanella et al., 1999; Cowieson and Adeola, 2005) and reduce anti-nutritive effects of poorly digested feed constituents (Ravindran et al., 1999; Cowieson et al., 2006); and their effect on growth performance has been well demonstrated (Campbell and Bedford, 1992; Friesen et al., 1992; Bedford and Schulze, 1998). Additionally, the products of the hydrolysis of indigestible feed constituents by exogenous feed-additive enzymes may produce substrates that promote the growth or activities of beneficial bacteria (Kiarie et al., 2013), which suggests the administration of particular enzyme blends may confer an additive benefit when combined with appropriate DFMs. This potential prebiotic-like effect on growth performance suggests the co-administration of enzyme blends with DFMs may be an important component of ABF management programs.

The co-administration of DFMs with feed-additive enzymes has been investigated previously. In addition to improving growth performance, co-administration of *Lactobacillus plantarum* and xylanase was demonstrated to reduce fecal shedding of *Salmonella* Typhimurium in experimentally challenged broilers (Vandeplas et al., 2009). Administration of a multi-strain DFM product containing *Bifidobacterium animalis* and several Lactic Acid Bacteria (**LAB**) in combination with xylanase improved growth performance when compared to either product individually (Murugesan and Persia, 2015). Dersjant-Li et al. (2015) demonstrated previously that administration of a multi-strain *Bacillus amyloliquefaciens* DFM product in combination with an enzyme complex composed of xylanase, amylase, and protease (**XAP**) improved growth performance in broilers fed a diet with reduced energy and digestible amino acids. Although the use of antibiotics in poultry production is continuing to decline, the use of non-medically relevant antibiotics, including bacitracin methylene dialicylate (**BMD**) and virginiamycin, has not been prohibited, and the effect of antibiotics on the efficacy of DFM and DFM-containing products is not well understood. In this study, we evaluated the effect of a feed additive containing 3 strains of *B. amyloliquefaciens* and XAP described previously, administered with or without AGP on the gastrointestinal microbiota and growth performance of broiler chickens.

MATERIALS AND METHODS

Experimental Design

Male broilers (Cobb 500, n = 2160) were obtained from a commercial hatchery on day of hatch, randomly assigned to treatment pens with similar starting weights, and provided experimental feed and water ad libitum for the duration of the study. Experimental animals were allocated to 6 experimental treatment groups with 9 replicate pens of 40 broiler chicks arranged as a randomized complete block design. Experimental treatment groups were fed experimental rations which contained combinations of an AGP [control (AGP), bacitracin methylene disalicylate (BMD), or virginiamycin (**VM**)] and a feed additive (**ADD**; DFM + XAP, Syncra AVI, Danisco Animal Nutrition/DuPont, Marlborough, Wiltshire, UK) composed of a DFM culture containing spores of 3 *Bacillus amyloliquefaciens* strains (7.5×10^7 cfu kg^{-1} feed) and an enzyme blend composed of *Trichoderma reesei* endoxylanase (2,000 U kg^{-1} feed), *Bacillus licheniformis* α-amylase (200 U kg^{-1} feed), and *Bacillus subtilis* serine protease (4,000 U kg^{-1} feed) (**XAP**) (Table 1). All animal care and experimental procedures were performed in accordance with protocols approved by the Texas A&M University Institutional Animal Care and Use Committee. Additional details including experimental design, experimental diets, animal husbandry, and growth performance measures are presented in a separate publication (Flores et al., n.d.)

Bacterial Enumeration

At 21 and 42 d posthatch, a single chicken of approximately mean pen weight (±5%) was selected from each replicate pen, euthanized, and necropsied for the collection of tissues for the enumeration of gastrointestinal microorganisms. The ceca and a section (~6 cm) of the ileum centered on the midpoint between Meckel's diverticulum and the ileocecal junction were dissected aseptically from each selected chicken.

Table 1. Feed Conversion of Broiler Chickens.

Treatment		FCR (Feed: Gain)		
AGP	ADD[1]	D 0–21	D 22–42	D 0–42
–	–	1.380[a]	1.875[a]	1.663[a]
–	+	1.358[b,c]	1.830[b]	1.625[b]
BMD[2]	–	1.357[b,c]	1.824[b]	1.625[b]
BMD	+	1.356[b,c]	1.807[b]	1.612[b]
VM[3]	–	1.371[a,b]	1.831[b]	1.636[a,b]
VM	+	1.352[c]	1.806[b]	1.612[b]
One-way *P* -values		0.007	0.018	0.003
Main Effects				
AGP				
Control		1.369	1.849[a]	1.644[a]
BMD		1.357	1.818[b]	1.619[b]
Virginiamycin		1.362	1.816[b]	1.624[b]
Feed Additive				
Control		1.369[a]	1.842[a]	1.641[a]
ADD		1.356[b]	1.813[b]	1.616[b]
***P* -values**				
AGP		0.079	0.016	0.015
Feed Additive		0.002	0.005	<0.001
AGP × *Feed Additive*		0.092	0.492	0.332
Pooled SEM		0.002	0.007	0.004

[a–c]different superscripts within columns indicate means are significantly different ($P \leq 0.05$).
[1]DFM + XAP; [2]Bacitracin methylene dialicylate (50 g t^{-1}).
[3]Virginiamycin (20 g t^{-1}).

Ileal specimens were homogenized and diluted using fluid thioglycolate medium (**FTM**; BD, Franklin Lakes, NJ), whereas cecal specimens were homogenized and diluted using sterile phosphate buffered saline (**PBS**; Fisher Scientific, Pittsburgh, PA). *Campylobacter jejuni*, *Escherichia coli*, *Salmonella*, and total Lactic Acid Bacteria (**LAB**) were enumerated from the ceca using Campy Cefex agar (Hardy Diagnostics, Santa Maria, California), Compact Dry EC plates (**EC**; Hardy Diagnostics), Xylose-Lysine-Tergitol-4 agar (**XLT-4**; BD), and deMan, Rogosa, and Sharpe agar (**MRS**; BD) supplemented with 100 μg mL^{-1} cycloheximide (Amresco, Solon, OH), respectively. *Clostridium perfringens* was enumerated from the ileum using Tryptose Sulphite Cycloserine Egg Yolk overlay agar (**TSC-EY**; BD). EC and XLT-4 were incubated aerobically at 37°C for 36 h. Campy Cefex and MRS were incubated in 10% CO_2 at 42°C and 37°C, respectively, for 36 h. TSC-EY was incubated at 37°C anaerobically (Coy Laboratory Products, Grass Lake, MI) for 36 h. *C. jejuni* was selectively enriched from cecal specimens using Bolton's Enrichment Broth (**BEB**, Hardy Diagnostic) incubated at 42°C for 24 h followed by Campy Cefex agar. *Salmonella* was selectively enriched from cecal from cecal specimens using Rappaport Vassiliadis R 10 broth (**RV**; BD) incubated at 42°C for 24 h and XLT-4 agar. *C. perfringens* was selectively enriched from the ileum using FTM incubated anaerobically at 37°C for 24 h followed by Iron Milk Medium incubated at 46°C for 3 h. Specimens from which there were no colonies appearing on enumeration plates but were positive by selective enrichment were assigned the lower limit of detection, 100 cfu g^{-1} for statistical analysis.

Presumptive *C. perfringens* were confirmed using Iron Milk Medium, whereas presumptive *C. jejuni*, *E. coli,* and *Salmonella* colonies were confirmed by PCR using species-specific primers (Table 2). *C. jejuni* ATCC 29428, *E. coli* ATCC 25922, and *Salmonella* Typhimurium ATCC 14028 were used as positive controls for PCR.

Statistical Analysis

Bacterial counts were $\log_{10}$ transformed for analysis and reported as the mean ± SEM $\log_{10}$ cfu g^{-1} digestive contents from 9 replicate pens per treatment. Data were analyzed using factorial analysis of variance (**ANOVA**) with main effects for AGP, Feed Additive, and AGP × Feed Additive. A one-way ANOVA was used to determine differences between individual treatment groups. Significantly different means ($P \leq 0.05$) were separated using Duncan's multiple range test. Associations between bacterial counts and feed conversion ratio (**FCR**) were evaluated by pens using Pearson's r. Analyses were conducted using IBM SPSS Statistics (V. 24.0, IBM Corp., Armonk, NY).

RESULTS

Gastrointestinal Microbiota

Gram-positive Bacteria Recovery of total LAB was greater from broilers treated with VM and ADD in combination than from the remaining treatment groups on d 21 and d 42 (Figure 1A-B). On d 21, recovery of *Clostridium perfringens* was greater from untreated broilers than from the remaining treatment groups (Figure 1C), whereas, on d 42, recovery of *C. perfringens* was greatest from broilers administered VM alone (Figure 1D). Administration of ADD increased counts of total LAB in the cecum of broiler chicks on d 21 ($P = 0.028$) but had no effect on d 42 (Table 3). Whereas no difference was observed on d 21,

Table 2. PCR primers used in this study.

Species	Gene	Primer	Sequence (5′-3′)	Reference
C. jejuni	*cadF*	cadF-F2B	TTG AAG GTA ATT TAG ATA TG	(Konkel et al., 1999)
		cadF-R1B	CTA ATA CCT AAA GTT GAA AC	
E. coli	*tuf*	TEcol553	TGG GAA GCG AAA ATC CTG	(Maheux et al., 2009)
		TEcol754	CAG TAC AGG TAG ACT TCT G	
Salmonella	*invA*	INVA-1	ACA GTG CTC GTT TAC GAC CTG AAT	(Chiu and Ou, 1996)
		INVA-2	AGA CGA CTG GTA CTG ATC GAT AAT	

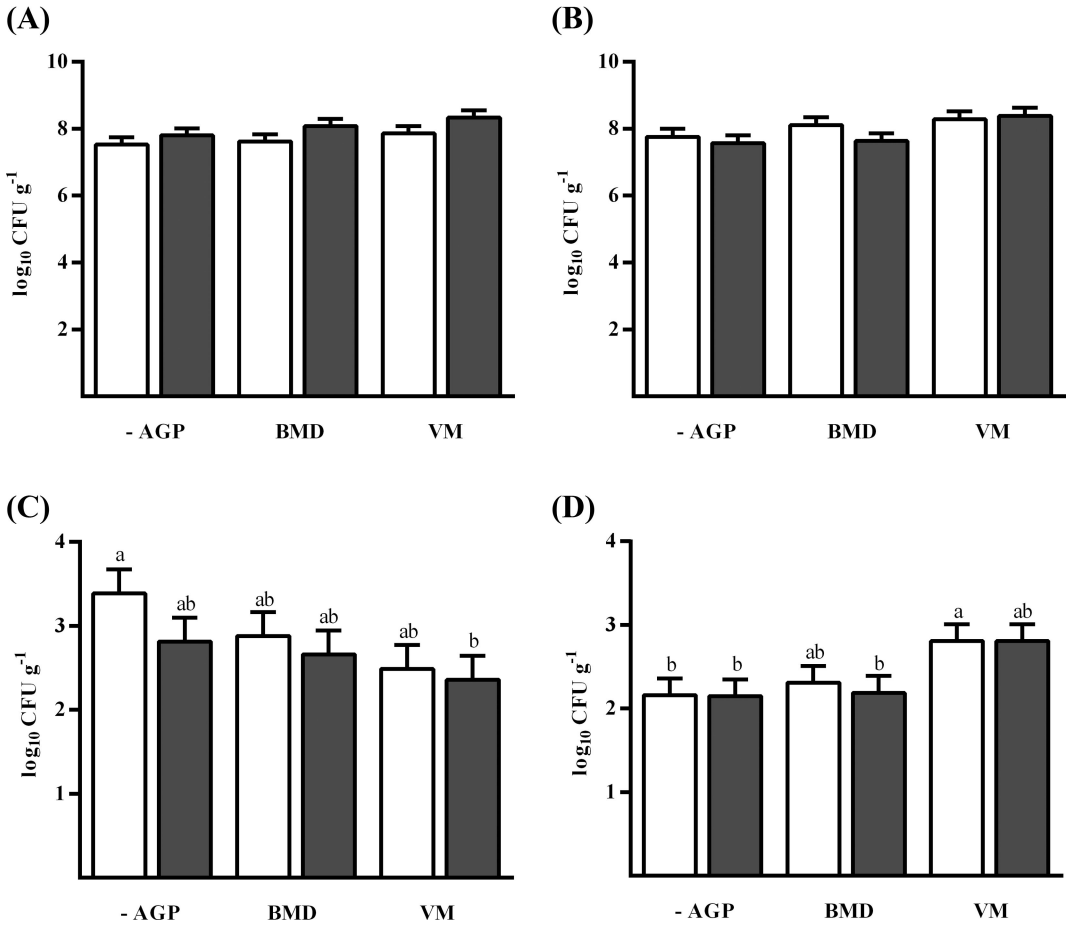

Figure 1. Enumeration of Gram-positive bacteria from broiler chickens. Total LAB were enumerated from the cecum of broiler chicks at (A) d 21 and (B) d 42 posthatch. *C. perfringens* was enumerated from the small intestine of broiler chicks at (C) d 21 and (D) d 42 posthatch. White bars (control); Gray bars (ADD). Counts are reported as the mean ± SEM $\log_{10}$ CFU g^{-1} digestive contents from 9 broiler chickens per treatment. Different letters above bars indicate means are significantly different ($P \leq 0.05$).

Table 3. Main effect of AGP and Feed Additive administration on gastrointestinal microbiota ($\log_{10}$ cfu g^{-1}).

Main Effect	Total LAB[4]		*C. perfringens*		*Salmonella*		*Campylobacter*		*E. coli*	
	d 21	d 42	d 21	d 42	d 21	d 42	d 21	d 42	d 21	d 42
AGP										
Control	7.67	7.67^{b}	3.11^{a}	2.16^{b}	0.24	0.00	2.01	2.05^{b}	7.10	6.48^{b}
BMD[1]	7.85	7.88$^{a, b}$	2.77$^{a, b}$	2.24^{b}	0.32	0.12	1.56	2.32$^{a, b}$	7.48	6.40^{b}
Virginiamycin[2]	8.10	8.33^{a}	2.43^{b}	2.73^{a}	0.01	0.00	2.12	3.27^{a}	7.39	7.34^{a}
Feed Additive										
Control	7.67^{b}	8.05	2.92	2.43	0.15	0.00	2.01	2.73	7.20	6.91
Feed Additive[3]	8.08^{a}	7.86	2.61	2.33	0.23	0.07	1.79	2.35	7.44	6.57
***P*-values**										
AGP	0.151	0.021	0.069	0.014	0.259	0.320	0.484	0.042	0.429	0.050
Feed Additive	0.028	0.330	0.183	0.544	0.636	0.321	0.567	0.351	0.350	0.313
AGP × Feed Additive	0.867	0.454	0.717	0.921	0.216	0.374	0.940	0.040	0.127	0.952
Pooled SEM	0.094	0.102	0.119	0.085	0.080	0.037	0.196	0.222	0.126	0.176

[a,b]Different superscripts within columns indicate means are significantly different ($P \leq 0.05$).
[1]Bacitracin Methylene Dialicylate; [2]Virginiamycin (20 g t^{-1}); [3]DFM + XAP;[4] LAB, Lactic Acid Bacteria.

administration of **AGP** was observed to have a significant effect on total LAB counts on d 42 ($P = 0.021$), with the recovery of total LAB being greater from broilers administered VM than from broilers which were not administered an AGP. Although a significant main effect was not observed for Feed additive administration on d 21 or d 42 (Table 3), recovery of *C. perfringens* in ADD treated broilers was similar to those administered AGPs on d 21 when compared to untreated broilers (Figure 1C). AGP administration was not observed to have a significant effect on d 21, but fewer *C. perfringens* tended to be recovered from broilers administered VM than from untreated broilers ($P = 0.069$). On d 42, more *C. perfringens* were recovered from broilers

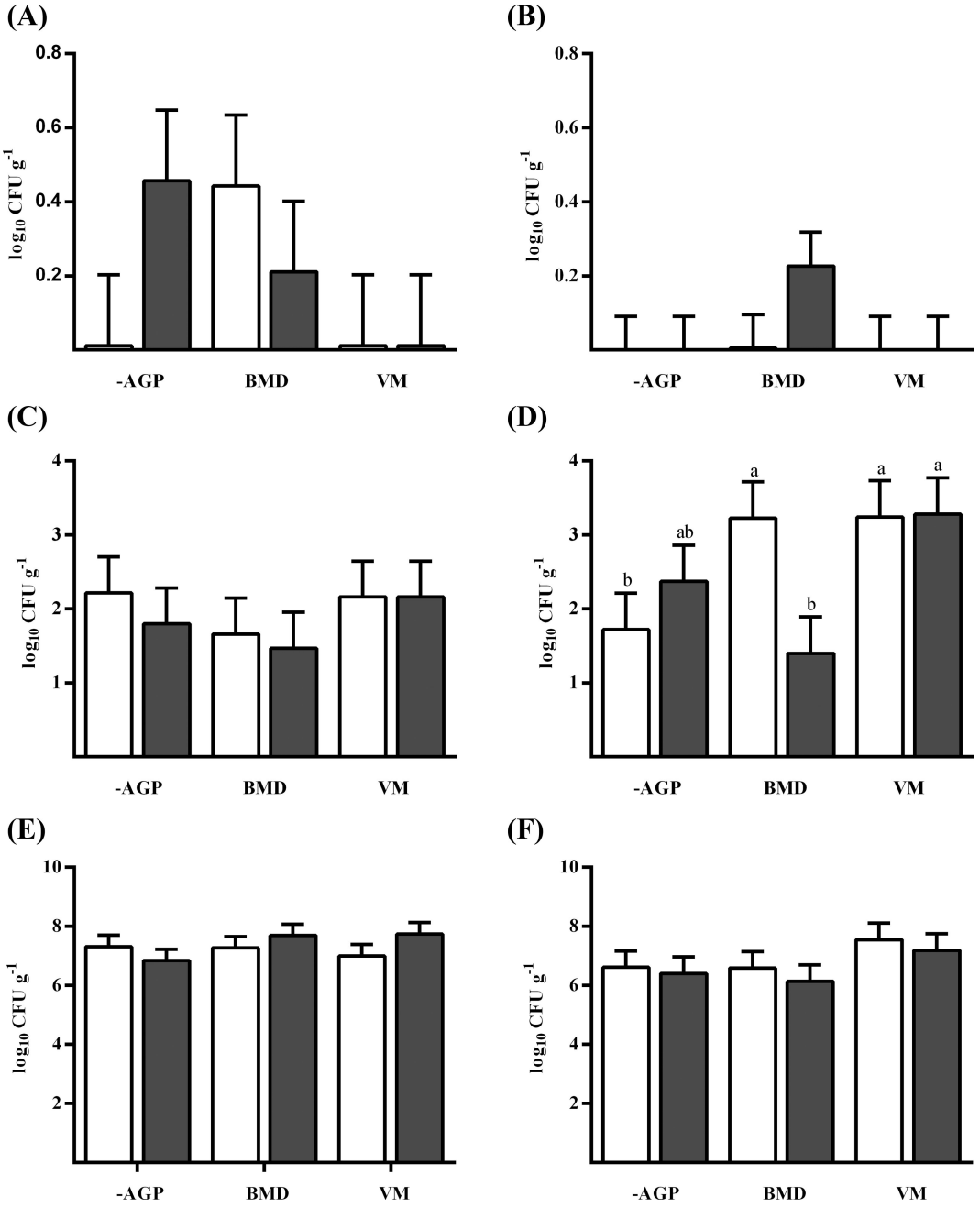

Figure 2. Enumeration of Gram-negative bacteria from broiler chickens. *Salmonella* were enumerated from the cecum of broiler chicks at (A) d 21 and (B) d 42 posthatch. *C. jejuni* were enumerated from the cecum of broiler chicks at (C) d 21 and (D) d 42 posthatch. *E. coli* were enumerated from the cecum of broiler chicks at (E) d 21 and (F) d 42 posthatch. White bars (control); Gray bars (ADD). Counts are reported as the $\log_{10}$ CFU g^{-1} digestive contents from 9 broiler chickens per treatment. Different letters above bars indicate means are significantly different ($P \leq 0.05$). Different letters above bars indicate means are significantly different ($P \leq 0.05$).

administered VM than from those administered BMD or untreated broilers ($P = 0.014$).

Gram-negative Bacteria The administration of AGPs or ADD resulted in no difference in the recovery of *Salmonella* (Table 3). Indeed, recovery of *Salmonella* was near the limit of detection for all treatment groups (Figure 2A-B). Although no significant difference was observed in the recovery of *Campylobacter* on d 21, a significant main effect for AGP administration was detected with more *Campylobacter* being recovered from broilers administered VM than from untreated broilers ($P = 0.042$) on d 42 (Table 3). Additionally, a significant AGP × ADD interaction ($P = 0.04$) was observed on d 42. In broilers administered BMD, fewer *Campylobacter* were recovered from ADD treated broilers ($P = 0.012$) than from those that did not (Figure 2D). Although, no significant difference was observed in the recovery of *E. coli* on d 21, recovery of *E. coli* was greater from broilers administered VM than from others ($P = 0.05$) on d 42.

Associations between the relative abundance of microorganisms in the gastrointestinal tract of chickens

Table 4. Correlation of bacterial counts with FCR.

Bacterial Counts (log_{10} CFU g^{-1})		FCR (Feed: Gain) d 0–21	FCR (Feed: Gain) d 22–42	FCR (Feed: Gain) d 0–42
Total LAB[1]				
d 21	r	−0.287	−0.237	−0.247
	P	0.035	0.085	0.072
d 42	r	−0.040	−0.278	−0.265
	P	0.773	0.042	0.053
C. perfringens				
d 21	r	0.186	0.339	0.405
	P	0.177	0.003	0.002
d 42	r	0.213	−0.019	0.014
	P	0.123	0.892	0.921
C. jejuni				
d 21	r	0.069	−0.114	−0.092
	P	0.621	0.410	0.509
d 42	r	−0.098	−0.428	−0.400
	P	0.479	0.001	0.003

[1]LAB, Lactic Acid Bacteria.

were also evaluated (not shown). Strong positive associations were detected between counts of total LAB and *E. coli* on d 21 ($r = 0.599$, $P < 0.001$) and d 42 ($r = 0.522$, $P < 0.001$). A moderate negative correlation was also detected between LAB and *Salmonella* on d 42 ($r = -0.290$, $P = 0.034$). Lastly, counts of LAB and *Campylobacter* on d 21 tended to correlate moderately ($r = 0.263$, $P = 0.055$), whereas LAB and *Campylobacter* counts were found to correlate moderately ($r = 0.362$, $P = 0.007$) on d 42. No other significant correlations between groups of microorganisms were observed.

Feed Conversion

The effect of the experimental treatments on the growth performance and feed conversion of broiler chickens in this study has been reported comprehensively in a separate publication (Flores et al., n.d.). Feed conversion ratio of broiler chickens reported previously is summarized in Table 1. Overall, administration of ADD improved early (D 0–21) ($P = 0.002$), late (D 22–42) ($P = 0.005$), and cumulative FCR (D 0–42) ($P < 0.001$) when compared to the control, whereas AGP administration improved only late ($P = 0.016$) and cumulative FCR ($P = 0.015$). Administration of ADD improved early feed conversion ($P = 0.007$) in unmedicated and VM-fed broilers but had no additional effect in broilers administered BMD.

Associations between populations of gastrointestinal microorganisms with feed conversion were evaluated (Table 4). Negative correlations ($P < 0.05$) were detected between total LAB counts on d 21 and early FCR (d 0–21) and between total LAB counts on d 42 and late FCR (d 21–42). Additionally, total LAB on d 21 tended to correlate negatively ($P < 0.1$) with late and cumulative FCR (d 0–42) and total LAB on d 42 tended to correlate with cumulative FCR. A moderate positive correlation was observed between counts of *C. perfringens* on d 21 ($P < 0.01$) with late and cumulative FCR. Overall, these data suggest that FCR is lowest in broilers with greater counts of total LAB in the cecum and fewer counts of *C. perfringens* in the ileum. No associations were detected between FCR and *Salmonella* or *E. coli*. However, a strong negative correlation was detected between counts of *Campylobacter* on d 42 with late and cumulative FCR.

DISCUSSION

The objective of this study was to investigate the co-administration of DFM and exogenous enzymes in broiler chickens as a potential alternative to and in addition to the use of AGP. Although AGP have been widely used in the production of poultry and other livestock, the demand for ABF livestock production has increased due to consumer and regulatory concerns over the development of antibiotic resistant bacteria (Dibner and Richards, 2005). Because the growth promoting activities of AGPs are a result of their effects on the gastrointestinal microbiota, the microbiota is likely to be an important target for the development of alternatives to antibiotics. The gastrointestinal microbiota is increasingly recognized as an important modulator of human and animal health (Askelson and Duong, 2015). Additionally, an important role of the microbiota is to augment host metabolism through the conversion of undigested feed components to bioavailable products that can subsequently be utilized by the host (Gibson and Roberfroid, 1995; Askelson et al., 2014). The effects of their administration on the gastrointestinal microbiota and in promoting growth performance suggests DFM and exogenous enzyme as potential alternatives to AGPs. The administration of DFMs in livestock has been demonstrated to improve growth performance at levels similar to AGPs (Awad et al., 2009; Mountzouris et al., 2010) and reduce colonization of human food-borne and poultry pathogens in the gastrointestinal tract of poultry (La Ragione et al., 2001; La Ragione and Woodward, 2003; Rahimi et al., 2011). Exogenous enzymes are used routinely in animal feeds to improve digestibility of poorly digested feed constituents (Zanella et al., 1999) and reduce their anti-nutritive effects (Choct et al., 2004). Additionally, the products of their hydrolysis may serve as substrates which promote the growth or activities of beneficial bacteria (Kiarie et al., 2013). Indeed, the potentially synergistic effects of the co-administration of DFM and exogenous enzymes on growth performance have been demonstrated previously (Murugesan and Persia, 2015). In this study, we evaluated the effect of the administration of a feed additive (ADD) composed of a DFM product containing spores of 3 *Bacillus amyloliquefaciens* strains and an XAP enzyme blend on the gastrointestinal microbiota and growth performance of broiler chickens fed diets with and without AGP.

Administration of ADD improved feed efficiency of broiler chickens at levels similar to AGP, suggesting the co-administration of DFM and enzyme blends may be

a potentially important component of an ABF management program. The growth promoting activities of DFM and exogenous enzymes have been widely demonstrated. Despite dramatic reductions in their use, AGPs are still widely administered in poultry production, and administration of products to further improve growth in AGP-fed animals is also of interest. In this study, administration of ADD did further improve feed efficiency in broilers administered VM suggesting co-administration of DFM and enzyme blends may provide additional benefits to growth performance in antibiotic-fed broiler chickens.

In this study, administration of ADD increased counts of LAB on d 21 ($P = 0.028$), whereas AGP administration increased LAB counts only on d 42 ($P = 0.021$). Although the difference was not significant in previously published work, ADD administration has been demonstrated to increase LAB counts in the gastrointestinal tract of broiler chickens (Dersjant-Li et al., 2015). Administration of direct-fed *B. amyloliquefaciens* (An et al., 2008) and xylanase (Nian et al., 2011) individually has been demonstrated previously to increase LAB in the gastrointestinal tract and improve growth performance of broiler chickens. Characterization of gastrointestinal microbiota of broilers fed conventional and ABF diets found no significant difference in total LAB counts between ABF broilers and those fed a diet containing BMD (Wise and Siragusa, 2007), suggesting AGP administration may have only minimal effect on total LAB. LAB isolated from non-animal environments, including starter cultures and fermented foods, are commonly found to be resistant to multiple antibiotics including bacitracin (Delgado et al., 2002; Danielsen and Wind, 2003; Liu et al., 2009) and virginiamycin (Temmerman et al., 2003; Bischoff et al., 2007), suggesting the resistance determinants are inherent rather than acquired (Mathur and Singh, 2005; Ammor et al., 2008).

In this study, the negative correlation of total LAB counts on d 21 and d 42 with early (d 0–21) and late (d 22–42) FCR, respectively, suggests an important association between LAB and more efficient feed conversion (Table 4). The LAB are important inhabitants of the gastrointestinal tract and are generally recognized as beneficial to poultry intestinal health (Gilliland, 1990; Patterson and Burkholder, 2003; Mountzouris et al., 2007). Cultures of LAB, particularly *Lactobacillus* species, have been used widely as probiotics and their administration to broilers has been demonstrated to improve growth performance (Kalavathy et al., 2003; Mountzouris et al., 2007; Loh et al., 2010). Administration of probiotic LAB has been shown to reduce colonization of bacterial pathogens, including *Clostridium* (La Ragione et al., 2004) and *Salmonella* (Pascual et al., 1999; Kizerwetter-Swida and Binek, 2009), in the gastrointestinal tract, likely through competition for shared attachment sites in the mucosa (Lu and Walker, 2001) and production of anti-microbial metabolites (Oelschlaeger, 2010; Neal-McKinney et al., 2012). Additionally, measures of improved epithelial barrier function including increased villus height and villus height: crypt depth ratio in the duodenum and ileum (Awad et al., 2009) and increased mucus production (Smirnov et al., 2005) have been observed in broilers administered probiotic LAB (Ohland and MacNaughton, 2010).

The positive correlation of *C. perfringens* counts on d 21 with late and cumulative FCR suggests that greater *C. perfringens* counts are associated with less efficient feed conversion (Table 4). In addition to promoting growth, BMD and VM are administered to control *C. perfringens*, suggesting the reduction of sub-clinical infections of this organism as a specific therapeutic target for the development of alternatives to AGP. Reduced weight gain and increased FCR have been reported when high numbers of *C. perfringens* were recovered from broilers (Van Immerseel et al., 2004; Gaucher et al., 2015), and negative effects on growth performance have been reported when broilers were experimentally infected with *C. perfringens* (Jia et al., 2009). Necrosis of epithelial tissues mediated by the multiple virulence factors of *C. perfringens*, including collagenolytic enzymes (Olkowski et al., 2008), NetB toxin (Keyburn et al., 2008), phospholipase C (α-toxin) results in reduced nutrient absorption through the intestinal epithelium (Al-Sheikhly and Truscott, 1977). Additionally, the subsequent immune response and repair of epithelial tissues further increases the nutritional cost of endogenous losses and results in decreased growth performance (Lochmiller and Deerenberg, 2000). Administration of ADD was demonstrated previously to significantly reduce *C. perfringens* in the ileum and cecum of broiler chickens (Dersjant-Li et al., 2015). Although a similar reduction was not observed in this study, ADD administration did reduce *C. perfringens* to levels similar to AGP administration. Administration of direct-fed *Bacillus* has been previously demonstrated to reduce *C. perfringens* and improve FCR to levels similar to AGP administration (Teo and Tan, 2007; Latorre et al., 2015). However, xylanase administration was previously demonstrated not to have an effect on the recovery of *C. perfringens* (Engberg et al., 2004).

A negative correlation was observed between *C. jejuni* counts and FCR (Table 4). However, overall, the treatments evaluated in this study were not observed to affect colonization by *Campylobacter* and *Salmonella*. In the absence of an experimental infection, it is difficult to assess the efficacy of an intervention in reducing colonization by these human food-borne pathogens. Administration of direct-fed *Bacillus* has been demonstrated previously to reduce *Campylobacter* (Aguiar et al., 2013) and *Salmonella* (La Ragione and Woodward, 2003; Shivaramaiah et al., 2011; Menconi et al., 2013) colonization in experimentally infected broilers. Additionally, co-administration of a DFM and xylanase was previously demonstrated to reduce shedding of *Salmonella* and improve FCR in experimentally infected broilers (Vandeplas et al., 2009). In the

current study, ADD administration reduced *Campylobacter* counts in broilers fed diets containing BMD. Although *C. jejuni* has been widely considered to be a commensal organism in poultry (Hermans et al., 2012; Sergeant et al., 2014), the understanding of its relationship with the avian host is complicated by reports of its ability to induce intestinal inflammation, reduce intestinal barrier function, and invade intestinal epithelial tissues in poultry (Smith et al., 2005; Humphrey et al., 2014, 2015; Awad et al., 2015). An improved understanding of the ecological niche filled by *Campylobacter* will inform the development of interventions to reduce colonization of this organism in the gastrointestinal tract of poultry in order to decrease the risk of *Campylobacter*-associated foodborne illness from poultry.

In this study, we investigated the effect of the co-administration of direct-fed *Bacillus* and an enzyme blend on the gastrointestinal microbiota and feed efficiency of broiler chickens. We have demonstrated the ability of the feed additive (DFM + XAP) to improve feed efficiency and modify the gastrointestinal microbiota to be similar to the use of antibiotic growth promoters suggesting this and other similar additives may serve as alternatives to sub-therapeutic use of antibiotics in poultry production. Additionally, we observed a potential additional benefit to growth performance from the co-administration of DFM and enzyme blends in antibiotic-fed broilers. We have observed moderate to strong associations of Lactic Acid Bacteria, *Clostridium perfringens*, and *Campylobacter jejuni* with feed conversion, suggesting potentially important roles of these organisms in gastrointestinal health or in the gastrointestinal fermentation community. Additional research will be required in order to determine the degree to which populations of these organisms should serve as therapeutic targets for the development of products intended to replace AGPs. Although we have not evaluated measures of intestinal barrier function, the effects on the microbiota observed in this study suggest improved intestinal barrier function associated with increased LAB counts and decreased nutritional costs associated with decreased sub-clinical infection by *C. perfringens* may be an important mode of action for the benefits of these antibiotic alternatives. Because of the reliability and effectiveness of antibiotic growth promoters, it is unlikely that a single alternative product will match their efficacy. Thus, the continued development of antibiotic free management programs is likely required to replace AGPs in poultry production.

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DECLARATION OF INTEREST

Y. Dersjant-Li, K. Gibbs, A. Awati are employees of DuPont Industrial Biosciences, Danisco Animal Nutrition, Marlborough, UK.

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