The effect of microbial-nutrient interaction on the immune system of young chicks after early probiotic and organic acid administration

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ABSTRACT: The combined effects of probiotics (Lactobacillus acidophilus, Lactobacillus casei, Streptococcus faecium, and Saccharomyces cerevisiae) and organic acids (sorbic and citric acid) on intestinal morphology and expression of immune-related genes were investigated. One-day-old chicks were randomly assigned to 1 of 3 groups: birds not receiving probiotic or organic acids (control; T1), or birds receiving an oral combination (1 g/L in water) of 10⁸ CFU/g of each of the aforementioned probiotics and organic acids (1% sorbic acid and 0.2% citric acid) for 7 (T2) or 14 d (T3). Each group was divided into 5 replicate pens of 20 birds each, and 5 birds from each group (1 from each pen) were killed on d 11 and 22. Intestinal sections were collected for histological assessment, and reverse transcriptase-PCR analysis was used to assess defensin and cathelicidins expression. Quantitative real-time PCR was used to assess toll-like receptors (TLR) and cytokine expression. Duodenal villus height was greater in T2 and T3 at d 11 (P ≤ 0.036) and 22 (P ≤ 0.015) compared with T1. At d 11, duodenal goblet cell/unit area was less in T3, whereas it was greater in T2 compared with T1 in the jejunum (P = 0.009). Ileal goblet cell/unit area was greater in T3 at d 22 compared with T1 (P < 0.001). Avian beta-defensin-3 was expressed in all tissues except the bursa of T3 birds at d 11, and TLR-2 was down regulated in the cecal tonsil of birds in T2 and T3 at d 11 compared with T1 (P = 0.020 and 0.003, respectively). Expression of IL-12p35 in the ileum at d 11 was down regulated in T2 and T3 compared with T1 (P = 0.030 and 0.012, respectively). Reduced expression of INF-γ was observed in the ileum in T3 compared with T1 at d 11 (P = 0.047). Ileal IL-6 and IL-10 and cecal tonsil interferon-gamma (INF-γ) expressions were greater T2 at d 22 (P ≤ 0.047) than T1. In conclusion, supplementation of combined probiotics and organic acids resulted in inconsistent gut morphology associated responses, and avian beta-defensins and cathelicidins expression were not associated with combined probiotics and organic acids supplementation. Birds supplemented with combined probiotics and organic acids for 7 d showing similar responses in TLR-2, IL-12p35, and IFN-γ compared with those supplemented for 14 d indicates that shorter periods of supplementation might be enough to elicit beneficial responses.

Key words: chickens, defensins, gut health, organic acids, probiotics, toll-like receptors

INTRODUCTION

The first 3 wk of life are a crucial time for gut maturation, and healthy birds perform at maximum potential without diverting energy towards immune response processes (Klasing, 2007; Yegani and Korver, 2008; Yin et al., 2010). Gut morphology affects nutri-
ent absorption, and greater villi length and crypt depth are associated with functional ability (Stokes et al., 2001; Yang et al., 2007; Choct, 2009).

Commensal bacteria live in harmony with and benefit the host in a variety of ways, including organic acid production and immunomodulation (Tse and Chadee, 1991). Organic acids inhibit pathogenic bacteria growth by disrupting bacteria cell membrane transport, preventing the bacteria from reaching equilibrium with their environment (Cherrington et al., 1991). Probiotics, beneficial microbial cultures, may be administered to stimulate the local immune system (Fuller, 1989; Gibson and Roberfroid, 1995; Netherwood et al., 1999) and enhance epithelial innate immunity-related gene expression through anti-inflammatory effects and reduced pro-inflammatory cytokine expression such as IL-6 (Revolledo et al., 2006; Amit-Romach et al., 2010; Pagnini et al., 2010). Furthermore, with the presence of microorganisms in the gut, innate immune system associated germ line-encoded pathogen-pattern recognition receptors called toll-like receptors (TLR) may induce expression of various pro-inflammatory cytokines (such as IL-6) and antimicrobial peptides (such as defensins), which are direct effector molecules of the innate immune response (Birchler et al., 2001; Ganz, 2003; Kaiser, 2010).

Therefore, we studied the combined effects of probiotics (a blend of Lactobacillus acidophilus, Lactobacillus casei, Streptococcus faecium, and Saccharomyces cerevisiae) and organic acids (sorbic and citric acids) on intestinal morphology and innate immunological responses of chickens.

**MATERIALS AND METHODS**

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Protocol Management and Review Committee, and chickens were handled according to the guidelines described by the Canadian Council on Animal Care (CCAC, 1993).

**Animal Trial**

A total of 300 Ross-308 chicks were obtained from a commercial hatchery (Carlton Hatchery, Grunthal, MB, Canada) and were divided into 3 groups of 100 chicks (20 birds/pen and 5 replications/treatment) in a randomized complete block design with the pen as the experimental unit, and location within the facility as a blocking factor. All birds were fed a standard corn-wheat-soybean meal diet (Table 1). Feed and water were provided ad libitum.

The 15 pens were randomly assigned to the 3 treatments where group 1 received only the basal diet (Control, T1) and group 2 received an oral blend of probiotic (108 CFU/g each of Lactobacillus acidophilus, Lactobacillus casei, Streptococcus faecium and Saccharomyces cerevisiae) and organic acids (1% sorbic acid and 0.2% citric acid; Alltech Inc., Nicholasville, KY) for 7 d (T2). The third group received a combination of the same treatments as T2 but for 14 rather than 7 d (T3). The probiotics and organic acids combination were provided via drinking troughs at a dosage of 1 g/L of water starting d 1 where chlorinated city water was used. On d 11 (4 d after stopping of treatment for T2 group) and d 22, 5 birds from each group (1 from each pen) were randomly chosen and killed by cervical dislocation, and intestinal sections (duodenum, jejunum, and ileum) and cecal tonsils were collected for histological and gene expression analysis. Sampling periods were cho-

<table>
<thead>
<tr>
<th>Item</th>
<th>Starter, d 1 to 14</th>
<th>Grower, d 15 to 22</th>
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<tbody>
<tr>
<td>Ingredient, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn</td>
<td>15.00</td>
<td>15.00</td>
</tr>
<tr>
<td>Wheat</td>
<td>41.59</td>
<td>47.70</td>
</tr>
<tr>
<td>Soybean meal, 46% CP</td>
<td>21.60</td>
<td>14.35</td>
</tr>
<tr>
<td>Porcine meat meal</td>
<td>6.55</td>
<td>5.80</td>
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<tr>
<td>Canola meal</td>
<td>6.00</td>
<td>6.00</td>
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<tr>
<td>Mineral/vitamin premix1</td>
<td>0.18</td>
<td>1.73</td>
</tr>
<tr>
<td>Animal/vegetable fat</td>
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<td>1.70</td>
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<tr>
<td>Broiler Micro HY2</td>
<td>0.50</td>
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<td>Limestone</td>
<td>0.53</td>
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<tr>
<td>Salt</td>
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<td>0.19</td>
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<tr>
<td>Biolyx3</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>ExtraPro4</td>
<td>6.00</td>
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<td>Sodium bicarbonate</td>
<td>0.10</td>
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<tr>
<td>DL-Met</td>
<td>0.15</td>
<td>0.11</td>
</tr>
<tr>
<td>Monensin premix</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td>Calculated nutrient analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME, kcal/kg</td>
<td>3,120.00</td>
<td>3,180.00</td>
</tr>
<tr>
<td>CP, %</td>
<td>23.39</td>
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<td>Fat, %</td>
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<td>Fiber, %</td>
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<td>3.67</td>
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<tr>
<td>Ca, %</td>
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<td>0.87</td>
</tr>
<tr>
<td>P, %</td>
<td>0.70</td>
<td>0.65</td>
</tr>
<tr>
<td>Na, %</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>Lys, %</td>
<td>1.31</td>
<td>1.15</td>
</tr>
</tbody>
</table>

1Provided per kilogram of diet: vitamin E, 20 IU; Thr, 17 mg; choline chloride, 100 mg; Cu as CuSO4, 43 mg; 9050 IU of vitamin A, 3000 IU of vitamin D3, 0.30 mg of Se as sodium selenite, 99.8 mg of Mn as manganese oxide, 79.7 mg of Zn as zinc oxide, 29.9 mg of Fe as ferrous sulfate, and 1.00 mg of I as calcium iodate

2Provided per kilogram of diet: organic trace minerals containing 20 mg of Zn as zinc oxide, 1.51 mg of Cu as copper sulfate, 20 mg of Mn as manganese oxide, and 0.09 mg of Se (Bioplex, Alltech Inc, ON, Canada), and 69 μg of 25-hydroxycholecalciferol HY-D (DSM, Nutritional Products Canada Inc, ON, Canada).

3Contains 50.7% L-Lys (Evonik Degussa Canada Inc., Burlington, ON, Canada).

4A blend of full fat canola and pulses extruded (Regina, SK, Canada).
sen on the basis of the immune system development status of birds, where the first collection was chosen to observe the response of a developing immune system to the treatments and the second collection was chosen to observe the effect of treatments on a developed immune system of birds (Bar-Shira et al., 2003).

**Histomorphological Studies**

The entire small intestine was rapidly removed and approximately 5 cm of intestinal sections from the duodenum (after the duodenal loop), jejunum (middle small intestine), and ileum (5 cm before the cecal tonsils) were collected. Sections were dehydrated, cleared, and embedded in paraffin where they were initially fixed in 10% phosphate-buffered formalin for at least 24 h, transferred to 50% ethanol solution for 15 min, and immersed in 70% ethanol until further processing. Formalin-fixed samples were embedded in paraffin, and 5-μm sections were sliced and stained with haematoxylin, eosin, and Alcian blue periodic acid-Schiff. The samples were analyzed under standard light microscope with special emphasis on gut architecture as described previously (Brady et al., 2010).

Histological characterization and enteric morphometric analysis were completed considering the following variables: villus height, crypt depth, and number of goblet cells to determine gut health status. Morphometric indices were determined using computer-aided light microscope image analysis as described by Bird et al. (1994). Sections from 5 birds per treatment per group were placed on each slide. A total of 5 digital images were taken from each tissue using a microscope equipped with digital camera (AxioCam ICc3, Carl Zeiss, Jena, Germany). From each slide, 5 areas were chosen, and 10 crypts were evaluated for crypt depth at each of these areas. Images were quantified (Imagej, US National Institute of Health, Bethesda, MD).

### Quantitative Real-Time PCR

Tissue samples from the ileum and cecal tonsil were used for total RNA isolation and cDNA synthesis as explained before. Real-time quantitative PCR (qRT-PCR) was performed for TLR-2, IL-12p35, interferon-gamma (IFN-γ), IL-6, IL-10, and β-actin using a thermocycler (StepOne; Applied Biosystems, Mississauga, ON, Canada) on a 48-well plate using a 25-μL total reaction volume as described by Pfaffl and Hageleit (2001). Primers (Table 2) for genes of interest were designed as described above, and primer concentrations for the target and housekeeping genes were adjusted according to the concentration of the standard curve for a gene target or housekeeping gene.

#### Table 2. Pairs of primers used for reverse transcriptase polymerase chain reaction and quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (3′-5′)</th>
<th>Accession number</th>
<th>Annealing temperature, °C</th>
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</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>AACACAGTGCTGTCTGGTGTA</td>
<td>TCGTACTCTGCTGTCTGATCC</td>
<td>NW_001486319</td>
<td>61</td>
</tr>
<tr>
<td>AvBD-3</td>
<td>ATGGGCGATGCGTCGACTCCTGCTC</td>
<td>AGGGAGGAGGTAATGGGG</td>
<td>NC_006090</td>
<td>58</td>
</tr>
<tr>
<td>CTHLB-1</td>
<td>CATGAGGTAGCTTACCTGAGTCG</td>
<td>CAGTGAAGTCAGGCCACCTG</td>
<td>AB307733</td>
<td>65</td>
</tr>
<tr>
<td>AvBD-6</td>
<td>AGGAGGAGGTTGCGAGGTTGC</td>
<td>GTCATGCTAGGAGTGGC</td>
<td>NC_006090</td>
<td>58</td>
</tr>
<tr>
<td>TLR-2</td>
<td>CGCTTGGAGGAGCAATCTGGAA</td>
<td>AGGCTATTTGAGAGTGTCAGAAT</td>
<td>NM_204278</td>
<td>59</td>
</tr>
<tr>
<td>IL-12p35</td>
<td>CAGCGAGACGACAGGAGGAGG</td>
<td>CCAGCTCGCTGATGCA</td>
<td>NC_006090</td>
<td>64</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>CTGAAAGACTGGAGACAGAG</td>
<td>CACGGCTCTGATAAGATGC</td>
<td>NC_006127</td>
<td>60</td>
</tr>
<tr>
<td>IL-6</td>
<td>CAGGAGGAGGAGCAAGAA</td>
<td>TAGCADAGAGACTCGAGT</td>
<td>NC_006089</td>
<td>59</td>
</tr>
<tr>
<td>IL-10</td>
<td>AGGAGGAGGAGGAGGAGGAGG</td>
<td>ATCCAGAGTACTCGAGGATT</td>
<td>NC_006113</td>
<td>55</td>
</tr>
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β-actin = beta actin; AvBD-3 = avian beta defensin 3; CTHLB-1 = cathelicidin 1; AvBD-6 = avian beta defensin 6; TLR-2 = toll like receptor-2; and IFN-γ = interferon gamma.
genes (Power SYBR Green PCR Master Mix containing AmpliTaq Gold DNA polymerase LD, SYBR Green I dye, and dNTPs with dTTP/dUTP; Applied Biosystems). The thermal cycling protocol consisted of an initial denaturation at 95°C for 10 min, followed by amplification for 40 cycles at 95°C for 10 s, annealing as described in Table 2 for each of the primer pairs, and elongation at 72°C for 10 s. The specificity of amplification for each product was determined by melting curve analysis at 95°C for 1 s and 65°C for 15 s. This was followed by progressive rising of the temperature to 95°C. The plates were then cooled at 40°C for 30 s. Alongside each qRT-PCR assay, a 10^-2 dilution of DNA plasmid encoding related genes and an empty plasmid medium were run to serve as a calibrator and a negative control, respectively.

**Calculations and Statistical Analysis**

Mean relative expression of triplicates of each gene were calculated on the basis of the expression of the housekeeping gene, β-actin, using Pfaffl’s formula as described by Parviz et al. (2009). Quantification of β-actin and gene of interest expression was estimated (Step One software; Applied Biosystems) from 5 birds per treatment and 3 technical replications per animal for qRT-PCR. Exact amplification efficiencies of target and reference genes were verified separately before normalizing the expression of the target gene to that of the housekeeping gene, and efficiency was calculated as E = 10^-1/slope of standard curve. With the gene of interest as the target and β-actin as the reference, the relative expression ratio was determined as follows:

$$R = \frac{(E_{target})^{ΔCP_{target} (Calibrator–Sample)}}{(E_{ref})^{ΔCP_{ref} (Calibrator–Sample)}}$$

where $E_{target}$ and $E_{ref}$ are the efficiencies of the target gene and β-actin, respectively, and $ΔCP$ is the difference of crossing points between calibrator and samples. The relative expression ratios, $R$, were used to determine differences in gene expression among different groups.

Performance, histological measures, and relative expression of each gene at each time point were analyzed as a randomized complete block design using the MIXED procedure (SAS Inst. Inc., Cary, NC) with the fixed effects of block and treatment and random effect of animal. Significance of the differences among means was assessed by the Scheffe test, and $P < 0.05$ was defined as the level of significance.

### RESULTS

The results of performance data are presented for d 0 to 7, 7 to 14, and 14 to 21 in Table 3. No significant difference was observed among treatments with regards to feed intake, BW, and feed efficiency. Histological observations indicated that probiotic and organic acid treatments had a mixed effect on intestinal villus height, crypt depth, and goblet cell numbers. Duodenal villus height was greater in both T2 and T3 compared with T1 at d 11 ($P = 0.022$ and 0.036, respectively) and 22 ($P = 0.015$ and 0.031, respectively; Figure 1A). Jejunal villus height was lower in birds receiving T2 and T3 on d 11 compared with T1 ($P = 0.003$ and 0.006, respectively), while no difference was observed among treatments at d 22 ($P = 0.675$). Ileal villus height was not affected by either T2 or T3 at both d 11 and 22. Duodenal crypt depth was not affected by treatment at both time points, but jejunal crypt depth was reduced ($P = 0.018$) in T2 at d 11 compared with T1 and T3, while no difference among treatments was observed at d 22 (Figure 1B). Only birds in T3 had greater ($P = 0.045$) ileal crypt depth at d 22 compared with control. Finally, duodenal goblet cell numbers were less ($P = 0.009$) in T3 at d 11 compared with T1 and T3, while no difference among treatments was observed at d 22 (Figure 1B).

### Table 3. Performance of broilers fed a blend of combination of probiotics and organic acids

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment^2</th>
<th>SEM^3</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADG</td>
<td>T1</td>
<td>18.2</td>
<td>20.2</td>
</tr>
<tr>
<td>ADFI</td>
<td>T2</td>
<td>21.5</td>
<td>22.3</td>
</tr>
<tr>
<td>G:F</td>
<td>T3</td>
<td>0.85</td>
<td>0.90</td>
</tr>
</tbody>
</table>

^1 Blend of probiotics (10^8 CFU/g each of *Lactobacillus acidophilus, Lactobacillus casei, Streptococcus faecium*, and *Saccharomyces cerevisiae*) and organic acids (1% sorbic acid and 0.2% citric acid; Alltech Inc., Nicholasville, KY). Values are means of 5 replicate pens per treatment with 20 broilers/pen.

^2 Treatments: T1 = birds that did not receive probiotics or organic acids (Control); T2 = birds received blend of probiotics and organic acids for 7 d, and T3 = birds received blend of probiotics and organic acid for 14 d.

^3 Pooled SEM.
The results of gene expression profiling by RT-PCR showed that at the first day post-hatch, only AvBD-3 and \( \beta \)-actin were expressed in all the tissue, while CTHLB-1 and AvBD-6 were not expressed in any of the tissues. The same pattern was observed at d 11 and 22 for all the genes for T1, T2, and T3, except for the expression of CTHLB-1, which showed a positive response in the bursa in all treatments at both sampling days. Furthermore, AvBD-3 showed a negative response at d 11 in T3 in the bursa (Table 4).

Toll-like receptor-2 expression in the ileum showed no difference among treatments on both d 11 and 22. However, TLR-2 down regulation was observed in both T2 and T3 compared with T1 on d 11 in the cecal tonsil (\( P = 0.020 \) and 0.003, respectively; Figure 2A). Expression of IL-12p35 in the ileum at d 11 was down-regulated in T2 and T3 compared with T1 (\( P = 0.030 \) and 0.012, respectively), while no difference was observed in the cecal tonsil at d 11 and in both tissues at d 22 (Figure 2B). Lower expression of INF-\( \gamma \) was observed in the ileum in T3 compared with T1 at d 11 (\( P = 0.012 \)), whereas no difference was observed among treatments at d 22.
Cecal tonsil expression of IFN-γ showed no difference at d 11, while an up-regulation of IFN-γ was observed in T2 compared with T1 and T3 at d 22 (P = 0.017 and 0.046, respectively; Figure 2C). Expression of IL-6 and IL-10 followed the same patterns, where, except in the ileum in T2 at d 22 (P = 0.022 and 0.019, respectively), all treatments in both time points showed no difference in both the ileum and cecal tonsil (Figure 3).

**DISCUSSION**

The overall health of the gastrointestinal tract in terms of digestion and absorption of nutrients, as well as defense against pathogens, depends on the cross-talk among diet, intestinal microbiota, gut architecture, and the gut-associated immune system (Patterson and Burkholder, 2003). No differences was observed among treatments in the current study in terms of ADFI, BW, and G:F. These findings seem to agree with that of Vicente et al. (2007) where supplementation of probiotics and organic acids in combination in 24 commercial chicken houses with 459,277 1-d-old broiler chickens did not result in different ADG and F:G. The results of the histomorphological assessment indicated that combining probiotics and organic acid supplementation improved duodenal villus height in both time points without any improvement on other intestinal regions. Previous studies using organic acids as feed additives in poultry have reported different findings. Adil et al. (2006) reported that different levels of organic acid supplementation (i.e., fumaric acid and lactic acid), increased ileal villus height in boilers, while Smulikowska et al. (2010) reported reductions in villus height and crypt depth as a result of supplementing broiler diets with organic acids (lactic, formic, and citric acids). Lack of any apparent change on duodenal, jejunal, and ileal crypt depth has also been reported in Cobb straight run commercial broiler chicks supplemented with organic acids (Adil et
Supplementation of the probiotic *Pediococcus acidilactici* has been reported to have increased duodenal and ileal villus height in treated birds compared with non-treated birds (Awad et al., 2006, 2009; Taheri et al., 2010). Dietary inclusion of live microbial feed additives (*Lactobacillus salivarius* and *Lactobacillus reuteri*) in broiler diets has also been shown to increase growth performance and intestinal nutrient absorption by improving intestinal architecture (Awad et al., 2010). In the current study, combined probiotic (a blend of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Streptococcus faecium*, and *Saccharomyces cerevisiae*) and organic acid (a blend of sorbic and citric acids) supplementation resulted in variable responses of intestinal histomorphology at both time points and intestinal locations. Whether these inconsistencies originate from the differences in the dose and type of probiotics or organic acids or both used and treatment duration needs further investigation.

One of the beneficial effects of probiotics is the stimulation of the local immune system against colonization by pathogenic microbes (Lan et al., 2005). Changes in mucin profile in response to bacterial colonization, as a function of goblet cell numbers, have been suggested to play a role against pathogenic invasion of the intestinal mucosa during early development (Forder et al., 2007). The current study showed greater goblet cell numbers in the ileum of birds treated for 14 d at d 22. Chichlowski et al. (2007a) reported that administration of probiotic mixtures containing *Lactobacilli*, *Bifidobacteria*, and *Enterococci* to broiler chicks resulted in a larger quantity of mucosal-adherent bacteria in the small intestine accompanied by a greater number of ileal goblet cells and larger mucous layer. In another study by Kum et al. (2010), broilers supplemented with organic acid had increased goblet cell counts and villus height at 21 and 42 d post treatment. The findings of the current study are also consistent with a previous report where probiotic treatment was associated with an increase in the number of goblet cells in mice at d 14 and 21 post-treatment (McClemens et al., 2010).

Defensins, a subset of antimicrobial peptides, are cysteine-rich peptides with broad spectrum antimicrobial activity against bacteria, fungi, protozoa, and enveloped viruses (Zasloff, 2002). Epithelial cells synthesize AvBD-3, which plays a role in the uterine innate immunity in the hen (Ahmad et al., 2011). In the present study, AvBD-3 was expressed in all tissues except in the bursa of Fabricius of birds receiving T3 at d11, while AvBD-6 was not detected at all, irrespective of treatment. Van Dijk et al. (2008) have reported that there was no detected AvBD3 and AvBD6 in the crop, proventriculus, and small intestine, while a strong AvBD3 was detected in the bursa of Fabricius. In another study, there were detectable AvBD6 and cathelicidin in the cecal tonsil of birds, with greater expression of both in birds infected with *Salmonella enterica* Serovar Typhimurium (Akbari et al., 2008). The absence of AvBD6 and cathelicidin gene expression in the current study can be explained by the association of expression of these genes during pathogenic infection and inflammation caused by infections (Mendez and Findley, 2007). Other studies (Wehkamp et al., 2004; Schlee et al., 2008) have reported that probiotics are able to induce antimicrobial peptide expression by host cells.

Toll-like receptors, also known as pathogen recognition receptors, are part of the innate immune system. These receptors recognize molecular patterns of pathogens called pathogen-associated molecular patterns, causing a chain reaction and stimulating the immune system to react (Aderem and Ulevitch, 2000). Greater expression of TLR-2 was found in the cecal tonsil of control birds at d 11 compared with birds receiving a combination of probiotics and organic acids, irrespective of treatment length. Greater TLR-2 expression in the cecal tonsil of non-treated birds as compared with the treated birds could be attributed to the bactericidal effect of organic acids, which control and limit the growth and colonization of numerous pathogenic and non-pathogenic bacteria in the gut (Hinton and Linton, 1988), and, thereby, reduce the immune response in the cecal tonsil.

Cytokines play a central role in immune response and maintenance of tissue integrity. Probiotics may offer protection against pathogenic infection via different mechanisms, including modulation of cytokine responses (Rakoff-Nahoum et al., 2004). Interleukin-12p35 was strongly expressed in the ileum of non-treated birds compared with treated birds irrespective of treatment duration at d 11. An up regulation of IL-12p35 has been reported in cecal tonsils on d 1 and 5 post exposure to *Salmonella* (Haghighi et al., 2008). In the present study, slightly greater expression of IFN-γ was observed at d 22 in the cecal tonsil of birds treated for 7 d compared with those that were treated for 14 d or to the control group. Probiotic effects have been reported to be more associated with changes in cytokine expression, particularly IFN-γ and IL-12 in the gut associated lymphoid tissues of the chicken, and correlate with protection against colonization with *Salmonella* Serovar Typhimurium (Haghighi et al., 2008). Interleukin-6 and IL-10 have been reported to be expressed in the cecal tonsils of broiler chicks challenged with different doses of *Salmonella enterica* Serovar Typhimurium at different time points (Haghighi et al., 2008). We found greater expression of IL-6 and IL-10 in the ileum of birds treated for 7 d compared with those that were treated for 14 d or those that received no treatment. Another study showed that in chicks fed probiotics for 21 d, there was a decreased and increased concentration of IL-6 and IL-10, respec-
tively (Chichlowski et al., 2007b). We propose that the presence of organic acids in some way could enhance a pro-inflammatory response. However, the balance between pro- and anti-inflammatory responses has been maintained in the current study.

Stimulatory effects of probiotics on the local immune system and bactericidal effects of organic acids are believed to be stronger during the first few days of life, when the birds are germ-free, compared with the later stages of life when the gastrointestinal tract is colonized by beneficial bacterial species (Barnes, 1972; Friedman et al., 2003). However, the results of the present study indicate that combined application of probiotics and organic acids led to treatment duration- or tissue-dependent expression differences or both among different immune related genes.

In conclusion, supplementation of a combined probiotic and organic acids to broiler diets resulted in inconsistent gut morphology associated responses where their effects were more apparent in the duodenum and ileum when the gut is fully developed. When considering the immune response, combination of probiotics and organic acids are capable of altering TLR-2 and cytokine profiles. They were able to down regulate cecal tonsil TLR-2, ileal IL-12p35, and IFN-γ at d 11 and up regulate cecal tonsil IFN-γ and ileal IL-6 and IL-10 at d 22. The down regulation of the cytokines implies that combined probiotics and organic acids supplementation support anti-inflammatory effect via Th-2 associated pathways involving cytokines such as IL-10. Furthermore, TLR-2, IL-12p35, and IFN-γ responses in birds supplemented with combined probiotics and organic acids for 7 d followed the same trend to those supplemented for 14 d, indicating that shorter periods of supplementation might be enough to elicit beneficial responses.

**LITERATURE CITED**


