Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses[1]

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ABSTRACT Direct-fed microbials (DFM) are used to improve livestock health and performance. The effects of 2 DFM products, a blend of 3 *Bacillus* strains (DFMB) and a *Propionibacterium* spp. (DFMP), on broiler performance, nutrient utilization, and immune responses were investigated. Day-old (n = 120) male broilers were divided into 24 groups of 5 birds and fed 3 wheat-based diets in mash form (8 groups per diet) from d 1 to 22. The control diet was fed without or with 7.5×10^4 cfu/g of either DFMB or DFMP. From d 19 to 21 fecal samples were collected for determination of total tract apparent retention (TTAR) of nutrients and AME_n. On d 21, feed intake and BW were determined. On d 22, 5 birds per treatment were killed by cervical dislocation to collect jejunal and ileal contents for determination of digesta viscosity and apparent ileal digestibility (AID) of nutrients, respectively, and ileum, cecal tonsil, and spleen tissues for Toll-like receptors (TLR) and cytokine expressions. Compared with the control, DFM did not affect BW gain and feed intake but DFMP reduced G:F ($P < 0.01$). Compared with the control (2,875 kcal/kg), birds fed on DFMB and DFMP had higher AME_n (2,979 and 2,916 kcal/kg, respectively; $P < 0.05$), whereas both DFM reduced the AID of DM ($P < 0.001$) and CP ($P < 0.01$). Furthermore, DFMP reduced TTAR of NDF (29.0 vs. 18.4%; $P < 0.001$), whereas both DFM increased TTAR of DM and fat ($P < 0.001$). Supplementing DFMP downregulated ileal expression of *TLR-2b*, *IL-2*, *IL-4*, *IL-6*, *IL-10*, and *IL-13*, whereas DFMB downregulated *TLR-2b*, *IL-2*, *IL-4*, and *IL-6* in all 3 tissues, *IL-10* in the spleen, and upregulated *IL-13* in the spleen. In conclusion, the DFM did not improve performance but increased the AME_n of diet by possibly increasing DM and fat retention. Overall, both DFM showed an antiinflammatory effect in the ileum, but DFMB had more effects on local and systemic immunity than DFMP.

Key words: broiler, direct-fed microbial, digestibility, performance, immunity

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INTRODUCTION

Probiotics are single or mixed cultures of live nonpathogenic microbes that when administered as feed supplements in sufficient numbers have beneficial effects on the health of the host by improving the properties of indigenous microflora (Fuller, 1989; Hong et al., 2005). Studies have demonstrated that probiotics may enhance host defenses in chickens as a result of the influence of bacteria on host immunity and intestinal integrity against enteric parasites (Dalloul et al., 2003; Farnell et al., 2003; Koenen et al., 2004).

The 2 types of antigenic molecules that confront the gut-associated lymphoid tissue in chickens include nonimmune-evoking innocuous antigens such as nutrients and antigens derived from intestinal or external pathogens that should evoke protective immune responses (Friedman et al., 2003). The balance between immune response to pathogens and tolerance to the fed protein in the gut must be finely kept and depends a great deal on the interaction between immune cells and the gut parenchyma (Bar-Shira and Friedman, 2006). The development and activation of the humoral and cellular gut-associated immune system are largely affected by the development of the gut microflora (Mwangi et al., 2010). Microbial communities can support the animal's defense against invading pathogens by stimulating gastrointestinal immune response (Brisbin et al., 2008). In an in vitro experiment designed to develop a safe microbial feed additive by isolating various bacte-

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rial strains out of the gastrointestinal tract of healthy chickens, 5 effective strains (*Pediococcus acidilactici*, *Enterococcus faecium*, *Bifidobacterium animalis* ssp. *animalis*, *Lactobacillus reuteri*, and *Lactobacillus salivarius* ssp. *salivarius*) exhibited the ability to inhibit a range of common pathogens (Klose et al., 2006; Hossain et al., 2012).

Generally, *Bacillus* sp. are used as probiotics for their antimicrobial and immune stimulation activity (Mongkolthanaruk, 2012). Sen et al. (2012) reported linear improvement in growth performance, apparent nutrient retention, villus height, and villus height to crypt depth ratio in the duodenum and ileum, and decreased cecal *Clostridium* and *Coliform* count in broilers fed diets supplemented with increasing levels of *Bacillus subtilis*. Generally, *Propionibacterium* spp. are preferred as probiotics for their production of propionic acid, bacteriocins, vitamin B_{12}, growth stimulation of other beneficial bacteria, and the ability to endure harsh gastric digestion (Mantere-Alhonen, 1995). Awaad et al. (2013) reported improved growth performance, immune response, and vaccine effectiveness in broilers fed diets supplemented with a combination of soluble β-1,3, D-glucan and *Propionibacterium granulosum*.

Probiotic feed additives generally consist of a single strain or a combination of several strains of bacteria and yeast species. Microorganisms that are to be used as probiotics are isolated from gastrointestinal content, mouth, and feces of animals and humans. The major microbial species presently used as probiotics in animal feeding are *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, *Enterococcus*, and *Saccharomyces* yeast (Patterson and Burkholder, 2003). Most commercial bacillus probiotics are single strain, but new multi-strain *Bacillus subtilis* products having strain composition/ratio optimized to enhance the health and performance of the chicken have been developed and tested for their ability to influence chicken health and systemic immune response (Lee et al., 2010a,b, 2011), but little is known about their effects on local immunity. Studies in chickens have shown that propionic bacteria may have beneficial effects on indices of gut health and function (El-Nezami et al., 2000; Gratz et al., 2005). However, implications of such effects on chicken performance as well as nutrient utilization and local immunity have not been evaluated.

Therefore, our study aims to investigate the effects of supplementing 2 direct-fed microbial (**DFM**) products: a blend of 3 *Bacillus* strains (**DFMB**) and a *Propionibacterium* (**DFMP**) spp. on performance, nutrient utilization, as well as local and systemic immune responses in broiler chickens.

MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Protocol Management and Review Committee, and birds were handled in accordance with the Canadian Council on Animal Care (2009) guidelines.

Experimental Diets

Two DFM supplements designated DFMB (Enviva Pro, a blend of 3 *Bacillus substilis* strains) and DFMP (*Propionibacterium acidipropionici*) were used in this study at an inclusion level of 7.5×10^4 cfu/g of DFM. The DFM supplements were supplied by Danisco Animal Nutrition (Marlborough, Wiltshire, UK). The control diet was formulated to meet NRC (1994) specifications for broiler chickens (Table 1). The diet was based on wheat, wheat middlings, barley, rye, and soybean meal and was formulated without or with either DFMB or DFMP. Each diet contained titanium dioxide (0.3%) as an indigestible marker and was assigned to 8 replicate cages each with 5 birds to give 40 birds per treatment. The diets were stored at 4°C throughout the experiment until fed to the birds.

Birds and Housing

One-day-old (n = 120) male broiler chicks (Ross 308, Carleton Hatcheries Ltd., Grunthal, Manitoba, Canada) were used in this experiment, which lasted for 22 d. The chicks were individually weighed upon arrival

Table 1. Composition of the basal diet

Item	Value
Ingredient (% of control diet)	
Hard wheat	43.9
Wheat middlings	2.8
Barley	10.0
Rye	5.0
Soybean meal (46% CP)	29.3
Tallow/animal fat	4.2
L-Lysine HCl	0.3
DL-Methionine	0.2
L-Threonine	0.1
Titanium dioxide[1]	0.3
Sodium bicarbonate	0.2
Salt	0.2
Limestone	1.3
Dicalcium phosphate	1.0
Minerals/vitamin premix[2]	1.0
Total	100.0
Analyzed composition	
AME (MJ/kg)	12.1
AME_n (MJ/kg)	12.0
CP (%)	22.9
Ca (%)	1.0
Total P (%)	0.7
Lys (%)	1.4
Met + Cys (%)	0.8

[1]Sigma T8141, Oakville, Ontario, Canada.

[2]Mineral premix provided per kilogram of diet: manganese, 55 mg; zinc, 50 mg; iron, 80 mg; copper, 5 mg; selenium, 0.1 mg; iodine, 0.36 mg; sodium, 1.6 g. Vitamin premix provided per kilogram of diet: retinyl acetate, 8,250 IU; cholecalcipherol 1,000 IU; DL-α-tocopherol, 11 IU; cyanocobalamin, 0.012 mg; phylloquinone, 1.1 mg; niacin, 53 mg; choline, 1,020 mg; folacin, 0.75 mg; biotin, 0.25 mg; riboflavin, 5.5 mg.

and stratified by BW into 5 groups of 24 chicks each. Twenty-four uniform groups of 5 chicks (one from each of the 5 groups) were formed. The chicks were then group-weighed and housed in a cage in an electrically heated Petersime battery brooder (Incubator Company, Gettysburg, OH). The brooder and room temperature were set at 32 and 29°C, respectively, during the first 7 d. Thereafter, heat supply in the brooder was switched off and room temperature was maintained at 29°C throughout the experiment. Light was on throughout the experiment. Birds had free access to feed and water throughout the experiment. Body weight and feed intake (**FI**) per cage were determined on d 21 after withdrawing feed for 4 h.

Sample and Tissue Collection

On d 18, 19, and 20, samples of excreta were collected, pooled within a pen, and stored frozen at −20°C for the determination of total tract apparent retention (**TTAR**) of nutrients. Care was taken during the collection of excreta samples to avoid contamination from feathers and other foreign materials. On d 22, all birds in each treatment were killed by cervical dislocation and contents of jejunum (from the end of duodenum to Meckel's diverticulum) and ileum (from Meckel's diverticulum to approximately 1 cm above the ileal-cecal junction) were obtained. Jejunal digesta was immediately prepared and analyzed for digesta viscosity whereas ileal digesta samples were stored frozen at −20°C until the analyses could be carried out. Tissue samples from the ileum, cecal tonsil and spleen were collected from 5 birds in each treatment and immediately frozen in liquid N, and thereafter stored at −80°C until required for analysis.

Sample Preparation and Chemical Analyses

Jejunal and ileal digesta samples from birds within a pen were pooled for viscosity and apparent ileal digestibility (**AID**) measurements respectively. Jejunal digesta was mixed to obtain a homogenous mixture, which was then centrifuged at 2,150 × *g* at 4°C for 15 min in duplicate tubes for 5 min to separate feed particles from the liquid phase. The supernatant (0.5 mL) from each tube was analyzed for viscosity, which was measured in centipoise units at 30 rpm and 40°C using the Brookfield digital viscometer (model LVDVII+ CP, Brookfield Engineering Laboratories, Stoughton, MA). Excreta samples were oven-dried at 60°C and finely ground to pass through a 1-mm screen using a Cyclotec 1093 Sample Mill (FOSS North America, Eden Prairie, MN); ileal samples were freeze-dried and finely ground in a grinder (CBG5 Smart Grind; Applica Consumer Products Inc., Shelton, CT); and the basal diet sample was finely ground to pass through a 1-mm screen in a Thomas-Wiley mill (Thomas Scientific, Swedesboro, NJ). Each was thoroughly mixed before analysis.

Dry matter was determined according to the AOAC International (1998) procedures (procedure 4.1.06), and gross energy was determined using the Parr adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, IL). Nitrogen was determined using a N analyzer (model NS-2000, Leco Corporation, St. Joseph, MI). Samples for Ca and P analyses were ashed and digested according to AOAC (1990) procedures (method 990.08) and read on a Varian inductively coupled plasma mass spectrometer (Varian Inc., Palo Alto, CA). Samples for titanium analysis were ashed and digested as described by Lomer et al. (2000) and read on a Varian inductively coupled plasma mass spectrometer (Varian Inc.). Samples for AA analysis were prepared by acid hydrolysis according to AOAC International (1998) procedures (procedure 4.1.11 alternative 3). Samples for analysis of sulfur containing amino acids (methionine and cysteine) were subjected to performic acid oxidation before acid hydrolysis. Tryptophan was not determined. The excreta samples were analyzed for NDF according to the method of Van Soest et al. (1991) using an Ankom 200 Fiber Analyzer (Ankom Technology, Fairport, NY) and for crude fat using hexane as the solvent according to the AOAC (1990; method 920.39). Starch content of feed and ileal contents were determined enzymatically as described by McCleary et al. (1997).

Total RNA Extraction and Reverse Transcription

Extraction of total mRNA from the ileum, cecal tonsil, and spleen was done using Trizol Reagent (Invitrogen Canada Inc., Burlington, ON, Canada) as instructed in the manufacturer's protocol. Methods of processing RNA and reverse transcription were performed as described by Rodríguez-Lecompte et al. (2012).

Quantitative Real-Time PCR

Quantitative real-time PCR was performed using the Step One thermo cycler (Applied Biosystems, Mississauga, ON, Canada) as described by Yitbarek et al. (2012). Primer sequences for β-actin, Toll-like receptor (***TLR***)-*2b*, *TLR-4*, *TLR-21*, *IL-2*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12p35*, *IL-13*, transforming growth factor β 4 (*TGF-β4*), interferon (***IFN***)-γ, *IFN-β*, and cluster of differentiation (***CD***)-*40* were obtained from gene databases (Table 2).

Calculations and Statistical Analyses

The digestibility of nutrients were calculated using the following equation:

$$\%\ \text{apparent nutrient digestibility} = \left\{1 - \left[\left(\frac{T_d}{T_f}\right) \times \left(\frac{N_f}{N_d}\right)\right]\right\} \times 100,$$

Table 2. Pairs of primers used for reverse-transcription PCR and quantitative real-time PCR

Gene[1]	Primer sequence (5′-3′)[2]	Fragment sizes (bp)	Annealing temperature (°C)	GenBank accession number
β-Actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	205	61	X00182
CD-40	F: CCTGGTGATGCTGTGAATTG R: CTTCTGTGTCGTTGCATTCAG	128	55	EF554721
IFN-β	F: GCCTCCAGCTCCTTCAGAATACG R: CTGGATCTGGTTGAGGAGGCTGT	224	55	GU119897
IFN-γ	F: CTGAAGAACTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	264	60	X99774
IL-2	F: TGCAGTGTTACCTGGGAGAAGTGGT R: ACTTCCGGTGTGATTTAGACCCGT	140	60	AJ224516
IL-4	F: TGTGCCCACGCTGTGCTTACA R: CTTGTGGCAGTGCTGGCTCTCC	193	57	GU119892
IL-6	F: CAGGACGAGATGTGCAAGAA R: TAGCACAGAGACTCGACGTT	233	59	AJ309540
IL-8	F: CCAAGCACACCTCTCTTCCA R: GCAAGGTAGGACGCTGGTAA	176	55	DQ393272
IL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	103	55	AJ621614
IL-13	F: ACTTGTCCAAGCTGAAGCTGTC R: TCTTGCAGTCGGTCATGTTGTC	129	55	GU119894
IL-12p35	F: CTGAAGGTGCAGAAGCAGAG R: CCAGCTCTGCCTTGTAGGTT	217	64	NM213588
TGF-β4	F: CGGCCGACGATGAGTGGCTC R: CGGGGCCCATCTCACAGGGA	113	55	AF459837
TLR-2b	F: CGCTTAGGAGAGACAATCTGTGAA R: GCCTGTTTTAGGGATTTCAGAGAATTT	90	59	NM204278
TLR-4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	190	55	AY064697
TLR-21	F: TGGCGGCGGGAGGAAAAGTG R: CACCGTGCTCCAGCTCAGGC	106	59	NM_001030558

[1]*CD-40* = cluster of differentiation-40; *IFN-β* = interferon β; *IFN-γ* = interferon gamma; *TGF-β4* = transforming growth factor β 4; *TLR-2b* = Toll-like receptor-2b; *TLR-4* = Toll-like receptor-4; *TLR-21* = Toll-like receptor-21.

[2]F = forward; R = reverse.

where T_d = the titanium dioxide (TiO_2) concentration in the diet, T_f = the TiO_2 concentration in the excreta or ileal digesta, N_f = the nutrient concentration in the excreta or ileal digesta, and N_d = the nutrient concentration in the diet.

The AME and AME_n content of experimental diets were calculated using the following equations:

$$\text{AME}\ (\text{kcal/kg}) = \text{GE}_{\text{kcal/kg of diet}} - \left[\text{GE}_{\text{kcal/kg of excreta}} \times \left(\text{TiO}_{2\%\ \text{diet}} \div \text{TiO}_{2\%\ \text{excreta}}\right)\right]; \text{ and}$$

$$\text{AME}_n\ (\text{kcal/kg}) = \text{GE}_{\text{kcal/kg of diet}} - \left[\text{GE}_{\text{kcal/kg of excreta}} \times \left(\text{TiO}_{2\%\ \text{diet}} \div \text{TiO}_{2\%\ \text{excreta}}\right)\right] - 8.22 \times \left\{\text{N}_{\%\ \text{diet}} - \left[\text{N}_{\%\ \text{excreta}} \times \left(\text{TiO}_{2\%\ \text{diet}} \div \text{TiO}_{2\%\ \text{excreta}}\right)\right]\right\},$$

where GE is gross energy, and 8.22 is the energy equivalent of uric acid N.

Performance data were analyzed using the GLM procedure in SAS statistical software (SAS Institute, Cary, NC) in a completely randomized design.

Performance, viscosity, and digestibility responses were analyzed using the following linear model (PROC GLM): $y_{ij} = \mu + t_i + e_{ij}$, where y_{ij} is the observation of the jth replicate ($j = 1$ to 8) in the ith treatment ($i = 1$ to 3), μ is the population mean, t_i is the treatment effect, and e_{ij} is the error deviation. Levels of expression for all genes were calculated relative to β-actin, the housekeeping gene, and gene expression was presented as fold change relative to the control diet (the control was used as the calibrator). Gene expression fold change, SE, and statistical significance were calculated using REST 2009 (Qiagen, Valencia, CA) based on the formula developed by Pfaffl et al. (2002). All data were considered significantly different at $P < 0.05$.

RESULTS

Effect of DFM on Growth Performance

Compared with control, supplementation of DFM did not affect FI and BW gain of the birds (Table 3). However, birds supplemented with DFMP had lower ($P < 0.05$) G:F than those supplemented with DFMB.

Effect of DFM on AID of Nutrients and Digesta Viscosity

Compared with control, birds offered the DFM diets had decreased AID of DM and CP ($P < 0.05$), but their AID of Ca, P, fat, starch (Table 3), and indispensable and dispensable AA (Table 4) were similar except that

Table 3. Growth performance, apparent ileal digestibility, digesta viscosity, total tract apparent retention, and AME_n of diets for broiler chickens fed diets supplemented with direct-fed microbials

	Diet[1]				
Item	Control	DFMB	DFMP	SEM	*P*-value
Initial BW (g/bird)	44.8	44.9	44.8	0.67	—
Final BW (g/bird)	936	948	916	18.0	NS
Feed intake (g/bird)	1,237	1,242	1,239	27.7	NS
BW gain (g/bird)	891	901	872	17.4	NS
G:F (g:g)	0.73[a]	0.72[a]	0.70[b]	0.004	**
Apparent ileal digestibility (%)					
DM	66.8[a]	56.5[c]	58.8[b]	0.08	***
CP	81.3[a]	75.2[b]	76.9[b]	0.63	**
Ca	53.8	53.8	60.1	3.79	NS
P	55.6	49.8	54.7	1.80	NS
Fat	78.2	75.2	77.4	1.54	NS
Starch	89.7	83.7	84.1	2.27	NS
Viscosity (cP)	4.3	5.1	4.5	0.25	†
Total tract apparent retention (%)					
DM	67.4[c]	69.4[a]	67.7[b]	0.05	***
NDF	29.0[a]	19.6[b]	18.4[b]	1.10	***
CP	62.2	62.6	60.3	0.88	NS
Ca	59.8	62.1	64.1	1.62	NS
P	52.4	50.7	47.9	1.45	NS
Fat	77.3[b]	84.2[a]	83.2[a]	0.42	***
AME (kcal/kg of diet)	2,894[c]	2,998[a]	2,934[b]	6.1	***
AME_n (kcal/kg of diet)	2,875[c]	2,979[a]	2,916[b]	6.2	***

[a–c]Means within a column lacking a common superscript differ ($P < 0.05$).

[1]DFMB = control supplemented with *Bacillus* spp.; DFMP = control supplemented with *Propionibacterium* spp.

†$P \leq 0.1$, **$P \leq 0.01$, ***$P \leq 0.001$, NS: $P > 0.1$.

of cysteine, which was reduced by DFMP ($P < 0.05$). The AID of phenylalanine tended to increase ($P < 0.1$) in birds offered the DFM, whereas that of proline tended to increase ($P < 0.1$) in birds offered DFMB. Birds offered DFMB tended to have higher ($P < 0.1$; Table 3) digesta viscosity than DFMP and control.

Effect of DFM on TTAR of Nutrients and AME_n of Diets

Birds offered the DFM-supplemented diets had similar TTAR of CP, Ca, and P (Table 4), decreased TTAR of NDF, increased TTAR of DM and fat and AME_n of

Table 4. Apparent ileal digestibility of indispensable and dispensable amino acids of broiler chickens fed diets supplemented with direct-fed microbials

	Diet[1]				
Item	Control	DFMB	DFMP	SEM	*P*-value
Indispensable amino acid (%)					
Arg	86.4	88.1	87.3	0.67	NS
His	68.1	69.0	65.9	1.54	NS
Ile	82.3	84.6	84.3	0.85	NS
Leu	83.8	85.5	84.7	0.61	NS
Lys	87.9	88.5	88.5	0.73	NS
Met	90.5	92.1	91.3	0.51	NS
Phe	84.9	86.9	86.1	0.55	†
Thr	79.7	81.4	79.7	0.82	NS
Val	80.5	82.0	81.5	0.85	NS
Dispensable amino acid (%)					
Ala	80.3	81.9	80.5	0.73	NS
Asp	79.7	81.6	80.1	0.76	NS
Cys	88.1[a]	87.0[a]	78.3[b]	2.25	*
Glu	90.1	91.0	90.1	0.35	NS
Gly	79.1	80.6	78.7	0.79	NS
Pro	88.1	89.3	87.8	0.45	†
Ser	81.9	83.4	81.8	0.67	NS
Tyr	83.2	84.7	83.9	0.73	NS

[a,b]Means within a column lacking a common superscript differ ($P < 0.05$).

[1]DFMB = control supplemented with *Bacillus* spp.; DFMP = control supplemented with *Propionibacterium* spp.

†$P \leq 0.1$, *$P \leq 0.05$, NS: $P > 0.1$.

the diet compared with the control (Table 3). Birds offered DFMB had greater ($P < 0.001$) TTAR of DM and AME_n (2,979 versus 2,916) than DFMP.

Effect of DFM on TLR and Cytokine Expression in the Ileum, Cecal Tonsil, and Spleen

Compared with the control, DFMB downregulated *TLR-2b* in the ileum, cecal tonsil, and spleen, DFMP downregulated *TLR-2b* only in the ileum, whereas both DFM had no effect on expression of *TLR-4* and *TLR-21* (Figure 1). Both DFMB and DFMP downregulated *IL-6* in a similar pattern as was *TLR-2b* and had no effect on expression of *IL-8, IFN-β*, and *IL-12p35* (Figure 2). Interleukin-10 was downregulated in the ileum and spleen by DFMP and DFMB, respectively (Figure 3). Both DFMB and DFMP downregulated *IL-4* (Figure 3) in a similar pattern as *TLR-2b*. Interleukin-13 was downregulated in the ileum by DFMP and upregulated in spleen by DFMB, but both DFM did not affect expression of *IFN-γ* in the tissues (Figure 3). Supplementation of DFMB and DFMP downregulated *IL-2* (Figure 4) in a similar pattern as was *TLR-2b*, whereas DFMB upregulated *TGF-β4* in the ileum and cecal tonsil, and both DFM had no effect on expression of *CD-40* (Figure 4).

DISCUSSION

This study was conducted to investigate the effects of supplementing 2 DFM products, DFMB and DFMP, on performance, nutrient utilization, and local and systemic immune responses in broiler chickens. Our results demonstrated that the DFM used in the study had no effect on growth performance of birds. Lee et al. (2010a) found no effect of DFMB on BW gain, whereas other studies have reported contrasting results on the effects of dietary DFM on broiler growth performance. Beneficial effects of DFM on broiler growth performance have been reported by Zhang et al. (2005) upon supplementation of yeast (*Saccharomyces cerevisiae*), Nayebpor et al. (2007) upon supplementation of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium thermophilum*, and *Enterococcus faecium*, Apata (2008) upon supplementation of *Lactobacillus bulgaricus* and Talebi et al. (2008) upon supplementation of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Enterococcus faecium*, and *Bifidobacterium bifidium* with a disease challenge. However, consistent with our results are studies of Mountzouris et al. (2007) who supplemented *Lactobacillus reuteri*, *Enterococcus faecium*, *Bifidobacterium animalis*, *Pediococcus acidilactici*, and *Lactobacillus salivarius*, Rodríguez-Lecompte et al. (2012) supplemented *Lactobacillus acidophilus*, *Lactobacillus casei*, *Streptococcus faecium*, and *Saccharomyces cerevisiae*, and Willis et al. (2007) and Willis and Reid (2008) supplemented

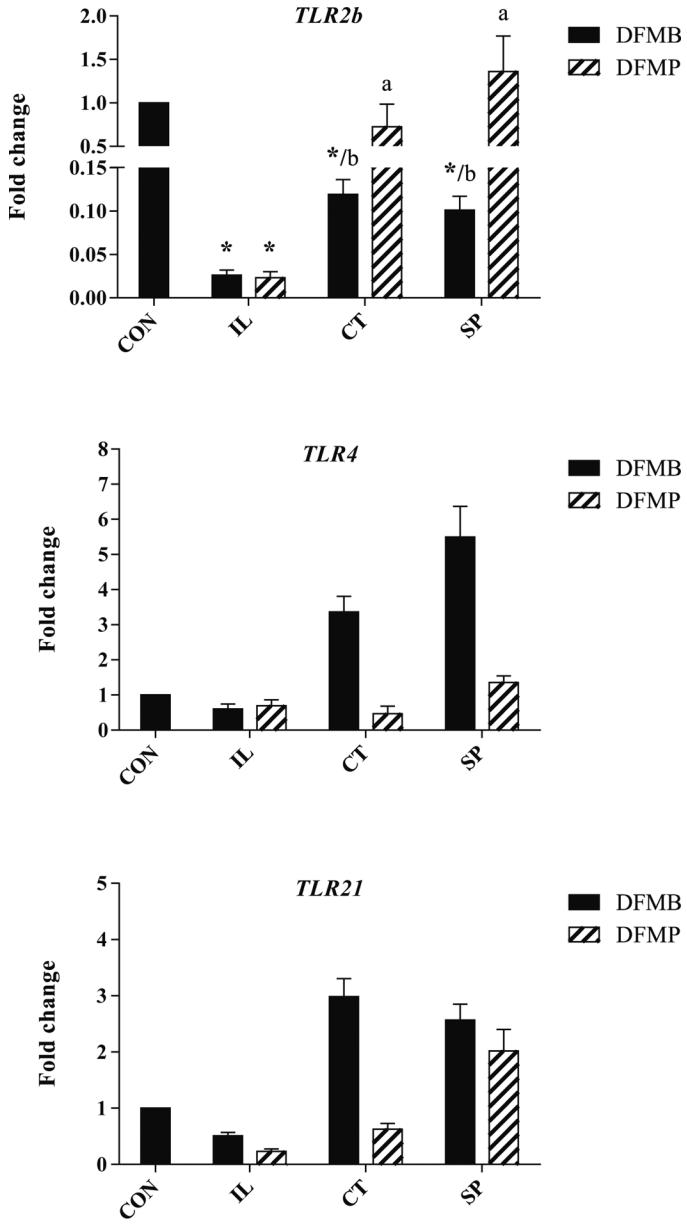

Figure 1. Fold change expression of Toll-like receptor (*TLR*)-*2b*, *TLR-4*, and *TLR-21* in the ileum (IL), cecal tonsil (CT), and spleen (SP) of broiler chickens fed diets supplemented with direct-fed microbials. *Bars with an asterisk differ significantly from the control (CON) at $P < 0.05$. Bars with different letters (a,b) differ significantly between treatments in a tissue ($P < 0.05$). DFMB = control supplemented with *Bacillus* spp.; DFMP = control supplemented with *Propionibacterium* spp.

similar DFM as Talebi et al. (2008) without a disease challenge but found no or minimal effect of DFM.

Our study shows that AID and TTAR of fat, DM, and CP were differentially affected in the ileum and hind gut of birds receiving DFM. Generally, the AID of DM and CP were decreased and that of fat not affected at the ileum, whereas the TTAR of fat and DM were increased and that of CP not affected in the excreta. The possible explanation of these observations is that the DFM ingested were more effective at the cecum and colon than at the ileum of the birds. Hence, the

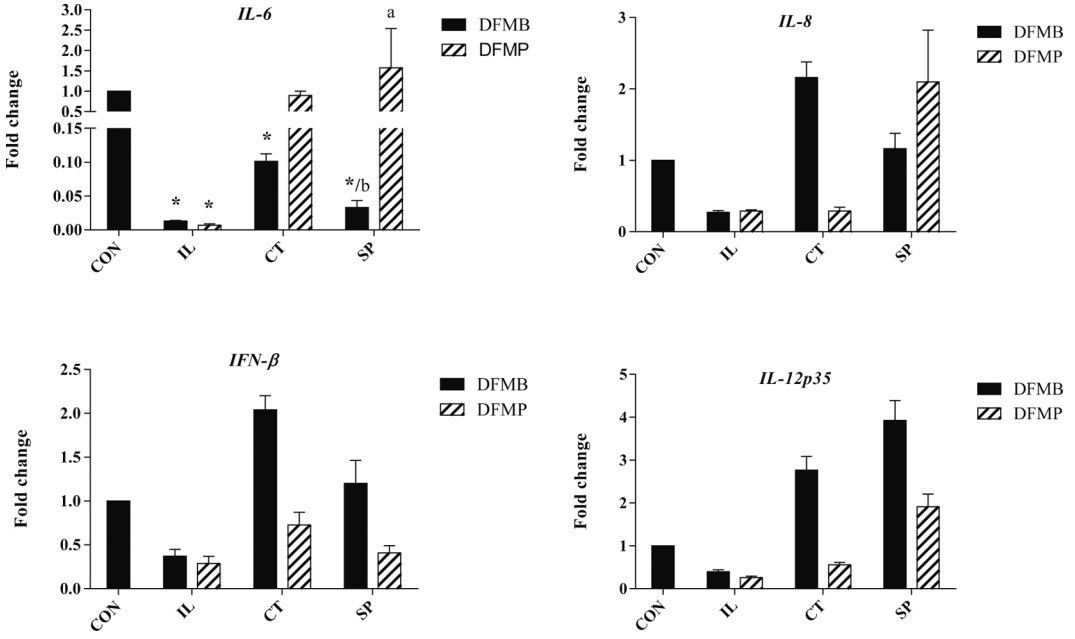

Figure 2. Fold change expression of *IL-6*, *IL-8*, interferon (*IFN*)-*β*, and *IL-12p35* in the ileum (IL), cecal tonsil (CT), and spleen (SP) of broiler chickens fed diets supplemented with direct-fed microbials. *Bars with an asterisk differ significantly from the control (CON) at $P < 0.05$. Bars with different letters (a,b) differ significantly between treatments in a tissue ($P < 0.05$). DFMB = control supplemented with *Bacillus* spp.; DFMP = control supplemented with *Propionibacterium* spp.

observed increase in TTAR of DM and NDF is due to fermentation aided by the DFM and other gut microflora residing at the cecum and colon of the birds. The decrease in AID of CP and lack of effect on AID of Ca, P, fat, starch, and AA in the DFM-supplemented birds could be due to nutrient requirements for growth and proliferation of the DFM and other beneficial gut microbes, hence providing a nutrient cost for the host. The increase in TTAR of fat is consistent with the study of Apata (2008) reporting increased TTAR of

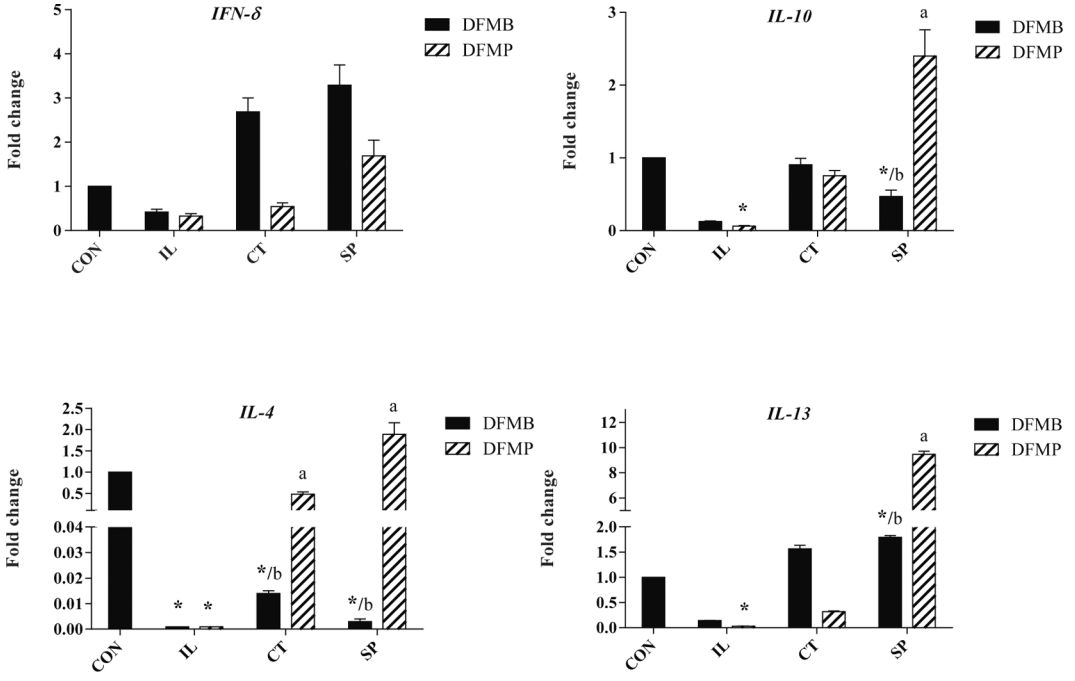

Figure 3. Fold change expression of interferon (*IFN*)-*γ*, *IL-10*, *IL-4*, and *IL-13* in the ileum (IL), cecal tonsil (CT), and spleen (SP) of broiler chickens fed diets supplemented with direct-fed microbials. *Bars with an asterisk differ significantly from the control (CON) at $P < 0.05$. Bars with different letters (a,b) differ significantly between treatments in a tissue ($P < 0.05$). DFMB = control supplemented with *Bacillus* spp.; DFMP = control supplemented with *Propionibacterium* spp.

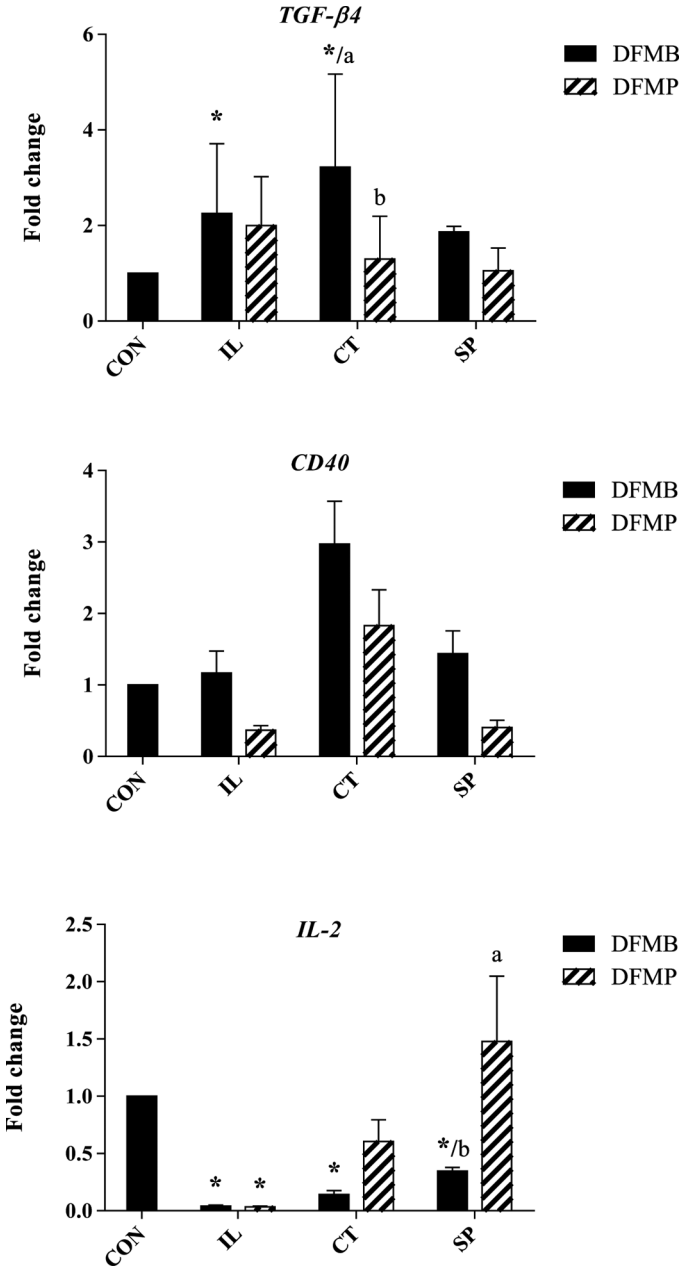

Figure 4. Fold change expression of transforming growth factor (*TGF*)-*β4*, cluster of differentiation (*CD*)*40*, and *IL-2* in the ileum (IL), cecal tonsil (CT), and spleen (SP) of broiler chickens fed diets supplemented with direct-fed microbials. *Bars with an asterisk differ significantly from the control (CON) at $P < 0.05$. Bars with different letters (a,b) differ significantly between treatments in a tissue ($P < 0.05$). DFMB = control supplemented with *Bacillus* spp.; DFMP = control supplemented with *Propionibacterium* spp.

fat after supplementing a *Lactobacillus* spp. in a broiler chicken starter diet.

Antinutritive effects of β-glucans (in barley) and arabinoxylans (in wheat and rye) could have affected digestibility of nutrients. These water-soluble nonstarch polysaccharides have been shown to detrimentally increase digesta viscosity (Almirall et al., 1995; Choct et al., 1995; Lazaro et al., 2003) and may have contributed to the similar AID and TTAR observed for most of the nutrients. There is compelling evidence indicating that broilers fed wheat, barley, and rye diets suffer from reduced digestibility of nutrients and energy, which results in depressed performance (Almirallet al., 1995; Choctet al., 1995; Bedford and Morgan, 1996; Langhout et al., 1999; Mathlouthi et al., 2002). It is not clear why birds offered DFMB tended to have higher digesta viscosity than those offered DFMP and control diet.

The lack of effect of the DFM on the TTAR of fiber observed in this study is consistent with the studies of Apata (2008). Though the lack of ability to degrade fiber can be associated to lack of enzymes with sufficient carbohydrase activities to cause differences, it is not clear why the DFM reduced fiber digestibility compared with the control. As reviewed by Rowland (1992), one of the most important ways in which a probiotic organism might exert beneficial effect on its host was to modify metabolic processes, particularly those occurring in the gut by either stimulating host digestive enzymes or provide a probiotic source of these enzymes. In this study intestinal colonization was not tested, and thus, the observed effects in digestibility of fiber and consequently other nutrients could not be conclusively attributed to competitive exclusion mechanisms, although the DFM could have negatively affected the normal gut bacteria responsible for fermenting the fiber.

We observed increased AME and AME_n in birds offered DFM-supplemented diets. The improved AME and AME_n might have been due to increased TTAR of fat and possibly starch (>10%) that escaped upper gut digestion. Although the DFM used in this study increased the AME_n of the diet as observed in the studies of Mohan et al. (1996) and Schneitz et al. (1998), this increase in AME_n did not improve growth performance, perhaps suggesting the basal diet was nutritionally adequate.

The primary function of the immune system is to identify and eliminate pathogens. This may be enhanced by administering probiotics that stimulate the local immune system (Fuller, 1989). In the presence of microorganisms in the gut, the TLR, also known as pattern recognition receptors, may induce expression of various proinflammatory cytokines (such as *IL-6*) and antimicrobial peptides (such as defensins), which are direct effector molecules of the innate immune response (Ganz, 2003; Kaiser, 2010). The TLR recognize microbial-associated molecular patterns, causing a chain reaction that stimulates the immune system (Aderem and Ulevitch, 2000). We found the control birds had greater expression of *TLR-2b* in the ileum than birds fed on DFMB and DFMP. This could be attributed to the ability of both *Baccillus* sp. and *Propionibacterium* sp. to produce bacteriocins that inhibit growth of other strains (Mantere-Alhonen, 1995; Mongkolthanaruk, 2012). Generally, *TLR-2* recognizes a broad range of microbial products including peptidoglycan and lipopeptides from gram-positive bacteria,

mycoplasmas, mycobacteria, and spirochetes (Lien et al., 1999; Schwandner et al., 1999; Takeuchi et al., 1999; Fukui et al., 2001), and zymosan from yeast (Underhill et al., 1999).

Another important effect of probiotics on barrier function is their ability to counteract the effects of proinflammatory cytokines. Interleukin-2 and IL-4 are produced by naïve and T-helper 2 cells, respectively, in response to antigenic stimulation; on activation by antigen recognition and stimulation naïve T cells produce IL-2, which binding to its receptor, initiates proliferation of T cells that recognize the antigen. For instance, upregulation of *IL-2* mRNA in chicken gut has been associated with *Eimeria* infection (Choi and Lillehoj, 2000). We observed a greater ileal expression of *IL-2* and *IL-4* in the control birds than in birds offered DFM. This suggests that the DFM could have neutralized the antigens in the gut that triggered *IL-2* and *IL-4* expression in the control group by the luminal gut microbiota. Chicken IL-6 is secreted by T cells and macrophages and acts as both a proinflammatory in association of the production of acute phase proteins and antiinflammatory cytokine. For instance, *IL-6* upregulation in chickens has been associated with *Salmonella* and *Eimeria* infection (Kaiser et al., 2000; Lynagh et al., 2000; Wigley and Kaiser, 2003). Therefore, a downregulation of *IL-6* coinciding that of *IL-2* and *IL-4* mostly favors an antiinflammatory response and shows that both DFM had an antiinflammatory effect in the gut.

Our results indicate that DFMB and DFMP stimulated the immune system differently, with DFMB having more effects than DFMP. Only DFMP downregulated ileal IL-10 and IL-13 compared with control showing its antiinflammatory effect in the ileum involved more genes than DFMB, given that both DFM also downregulated ileal IL-2, IL-4, and IL-6 expression. However, DFMB downregulated cecal tonsil *IL-2*, *IL-4*, and *IL-6*, and splenic *IL-2* and *IL-4* expression compared with DFMP. This shows that DFMB had both local and systemic immunity effects. In addition, DFMB had a higher ileal and cecal tonsil expression of *TGF-β_4* than DFMP. Although the roles of chicken *TGF-β_4* in vivo have not yet been well established (Pan and Halper, 2003), its upregulation in the chicken gut has been associated with *Eimeria* infection (Choi et al., 1999), presumably as part of an antiinflammatory response.

In conclusion, supplementing the DFM in the diet did not have beneficial effects on performance but increased the AME_n of diet by possibly increasing DM and fat retention. Both DFM downregulated ileal *TLR-2b* showing their ability to inhibit the adhesion of other gut microflora that compete for available nutrients. The downregulation of ileal, cecal tonsil, and splenic cytokines by both DFM suggests they have antiinflammatory responses in broiler chickens. Comparing the 2 DFM products, DFMB had more effects on both local and systemic immunity than DFMP.

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