ABSTRACT This study was conducted to compare growth performance, gut morphometry, and parameters of local and systemic immunity in broiler chickens fed for 22 consecutive days with a diet supplemented with Bacillus spp. as direct-fed microbials (DFM), a commercial product incorporating 3 DFM, or a non-supplemented diet. Direct-fed microbials did not significantly modify BW gain and most failed to affect serum antibody levels in response to immunization with a recombinant Eimeria protein. However, altered intestinal morphometric measurements were readily apparent in DFM-fed chickens as revealed by increased villus height and crypt depth compared with non-DFM-fed controls. In addition, serum levels of α-1-acid glycoprotein as an inflammatory marker were reduced in DFM-fed birds, whereas splenic lymphocyte proliferation, intestine intraepithelial lymphocyte subpopulations, and cytokine mRNA levels in intraepithelial lymphocytes were increased, decreased, or unchanged compared with controls depending on the DFM used. These results provide a rational scientific basis for future studies to investigate DFM as immunomodulating agents to enhance host protective immunity against enteric pathogens in broiler chickens.

Key words: chicken, direct-fed microbial, immune response, growth performance, intestinal morphometry

INTRODUCTION

Enteric diseases in commercial poultry contribute to losses in productivity, increased mortality, and contamination of products for human consumption (Dekich, 1998; Patterson and Burkholder, 2003). Among the diseases of high concern are those due to Clostridium-related infections (such as gangrenous dermatitis or turkey cellulitis in pouls and necrotic enteritis), enteritis of unknown etiologies, and colibacillosis (Smith and Helm, 2008). A variety of different approaches to enhance intestinal immunity have been explored to reduce the incidence of poultry enteric disorders (Reid and Friendship, 2002; Callaway et al., 2008). Among these, direct-fed microbials (DFM), or probiotics, have shown promise as an alternative to in-feed antibiotics in reducing enteric diseases and eliminating subsequent contamination of poultry products. Although the manner by which DFM act remains to be clarified, they are thought to function by maintaining the presence of beneficial commensal microorganisms in the gut thereby providing an optimally balanced population among the diverse microfloral species (Reid and Friendship, 2002; Patterson and Burkholder, 2003; Dahiya et al., 2006; Callaway et al., 2008). Mechanistically, DFM influence the intestinal microbiota in multiple and diverse ways, including competitive exclusion of pathogenic bacteria, regulation of the local mucosal cell-mediated immune responses, increasing antibody production, promoting epithelial barrier integrity, reducing epithelial cell apoptosis, enhancing dendritic cell-induced T cell hyporesponsiveness, improving T cell homing to mesenteric lymph nodes, and augmenting toll-like receptor signaling (Ng et al., 2009).

The most common DFM are live bacteria or yeast used as feed supplements (Patterson and Burkholder, 2003). Bacteria frequently used as DFM in poultry pro-
duction include species of *Bacillus, Bifidobacterium, Enterococcus, Escherichia, Lactobacillus, Lactococcus,* and *Streptococcus.* Among these, nonpathogenic *Bacillus* spp. have been extensively studied and widely employed in many commercial applications (Hong et al., 2005). Spores of *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus clausii* have been used as DFM in animals and humans. Strains of *B. subtilis* have been selected as candidate DFM on the basis of their in vitro inhibitory effect on avian pathogenic *Escherichia coli* or *Clostridium perfringens* (Gebert et al., 2006). Supplementation with a *Bacillus*-based DFM was shown to improve feed conversion in poultry (Gebert et al., 2007) and pigs (Davis et al., 2008) and to beneficially alter the gastrointestinal microflora to reduce colonization by avian pathogenic *E. coli* and *C. perfringens* type A (Gebert et al., 2007). In addition, it has been proposed that *Bacillus*-based DFM enhance immune function and promote the synthesis of endogenous antimicrobial peptides in the gut (Hong et al., 2005). In spite of these reports, limited information exits concerning the mechanisms through which DFM influence host immunity in chickens. Therefore, this study was conducted to investigate the effects of selected DFM on the growth performance and gut morphology in broiler chickens and to correlate these effects with changes in various parameters of intestinal immunity that are associated with protection against infection by enteric pathogens.

**MATERIALS AND METHODS**

**DFM**

Eight individual *B. subtilis* strains, designated Bs2084, LSSAO1, 3AP4, Bs18, 15AP4, 22CP1, Bs27, and Bs278, and 1 multiple-strain DFM product, Avicorr (Danisco/Agtech Products Inc., Waukesha, WI) were used. These *Bacillus* strains were isolated from various sources including poultry litter, swine lagoon, rumen fluid, and other agricultural environments. Direct-fed microbials were selected based on their inhibitory effects on avian pathogenic *E. coli* or *C. perfringens* type A (Rehberg-er and Jordan-Parrott, 2005). Avicorr contains equal amounts of Bs2084, LSSAO1, and 15AP4; is generally recognized as safe by the US Food and Drug Administration; and is approved for feeding to animals by the Association of American Feed Control Officials.

**Birds, Diets, and Experimental Design**

Two hundred fifty 1-d-old broiler chickens hatched at Longenecker’s Hatchery (Elizabethtown, PA) were wing-banded upon arrival, weighed, and randomly placed in Petersime starter brooder units. At 1 and 2 d posthatch, 5.0 × 10⁶ cfu of DFM suspended in 0.5 mL of sterile distilled water were administered to chicks (n = 24/group) by oral gavage. Controls (n = 34) were given carrier alone dissolved in water. All chicks at 1 and 2 d were provided with nonmedicated mash base diets. Beginning at 3 d, chicks were fed ad libitum with nonmedicated mash base diets supplemented with 1.5 × 10⁵ cfu/g of DFM until the end of the experimental period. The control diet was formulated by mixing the base diet with carrier alone. At 15 d posthatch, all chickens were transferred to larger hanging cages. No adverse events on the chicks were observed throughout the 22-d experimental period. Body weights were measured at 7, 14, and 21 d. All experimental protocols were approved by the Small Animal Care Committee of the Beltsville Agricultural Research Center.

**Immunization with Recombinant Eimeria Profilin Protein**

To evaluate the effect of dietary DFM on an antibody response, 3 birds/group were immunized subcutaneously with 50 μg/mL of the 3-1E recombinant profilin coccidial protein (Lillehoj et al., 2005, 2007) in 0.1 mL of Freund’s complete adjuvant at 8 d and boosted with the equivalent amount in Freund’s incomplete adjuvant at 15 d (Figure 1).

**Collection of Samples**

Five birds per group at 7 d and 3 birds/group at 14 and 21 d posthatch were selected for collection of blood, spleen, and small intestine samples. Cervical dislocation was used to euthanatize the birds by the well-trained personnel as proposed by AVMA (2007). Immediately after euthanasia, blood was obtained by cardiac puncture, and the spleen and intestines were immediately removed. In addition, at 22 d, blood was drawn from 3 birds/group that had been immunized with the 3–1E protein. Spleens were processed to measure splenocyte proliferation on the day of euthanasia. A section of tissue from the duodenum to the ileum was used to measure intestinal intraepithelial lymphocyte (IEL) subpopulations, cytokine mRNA levels, and intestinal villus-crypt morphometry. For measurement of cytokine mRNA, sections approximately 20 cm long that were anterior and posterior to the diverticulum were taken at 14 d. The remainder of the intestine was used for IEL subpopulation measurements, except for those sampled at 21 d when 1.0 cm was taken for morphometry from the duodenum (midpoint of ascending duodenum), the jejunum (10 cm anterior to the diverticulum), and the ileum (10 cm posterior to the diverticulum).

**Villus and Crypt Morphometry**

Intestinal samples were fixed in 10% phosphate-buffered formalin for a minimum of 48 h, and 4.0-μm sections were prepared commercially (American Histolab Inc., Gaithersburg, MD). The sections were stained with standard hematoxylin-eosin solution and observed
for villus height and crypt depth at 100× magnification by light microscopy (CH30, Olympus, Tokyo, Japan) using a calibrated ocular micrometer. Ten microscopic fields per bird were measured.

**Spleen Lymphocyte Proliferation**

Spleen lymphocyte proliferation in response to medium alone (control), concanavalin A (ConA, 5.0 μg/mL; Sigma, St. Louis, MO), or Salmonella Typhimurium lipopolysaccharide (LPS, 5.0 μg/mL; Sigma) was measured by cellular incorporation of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5(2,4-disulfophenyl)-2H-tetrazolium as described by Lee et al. (2008). The stimulation index was calculated as the ratio of the mean optical density (OD450) value of mitogen-stimulated cells divided by the OD450 value of medium alone-stimulated cells.

**Intestinal IEL Subpopulations**

Intestinal sections between the duodenum and the ileum were excised, cut longitudinally, and washed with ice-cold Hanks’ balanced salt solution without calcium chloride and magnesium sulfate (Sigma). The IEL were isolated by density gradient centrifugation as described (Dalloul et al., 2002).

The IEL obtained were then analyzed using a FACSAria II flow cytometer (BD Biosciences, San Jose, CA). The cells were stained using monoclonal antibodies (mAb; USDA-Agricultural Research Service, Beltsville, MD) against the following surface markers: HB2 (human T cells, negative control), K55 (total chicken lymphocytes, positive control), K1 (chicken macrophages-thrombocytes), CD4 (chicken T helper lymphocytes), CD8 (chicken cytotoxic T lymphocytes), TCR1 (chicken $\gamma\delta$ T cell receptor), TCR2 (chicken $\alpha\beta$ TCR), CD3 (chicken T cells), and BU1 (chicken B cells). Lymphocyte subpopulations were expressed as the percentage of total lymphocytes stained with mAb K55.

**Cytokine mRNA Levels in Intestinal IEL**

Total RNA extraction, cDNA synthesis, and quantitative reverse transcription-PCR were performed as described (Hong et al., 2006a,b; Lee et al., 2008; Park et al., 2008). Standard curves were generated by using log10-diluted standard RNA, and levels of individual transcripts were normalized to those of glyceraldehyde 3-phosphate dehydrogenase analyzed by the Q-gene program (Hong et al., 2006a,b). The PCR primers for interferon (IFN)-α, IFN-γ, interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17α, tumor necrosis factor superfamity 15 (TNFSF15), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, negative control) are listed in Table 1.

**Chicken α-1-Acid Glycoprotein ELISA**

Chicken α-1-acid glycoprotein (α-1-AGP) in serum was measured by ELISA (Life Diagnostics Inc., West Chester, PA) according to the instructions of the manufacturer. The OD450 values were determined with an automated microplate reader (Bio-Rad, Richmond, CA).

**Measurement of Eimeria Profilin-Specific Antibody Responses**

An in-house ELISA was used to measure antibody levels against *Eimeria* profilin (3-1E) in sera collected at 22 d posthatch (7 d after secondary immunization; Lillehoj et al., 2005). Briefly, microtiter plates were coated overnight with 10 μg/well of purified recombinant 3-1E protein, washed with PBS containing 0.05% Tween 20, and blocked with PBS containing 1% BSA. Sera (100 μL/well) were incubated for 1 h at
room temperature with gentle agitation, the wells were washed, and bound antibody was detected with peroxidase-conjugated rabbit anti-chicken IgG (Sigma) and peroxidase-specific substrate. The OD₄₅₀ values were measured with a microplate reader (Bio-Rad).

**Statistical Analysis**

All data were subjected to 1-way ANOVA using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). Mean values of treatment groups were compared using Duncan's multiple range test with P < 0.05 considered statistically significant.

**RESULTS**

**Effect of DFM on Growth Performance and Gut Morphometry**

Chickens that were fed a diet containing any of the 9 DFM tested in this study did not statistically exhibit altered BW gains between 0 and 21 d posthatch compared with birds fed the nonsupplemented control diet (Table 2). Although not statistically significant, chickens fed a diet containing Bs27 tended to have the greatest BW gains among the treatment groups, followed by the LSSAO1 and 15AP4 groups, respectively. Birds on the 22CP1-supplemented diet had the least weight gain. Chickens fed diets supplemented with 15AP4 or Avicorr had greater duodenal villus height compared with birds given the control diet (Table 2). Villus height in the jejunum was significantly higher in birds fed diets supplemented with 3AP4, Bs18, 15AP4, 22CP1, Bs27, Bs278, or Avicorr compared with nonsupplemented controls. Ileum villus height was increased in birds fed diets supplemented with Bs27, Bs278, or Avicorr versus the control group. Increased crypt depth was observed in the duodenum of birds fed 15AP4, 22CP1, or Bs27; in the jejunum of chickens fed 22CP1 or Bs27; and in the ileum of birds fed with 15AP4 or Bs278 compared with the control group. On the other hand, crypt depth was significantly decreased in the jejunum of birds fed diets containing LSSAO1 as well as in the ileum of those given Bs2084, 3AP4, or Bs18 compared with controls. Ratios of villus height to crypt depth were decreased in the duodenum of birds fed Bs27 or 22CP1 and in the ileum of chickens fed 15AP4 but were increased in the jejunum of birds provided with LSSAO1 or 15AP4 and in the ileum of chickens fed Bs2084, 3AP4, Bs18, or Bs27 compared with controls.

**Effect of DFM on Serum α-1-AGP Levels**

In general, serum α-1-AGP concentrations increased between 7 and 21 d posthatch in the control and treatment groups (Table 3). However, the increase in α-1-AGP levels was attenuated in the DFM-fed groups compared with the control group. At 7 d posthatch, serum α-1-AGP levels were equal in the control and DEM birds. At 14 d, α-1-AGP levels were lower in chickens

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**Table 1. Oligonucleotide primers used for quantitative reverse transcription-PCR of chicken cytokines**

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<th>Type†</th>
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<th>Primer sequence²</th>
<th>PCR product size (bp)</th>
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<tr>
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</table>

¹Th = T helper cell.
²GAPDH = glyceraldehyde 3-phosphate dehydrogenase; IFN = interferon; IL = interleukin; TNFSF15 = tumor necrosis factor superfamily 15.
³F = forward primer; R = reverse primer.
fed Bs2084, LSSAO1, 3AP4, 15AP4, 22CP1, Bs27, or Bs278 but were higher in chickens fed Bs18 or Avicorr compared with controls. At 21 d, α-1-AGP levels were lower in chickens fed all of the DFM diets compared with the control group.

**Effect of DFM on Serum Antibody Response to Eimeria Profilin Protein**

The effect of DFM on humoral immunity was assessed by measuring serum antibody responses after immunization and boosting with an *Eimeria* profilin recombinant protein. As shown in Figure 2, only birds fed 15AP4 showed elevated profilin-specific antibody levels compared with the unmodified control diet.

**Effect of DFM on Spleen Lymphocyte Proliferation**

At 7 d posthatch, splenocyte proliferation induced by the T cell mitogen ConA was greater in chickens fed 15AP4, 22CP1, or Avicorr compared with controls and was greater after stimulation with the B cell mitogen LPS in the Bs2084, LSSAO1, 3AP4, Bs18, and Avicorr groups compared with nonsupplemented controls (Figure 3). On the contrary, significantly low LPS-induced splenocyte proliferation was seen in birds fed Bs27 compared with the nonsupplemented control group. At 14 d, spleen cell proliferation in response to ConA was decreased in birds given Bs2084 or LSSAO1 diets but increased after ConA stimulation in birds fed Avicorr and after LPS stimulation in birds fed Bs278.
or Avicorr. At 21 d, ConA-stimulated proliferation was increased in the 15AP4, Bs27, and Bs278 groups and decreased in the LSSAO1, Bs18, and 22CP1 groups, whereas LPS-stimulated proliferation was increased in the LSSAO1 group and decreased in the 3AP4, 22CP1, and Bs27 groups.

**Effect of DFM on Intestinal IEL Subpopulations**

At 7 d posthatch, TCR2\(^+\) IEL were undetectable in chickens fed the nonsupplemented diet but were detected in all of the DFM-fed groups, ranging from 5.1 to 17.7% of total IEL (Table 4). The K1\(^+\) and BU1\(^+\) IEL were relatively low. At this time, CD3\(^+\), CD4\(^+\), CD8\(^+\), and TCR1\(^+\) subpopulations also were increased in 3 or 4 of the 9 groups on DFM diets compared with the control group. Of note, chickens fed the Avicorr-supplemented diet exhibited 84.8% CD8\(^+\) cells compared with 14.8% in the control group, a 5.7-fold increase. At 14 d posthatch, CD8\(^+\) IEL remained greater than 50% of the total lymphocyte population in 7 of the 9 experimental groups (Bs2084, LSSAO1, Bs18, 15AP4, Bs27, Bs278, and Avicorr; Table 5). With the exception of the macrophage-thrombocyte K1\(^+\) subpopulation, the majority of changes observed in the CD4\(^+\), TCR1\(^+\), TCR2\(^+\), and BU1\(^+\) cells of DFM-fed chickens were decreased compared with birds given the control diet. The percentage of CD3\(^+\) IEL was high in Bs27 and Bs278 groups but low in LSSAO1, Bs18, and 22CP1 groups compared with the control group. At 21 d posthatch, all subpopulation changes that were induced by the supplemented diets were increased compared with controls and none of the subpopulations examined were decreased (Table 6). The 30.4-fold increase in CD4\(^+\) cells from 0.7% in the control group to 21.3% in the Bs27 group represented the greatest relative increase observed among all treatment groups at all time points examined. As an additional negative control, the percentage of cells stained by the human T cell-specific HB2 mAb was consistently less than 2% in all treatment groups (data not shown).

**Effect of DFM on Cytokine mRNA Levels in IEL**

The levels of mRNA encoding proinflammatory (IFN-\(\gamma\), IL-13, IL-6, IL-17\(\alpha\), TNFSF15), T helper (Th)-1 type (IFN-\(\gamma\), IL-2, IL-12), and Th-2 type (IL-4, IL-10, IL-13) cytokines were quantified by real-time reverse transcription-PCR in IEL at 14 d posthatch from chickens fed control or DFM-supplemented diets. For the proinflammatory cytokines (Figure 4), IFN-\(\gamma\) transcripts were increased by Bs2084, LSSAO1, 3AP4, and 22CP1 and were decreased by 15AP4 and Bs27. The IL-13 transcripts were increased by LSSAO1 and 3AP4 and were decreased by Bs27. The IL-6 transcripts were increased by LSSAO1, 22CP1, and Avicorr. The IL-17\(\alpha\) transcripts were increased by Avicorr, and TNFSF15 transcripts were increased by 3AP4, Bs278, and Avicorr. In particular, the 294-fold increase in IL-17\(\alpha\) transcripts in IEL from chickens fed Avicorr represented the greatest increase observed among all cytokines in all treatment groups. For the Th-1-type cytokines (Figure 5), IFN-\(\gamma\) mRNA were increased by 22CP1 and were decreased by all remaining DFM. The IL-2 mRNA were increased by 22CP1 and were decreased by Bs18, 15AP4, and Bs27. The IL-12 mRNA were increased by Bs278 and Avicorr. For the Th-2-type cytokines (Figure 6), IL-4 transcripts were increased by LSSAO1, 22CP1, and Avicorr; IL-10 transcripts were increased by LSSAO1, Bs18, and 22CP1; and IL-13 transcripts were increased by LSSAO1, 22CP1, and Avicorr. None of the Th-2 cytokines analyzed were decreased by any of the DFM diets.

**DISCUSSION**

This study was conducted to investigate the effects of 9 selected DFM on broiler chicken growth performance, gut villus and crypt morphometry, and local and systemic inflammation-immunity. Our results demonstrated that although BW gains were unaffected by any of the DFM, chickens that were fed 7 of the 9 experimental diets displayed increased villus height or crypt depth, or both, compared with control diet-fed birds. In addition, serum concentrations of the \(\alpha\)-1-AGP acute phase protein as an index of nonspecific inflammation were generally depressed in DFM-fed birds compared with birds on the control diet, especially at 14 and 21 d posthatch, although antibody responses after immunization with _Eimeria_ profilin protein as a measure of humoral immunity were, by large, unaffected by DFM diets, spleen cell proliferative responses to T and B cell
mitogens were increased or decreased compared with control diet birds. However, no consistent patterns of altered mitogen-induced proliferation were evident at 7, 14, and 21 d posthatch with any particular DFM. At 7 d posthatch, chickens fed diets containing 15AP4, Bs27, or Avicorr had a higher percentage of intestinal IEL expressing T cell surface markers (CD3, CD4, CD8, TCR1, TCR2), whereas most DFM decreased the percentage of IEL expressing the BU1 B cell marker at this time. Interestingly, cells expressing the K1 macrophage-thrombocyte marker were increased by Bs18, 15AP4, 22CP1, Bs27, and Avicorr at 14 d posthatch but were unaffected at 7 and 21 d. Finally, dietary DFM generally increased the levels of IEL transcripts for proinflammatory, Th-1-type, and Th-2-type cytokines, with the notable exception of IFN-γ, which was reduced by

![Figure 3](image-url)

Figure 3. Effect of dietary direct-fed microbials on spleen lymphocyte proliferation. Spleen lymphocytes obtained at 7 d (A, B), 14 d (C, D), or 21 d (E, F) posthatch were stimulated with concanavalin A (ConA) or lipopolysaccharide (LPS), and the stimulation index (SI) for each treatment was calculated as described in the Materials and Methods. Each bar represents the mean ± SD of triplicate samples. The asterisk (*) denotes significantly increased proliferation compared with the control group and the dagger (†) denotes significantly decreased proliferation compared with the control group (P < 0.05). Avicorr was provided by Danisco/Agtech Products Inc. (Waukesha, WI).
this study modulate a diverse set of physiologic and immune-related parameters in broiler chickens. Growth performance have been controversial. Although others have found no or minimal effect (Lee et al., 2008), others have found their effects to be beneficial (Zhang et al., 2005a, b; Nayebpor et al., 2007; Talebi et al., 2008). Our results tend to support the latter group of studies, although the increase in BW gain produced by the Bs27-containing diet (842 g vs. 760 g for the control) may require further dose-response and time course analyses to achieve statistical significance.

Because the modulation of gut microflora by dietary DFM in chickens is well-documented (Hariharan et al., 2004; Nava et al., 2005; Yang et al., 2009), the DFM used in this study were selected under the presumption that they act by decreasing the intestinal load of harmful bacteria, thereby promoting gut function, increasing immunity-mediated pathogen protection and nutrient absorption, and enhancing weight gain. In this regard, future studies using broilers fed with less digestible diets, reared under field conditions or exposed to common enteric infections, or both, may delineate a positive effect of dietary DFM on growth performance.

Given the paucity of published literature reports documenting the effects of DFM on immune responses in naïve broilers, the main focus of the current investigation was to ascertain the manner by which these

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| P-value   | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

1Each value represents the mean percentage of cells expressing the indicated surface marker compared with cells expressing the K55 pan lymphocyte marker from 5 birds/treatment group measured at 14 d posthatch.
2Danisco/Agtech Products Inc., Waukesha, WI.
*Significantly increased percentage of cells compared with the control group (P < 0.05).
†Significantly decreased percentage of cells compared with the control group (P < 0.05).
The antibody response of birds that had been immunized with the coccidial 3-1E protein while receiving a 15AP4-supplemented diet was significantly enhanced compared with nonsupplemented diets (Haghighi et al., 2005; Khajsefidi and Ghoorchi, 2006; Li et al., 2009). Increased humoral immunity in DFM-fed chickens has been attributed to increased proliferative and functional activities of antibody-producing B cells (Panda et al., 2008). In this study, we used Freund’s complete or incomplete adjuvant for primary and secondary immunization with the coccidial 3-1E protein. Although Freund’s adjuvant has been used in recombinant Eimeria vaccine (Subramanian et al., 2008), subtle effects derived from the DFM might be masked because Freund may induce strong immune response. In this regard, a less active adjuvant such as aluminum hydroxide (Asif et al., 2004) would be more appropriate to see, if any, the immune adjuvant role of DFM in poultry.

Cell proliferation is an important indicator of lymphocyte function in DFM-fed chickens (Erickson and Hubbard, 2000; Brisbin et al., 2008a). Dietary DFM exhibited strain-specific effects on both B and T cell mitosis (Kirjavainen et al., 1999), whereas other investigators reported no effect of DFM on proliferation (Koenen et al., 2004; Roller et al., 2004). According to in vitro studies, DFM components stimulated splenocyte proliferation (Amrouche et al., 2006) or altered gene expression profiles (Brisbin et al., 2008b). In the current study, single DFM strains as well as the Avicor 3 component mixture affected both T and B cell responses. The underlying mechanism by which dietary DFM influence splenocyte proliferation has been postulated to involve “cross-talk” between the bacterial cells and the host immune system (Koenen et al., 2004; Corthesy et al., 2007).

Intestinal IEL subpopulations expressing macrophage, T cell, or B cell surface markers were measured on a weekly basis to assess whether dietary DFM affected development of the local immune system in broiler chicken. Intraepithelial lymphocytes are chosen because they constitute the primary immune effector cells in the gut.

### Table 6. Effects of direct-fed microbials on intestinal intraepithelial lymphocyte subpopulations at 21 d posthatch

<table>
<thead>
<tr>
<th>Treatment</th>
<th>K1</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>TCR1</th>
<th>TCR2</th>
<th>BU1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.2</td>
<td>12.8</td>
<td>0.7</td>
<td>13.7</td>
<td>10.0</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Bs2084</td>
<td>0.0</td>
<td>15.6</td>
<td>4.1*</td>
<td>19.2</td>
<td>9.1</td>
<td>5.0</td>
<td>7.1</td>
</tr>
<tr>
<td>LSSAO1</td>
<td>0.0</td>
<td>33.6*</td>
<td>2.9</td>
<td>21.3</td>
<td>36.4*</td>
<td>16.9*</td>
<td>8.4</td>
</tr>
<tr>
<td>3AP4</td>
<td>0.0</td>
<td>50.9*</td>
<td>5.1*</td>
<td>44.7*</td>
<td>23.8*</td>
<td>18.6*</td>
<td>12.0</td>
</tr>
<tr>
<td>Bs18</td>
<td>0.6</td>
<td>41.6*</td>
<td>12.4*</td>
<td>22.6</td>
<td>22.4*</td>
<td>10.5</td>
<td>16.3*</td>
</tr>
<tr>
<td>15AP4</td>
<td>0.0</td>
<td>35.4*</td>
<td>0.9</td>
<td>25.8*</td>
<td>25.8*</td>
<td>30.8*</td>
<td>28.4*</td>
</tr>
<tr>
<td>22FC1</td>
<td>0.4</td>
<td>47.9*</td>
<td>12.9*</td>
<td>56.8*</td>
<td>23.7*</td>
<td>46.0*</td>
<td>26.5*</td>
</tr>
<tr>
<td>Bs27</td>
<td>0.0</td>
<td>71.4*</td>
<td>21.3*</td>
<td>60.5*</td>
<td>14.2</td>
<td>27.0*</td>
<td>22.9*</td>
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<tr>
<td>Avicor2</td>
<td>0.4</td>
<td>46.9*</td>
<td>6.6*</td>
<td>31.1*</td>
<td>20.4*</td>
<td>34.9*</td>
<td>9.4</td>
</tr>
<tr>
<td>Pooled SEM</td>
<td>0.01</td>
<td>1.362</td>
<td>0.296</td>
<td>1.160</td>
<td>0.692</td>
<td>0.811</td>
<td>0.600</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Significantly increased percentage of cells compared with the control group ($P < 0.05$).
and play a critical role in eliciting protective immunity to enteric pathogens (Lillehoj et al., 2004). Among the DFM examined, 15AP4, Bs27, Bs278, and Avicorr were most effective in enhancing the percentage of IEL T cell subpopulations, particularly CD8+ cells. These results complement our earlier report demonstrating increased CD3+, CD4+, CD8+, and TCR2+ IEL subpopulations in broiler chickens fed a Lactobacillus-supplemented diet compared with birds given a DFM-free diet (Dalloul et al., 2003). Similarly, Novak et al. (2007) reported that early application of Lactobacillus brevis to turkey poults had the potential to enhance immune development in both the intestine and peripheral blood mononuclear cells. Although IEL percentages are changed by DFM compared with controls, these changes were unlikely to reflect alterations in total IEL numbers based upon the results of our previous probiotic studies (Dalloul et al., 2003; Lee et al., 2007a,b). Stimulation of specific IEL subsets by DFM likely contributes to increased host resistance to enteric pathogens that would otherwise cause clinical disease (Lillehoj and Trout, 1996). Indeed, Lee et al. (2007a,b) demonstrated such an effect in the context of experimental avian coccidiosis using Pediococcus- or Saccharomyces-based DFM. Given that young chicks are particularly susceptible to infection by opportunistic pathogens due to their immature immune system (Lowenthal et al., 1994; Koenen et al., 2002), application of dietary DFM at an early age would appear to provide the optimal effect on enhancing immune competence.

Figure 4. Effects of dietary direct-fed microbials (DFM) on proinflammatory cytokine mRNA levels. Chickens were fed diets with or without DFM, intestinal intraepithelial lymphocytes were isolated at 14 d posthatch, and transcripts for the indicated cytokines were quantified by real-time reverse transcription-PCR and normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts. Each bar represents mean ± SD of triplicate samples. The asterisk (*) denotes significantly increased mRNA levels compared with the control group and the dagger (†) denotes significantly decreased mRNA levels compared with the control group (P < 0.05). Avicorr was provided by Danisco/Agtech Products Inc. (Waukesha, WI). IFN-α = interferon-α; IL = interleukin; TNFSF-15 = tumor necrosis factor superfamily 15.
Figure 5. Effects of dietary direct-fed microbials (DFM) on T helper 1-type cytokine mRNA levels. Chickens were fed diets with or without DFM, intestinal intraepithelial lymphocytes were isolated at 14 d posthatch, and transcripts for the indicated cytokines were quantified by real-time reverse transcription-PCR and normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts. Each bar represents mean ± SD of triplicate samples. The asterisk (*) denotes significantly increased mRNA levels compared with the control group and the dagger (†) denotes significantly decreased mRNA levels compared with the control group (P < 0.05). Avicorr was provided by Danisco/Agtech Products Inc. (Waukesha, WI). IFN-γ = interferon-γ; IL = interleukin.

Figure 6. Effects of dietary direct-fed microbials (DFM) on T helper 2-type cytokine mRNA levels. Chickens were fed diets with or without DFM, intestinal intraepithelial lymphocytes were isolated at 14 d posthatch, and transcripts for the indicated cytokines were quantified by real-time reverse transcription-PCR and normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts. Each bar represents mean ± SD of triplicate samples. The asterisk (*) denotes significantly increased mRNA levels compared with the control group (P < 0.05). Avicorr was provided by Danisco/Agtech Products Inc. (Waukesha, WI). IL = interleukin.
Based on the fact that IEL subpopulations were altered by DFM feeding, it is not surprising that markedly altered levels of transcripts encoding proinflammatory, Th-1-type, and Th-2-type cytokines also were detected in these cells. Cytokine mRNA levels were increased, decreased, or unchanged compared with controls depending on the DFM used. Chichlowski et al. (2007) reported that chicks fed a diet supplemented with Lactobacillus casei, Lactobacillus acidophilus, Bifidobacterium thermophilum, and Enterococcus faecium (PrimaLac, Star Labs Inc., Clarksdale, MO) for 21 d exhibited decreased intestinal mRNA levels of the proinflammatory cytokine IL-6 and increased expression of the antiinflammatory cytokine IL-10. On the other hand, another proinflammatory cytokine, IL-13, was not affected by the DFM diet. The authors stated, however, that the significance of these alterations was not clear due to the small sample size employed in their study. Repression of IFN-γ and IL-12 expression in the chicken gut was reported to be associated with DFM-mediated reduction in intestinal colonization by Salmonella enterica serovar Typhimurium (Haghighi et al., 2008). By contrast, Fuijiwara et al. (2009) observed no statistically significant differences in the expression of IFN-γ, IL-3, and IL-4 when birds were fed diets with or without Bacillus subtilis-fermented soybean. In a mouse study by Huang et al. (2008), dietary DFM increased IL-6 and tumor necrosis factor-α (TNF-α) in spleen and mesenteric lymph nodes.

Cytokines are immunoregulatory peptides with relatively small molecular weights that participate in innate and adaptive immune responses. Interleukin-2 (IL-2) is a Th-1-associated cytokine that plays a central role in adaptive immunity. Interleukin-12 (IL-12) is an important cytokine required for the initiation and regulation of cellular immunity through the differentiation of naive T cells into Th-1 cells, which is crucial for host resistance to many microbial pathogens (Park et al., 2008). Interferon-γ (IFN-γ) regulates acquired immunity by activating lymphocytes and enhancing the expression of MHC class II antigens. In addition, IFN-γ is a common marker of cellular immunity and high levels have been correlated with protective immune responses to coccidial infections (Lee et al., 2008). Members of the tumor necrosis factor superfamily play crucial roles in both innate and adaptive immunity, including inflammation, apoptosis, and cell proliferation (Kaiser and Stäheli, 2008). Interleukin-17α (IL-17α) has been implicated in host defense against bacteria, parasites, and viruses including Mycoplasma pneumoniae, Toxoplasma gondii, human immunodeficiency virus, and Eimeria (Min and Lillegard, 2002). Therefore, because Bacillus-based DFM affected the expression of numerous IEL cytokines, it can be expected that they would also influence a diverse array of immune functions.

In conclusion, 8 Bacillus spp. and 1 commercial DFM preparation were tested as in-feed supplements for their effect on BW gain, intestinal villus-crypt length-depth, and various parameters of innate and adaptive immuno-

ty. Although growth performance was not statistically significant among the different dietary groups and most failed to affect serum antibody levels after immunization with a recombinant Eimeria protein, altered intestinal morphometric measurements were readily apparent in DFM-fed chickens compared with controls. In addition, levels of serum acute phase protein α-1-AGP were reduced in DFM-fed birds, whereas mitogen-induced splenic lymphocyte proliferation, intestine IEL subpopulations, and cytokine mRNA levels in IEL were increased, decreased, or unchanged compared with controls depending on the DFM used. Based on these results, further studies are needed to determine whether Bacillus-based DFM can augment protective immunity against enteric pathogens in chickens and, if so, whether this protection is induced by alterations in particular intestinal lymphocyte subpopulations or cytokine expression profiles, or both.

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