Introduction

Prebiotics are nondigestible food ingredients that stimulate the growth of beneficial bacteria in the gut of animals. The major prebiotics of interest are fructooligosaccharide and mannan-oligosaccharide. Fructooligosaccharide and mannan-oligosaccharide have been successfully applied to increase broiler BW gain, feed efficiency, and gut microbiota population (Kim et al., 2011). Prebiotics, such as fructo-oligosaccharide, alter the gut microbiota population to facilitate the growth of lactobacillus and bifidobacteria (Jung et al., 2008). Inulin increased the digestibility of CP and fat and improved production performance in broiler birds (Alzueta et al., 2010).

Prebiotics are being heavily promoted as an alternative to antibiotics because prebiotics decrease pathogen infection in the gut (Baurhoo et al., 2007b). Several potential mechanisms through which prebiotics act to exclude enteric pathogens have been proposed, including competition for receptor sites, competition for limiting nutrients, and production of antimicrobial metabolites (Vandeplas et al., 2010). Prebiotics, by reducing the pathogen load in the gut, modify immune parameters in the host (Robeरfroid et al., 2010). Though several articles have reported studies on the effect of prebiotic supplementation on systemic immune function, understanding of the effect of prebiotic supplementation on local immune organs in the gut, such as gut-associated lymphoid tissue, is lacking (Roller et al., 2004).

Regulatory T cells (Tregs) are a subset of T cells that specialize in immune suppression. Regulatory T cells protect the host from excessive immune responses and maintain self tolerance and mucosal tolerance (Workman et al., 2009). Immune cells produce proinflammatory cytokines, such as IL-1, which increase immune cell migration to the site of infection and activity of other immune cells. On the other hand, Tregs produce antiinflammatory cytokines, such as IL-10, to dampen immune responses. An immune response is a balance between Tregs and other immune cells. Prebiotics have been shown to alter gut Treg population in humans and mice (Round and Mazmanian, 2010). Our laboratory recently identified and characterized Tregs in chickens (Shanmugasundaram and Selvaraj, 2011).

ABSTRACT Two experiments were conducted to study the effect of CitriStim, a commercial killed whole yeast cell prebiotic, on broiler performance, regulatory T cells, CD4+ and CD8+ percentages, and IL-10 and IL-1 mRNA contents of the spleen and cecal tonsils. No immune challenges were imposed in either of the 2 experiments. One-day-old broiler chicks were fed a corn- and soybean meal-based diet supplemented with 0, 0.1, or 0.2% CitriStim (ADM, Decatur, IL) for 35 d. At 21 (P = 0.03) and 35 d (P = 0.02) of age, CitriStim supplementation at 0.2% increased regulatory T cell percentage in the cecal tonsil compared with that of the 0% CitriStim-supplemented group. At 21 (P = 0.08) and 35 d (P = 0.01) of age, CitriStim supplementation at 0.2% increased IL-10 mRNA content of the cecal tonsil compared with that of the 0% CitriStim-supplemented group. At 21 (P = 0.13) and 35 d (P < 0.01) of age, CitriStim supplementation at 0.2% decreased IL-1 mRNA content compared with that of the 0% CitriStim-supplemented group. CitriStim supplementation did not (P > 0.05) alter the IL-10 and IL-1 mRNA contents in the spleen. CitriStim supplementation did not (P > 0.05) alter the CD4+ and CD8+ cell percentages in the spleen and cecal tonsil at 21 and 35 d of the experiment. CitriStim supplementation increased regulatory T cell percentage and IL-10 mRNA content and decreased IL-1 mRNA content in the cecal tonsil to produce a net antiinflammatory milieu. The immunomodulatory effect of CitriStim supplementation was a local effect rather than a systemic effect.

Key words: prebiotic, whole yeast cell, CitriStim, immunity

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Whole yeast cells or some yeast cell wall products are a good source of mannan-oligosaccharides and, hence, are considered prebiotics (Kim et al., 2011). Yeast cell wall by-products are studied extensively as prebiotics in several species, such as mice, humans (de Vrese and Schrezenmeir, 2008), and chickens (Jacobs and Parsons, 2009). CitriStim (ADM, Decatur, IL) is a commercial killed whole yeast cell that is considered to be a good source of mannan-oligosaccharide, β-glucan, d-mannose, and α methyl-d-mannoside. CitriStim contains a proprietary mixture of partially fermented yeast (Pichia guilliermondii), which is leftover following citric acid extraction from the yeast culture. The product provides the whole yeast cell and all of its components. The present experiment was conducted to study the effects of CitriStim supplementation on broiler production performance, Treg properties, and other immune cell parameters in the cecal tonsil and spleen.

MATERIALS AND METHODS

Two experiments were conducted to study the effect of CitriStim supplementation on broiler production performance and immune cell parameters. All animal protocols were approved by the Ohio Agricultural Research and Development Center Animal Care and Use Committee.

Experiment 1

Experiment 1 was conducted to study the effects of CitriStim supplementation on broiler BW gain and feed efficiency. In total, 90 one-day-old chicks (Ross × Ross; Orrville Chick Hatchery, Orrville, OH) were randomly distributed to 1 of the 3 dietary treatments (0, 0.1, and 0.2% CitriStim). Each treatment was replicated in 5 pens of 6 chicks per replication (n = 5). The basal diet was based on corn and soybean meal (Table 1). Feed intake and BW were measured at weekly intervals until 5 wk of age.

Experiment 2

Experiment 2 was conducted to study the effects of CitriStim supplementation on broiler immune parameters. In total, 72 one-day-old chicks were randomly distributed to 1 of the 3 dietary treatments (0, 0.1, and 0.2% CitriStim). Each treatment was replicated in 4 pens of 6 chicks per replication (n = 4). Dietary treatments were similar to experiment 1. Cecal tonsils and spleens were collected from 2 birds per pen on d 21 and 35. One of the spleens and one of the cecal tonsils were frozen immediately with liquid nitrogen and stored at −80°C until further analysis. One spleen and one cecal tonsil were used for CD4+ CD8+, and Treg analysis.

RNA Isolation and Real-Time PCR

The total RNA was collected from the spleens and cecal tonsils and reverse transcribed into complementary DNA (Selvaraj and Klasing, 2006). The mRNA was analyzed for IL-10 (5′-CATGCTGCTGGCCCTGAA-3′ and 5′-CTTCTTGTATCGTTAGT-3′; Rothwell et al., 2004) and IL-1β (5′-TCTCCAGCCAGAAAGTGA-3′ and 5′-CAGCGGTTAGAAGATTGAAGC-3′) mRNA by real-time PCR (iCycler, Bio-Rad, Hercules, CA) using SyBr green (Qiagen, Valencia, CA) after normalizing for β-actin mRNA (5′-ACCGGACTGGTTACCAACACC-3′ and 5′-GACGTGTGCTGACACCTTCA-3′; Shannugasundaram and Selvaraj, 2010). The annealing temperature for IL-10 was 55°C, for IL-1β it was 60°C, and for β-actin it was 57°C. Fold change from the reference was calculated (Selvaraj et al., 2010) as ES (CT sample)/ER (CT reference), where ES and ER are the sample and reference PCR amplification efficiencies, respectively, as determined by a LinRegPCR program (Ramakers et al., 2003), and C T is the threshold cycle. The C T was determined by the iQ5 software (Biorad) when the fluorescence rose exponentially 2-fold times above background. The reference group was the group that had the lowest expression of that particular cytokine.

Sampling for CD4+, CD8+, and Treg Analysis

Single cell suspensions of the cecal tonsils or spleen were concentrated for mononuclear cells by density centrifugation at 400 × g for 20 min over Histopaque (1.077 g/mL; Sigma Aldrich, St. Louis, MO). Production and phycoerythrin-linking of mouse anti-chicken CD25+ were conducted as described earlier (Shannugasundaram and Selvaraj, 2010). Approximately 1 × 10⁶ cells were incubated with 10 μg/mL of primary phycoerythrin-linked mouse anti-chicken CD25+, 1:200 dilution of fluorescein isothiocyanate conjugated mouse anti-chicken CD4 (Southern Biotech, Birmingham, AL) or 1:200 fluorescein isothiocyanate conjugated mouse anti-chicken CD8 (Southern Biotech), and 1:200 unlabelled mouse IgG for 45 min. Unbound primary antibodies were removed by centrifugation at 400 × g for 20 min. The percentages of CD4+, CD8+, and CD4+CD25+ cells (Tregs) in cecal tonsils and the spleen were analyzed by flow cytometry (Guava EasyCyte, Millipore, Billerica, MA). The CD4+ and CD8+...
cells were expressed as a percentage of mononuclear cells. The percentage of Tregs was expressed as a percentage of CD4+ cells to facilitate comparison between samples.

**Statistical Analysis**

A one-way ANOVA (JMP software, Cary, NC) was used to examine the effect of CitriStim supplementation on dependent variables. When main effects were significant ($P < 0.05$), differences between means were analyzed by Tukey’s least squares means comparison.

**RESULTS AND DISCUSSION**

**Experiment 1**

Final BW ($P = 0.54$), feed intake ($P = 0.89$), and feed efficiency ($P = 0.56$) were not significantly affected by the treatment diets at both the 21 and 35 d intervals (Table 2). Earlier studies with a different yeast wall product did not alter broiler production performance (Jacobs and Parsons, 2009).

**Experiment 2 Treg, CD4+, and CD8+ Cell Percentages**

At both 21 ($P = 0.03$) and 35 d ($P = 0.02$) of age, CitriStim supplementation at 0.2% increased Treg percentage in the cecal tonsil compared with that in the 0% CitriStim-supplemented group (Figure 1). In chickens, mucosal immune organs, such as lung and cecal tonsils, have a higher percentage of Tregs than do other immune organs, such as the spleen, bursa, or thymus (Shanmugasundaram and Selvaraj, 2011). Such a high number of Tregs in mucosal immunology reflects the importance of Tregs in maintaining mucosal tolerance in an animal. The gut is the organ through which the body encounters exogenous antigens. Tregs regulate the local immune response to avoid an immune response against food antigens and gut microbiota (Izcue et al., 2006). Though CitriStim supplementation at 0.2% increased the Treg percentage in the cecal tonsil, the Treg percentage was not altered in the spleen. The spleen is a systemic immune organ; thus, alteration in Treg percentage in the cecal tonsil but not in the spleen clearly suggests that the immune effect of CitriStim is a local effect rather than a systemic effect.

Compared with the control group, CitriStim supplementation did not ($P > 0.05$) alter the CD4+ and CD8+ cell percentages in the spleen and the cecal tonsil at both 21 and 35 d of the experiment. The mean cecal tonsil percentage of CD4 was 14.3, 13.9, and 14.7% at 21 d and 13.3, 15.2, and 15.3% at 35 d of age in birds fed 0, 0.1, and 0.2% CitriStim, respectively. The mean cecal tonsil percentage of CD8 was 28.8, 27.4, and 33.3% at 21 d and 24.8, 20.7, and 24.8% at 35 d of age in birds fed 0, 0.1, and 0.2% CitriStim, respectively.

**IL-10 mRNA**

At both 21 ($P = 0.08$) and 35 d ($P = 0.01$) of age, CitriStim supplementation at 0.2% increased IL-10 mRNA content of the cecal tonsil compared with that of the 0% CitriStim-supplemented group (Figure 2). At 35 d of age, birds fed 0.2% CitriStim had a 9-fold higher IL-10 mRNA content than the 0% CitriStim-fed

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**Table 2. Body weight and feed consumption of broiler birds fed experimental diets (n = 5)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0</th>
<th>0.10</th>
<th>0.20</th>
<th>SEM</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 d</td>
<td>688</td>
<td>749</td>
<td>722</td>
<td>45</td>
<td>0.63</td>
</tr>
<tr>
<td>35 d</td>
<td>1,861</td>
<td>1,941</td>
<td>1,963</td>
<td>66</td>
<td>0.54</td>
</tr>
<tr>
<td>Feed consumption (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–21 d</td>
<td>981</td>
<td>1,043</td>
<td>999</td>
<td>55</td>
<td>0.72</td>
</tr>
<tr>
<td>0–35 d</td>
<td>2,805</td>
<td>2,891</td>
<td>2,891</td>
<td>146</td>
<td>0.89</td>
</tr>
<tr>
<td>Feed efficiency</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–21 d</td>
<td>1.54</td>
<td>1.48</td>
<td>1.51</td>
<td>0.04</td>
<td>0.27</td>
</tr>
<tr>
<td>0–35 d</td>
<td>1.55</td>
<td>1.52</td>
<td>1.53</td>
<td>0.04</td>
<td>0.56</td>
</tr>
</tbody>
</table>

$^1$CitriStim (ADM, Decatur, IL) is a commercial killed whole yeast cell prebiotic.
group. The IL-10 is an antiinflammatory cytokine that is produced by several immune cells, including Tregs (Sabat, 2010). Chicken Tregs have 29-fold higher IL-10 mRNA content than do T cells (Shanmugasundaram and Selvaraj, 2011). The Treg immunosuppressive effects are through IL-10; silencing IL-10 mRNA in human Tregs reverses their suppressive properties (Sun et al., 2010). CitriStim supplementation increased Treg percentage in the cecal tonsil. The increase in IL-10 mRNA content of the cecal tonsil by CitriStim likely occurs through increased Treg percentage.

Both IL-10 and Tregs play a major role in immune tolerance and in maintaining mucosal integrity (Lan et al., 2007). The Treg percentage in gut-associated lymphoid tissue is highly dynamic, and several factors (Lan et al., 2007; Feng et al., 2011) are involved in altering Treg percentage in the gut. One important factor that can alter Treg numbers in humans is probiotic Lactobacillus bacteria (Smits et al., 2005). In chickens, mannan-oligosaccharide supplementation increases the load of Lactobacillus (Baurhoo et al., 2007a). The Treg percentage in gut-associated lymphoid tissue is very sensitive to gut microbiota. In the absence of infection, Tregs are present in high numbers in the gut, which facilitate immune tolerance. In the presence of pathogenic bacteria, an inflammatory response mediated by Th17 cells predominates (Ivanov et al., 2009). Some gut microbes have evolved to escape host immune regulation by upregulating Treg population in the host. For example, Bacteroides fragilis, a human commensal bacteria, directs the development of Tregs through an epitope (Round and Mazmanian, 2010). Thus, CitriStim could have an effect on Tregs and IL-10 by altering the gut microbial population. Increased Treg percentage and IL-10 mRNA content strongly suggest that CitriStim stimulates an antiinflammatory milieu. Compared with the control group, CitriStim supplementation did not \( (P > 0.05) \) alter the IL-10 mRNA content in the spleen at both 21 and 35 d of the experiment.

**IL-1 mRNA**

CitriStim supplementation at 0.2% decreased IL-1 mRNA content compared with that of the supplemented group at 21 d of age \( (P = 0.13) \), and significantly so at 35 d \( (P < 0.01) \) of age (Figure 3). At 35 d of age, the amount of IL-1 mRNA in birds fed 0.2% CitriStim was 0.04% of that of the 0% CitriStim-fed group. The IL-1 is a proinflammatory cytokine. An immune response is a balance between antiinflammatory and proinflammatory responses. The ultimate goal of the immune system is to eliminate the pathogen with minimal damage to the host, and the immune system achieves this goal by using a unique combination of different components of the immune systems. The immune system has evolved to include several inherent balances and counterbalances that regulate it. Proinflammatory cytokines, although essential to eliminate the pathogen, will increase the nutritional cost of the immune response (Klasing, 1988; Klasing, 1998). As a prebiotic, CitriStim might increase the load of beneficial bacteria and decrease the load of pathogenic bacteria, which will act to remove the proinflammatory immune signals and decrease IL-1 production. Compared with the control group, CitriStim supplementation did not \( (P > 0.05) \) alter the IL-1 mRNA content in the spleen at both 21 and 35 d of the experiment.

In this experiment, with no immune challenge, CitriStim supplementation did not alter broiler performance. Preliminary trials produced similar results (data not shown). CitriStim supplementation increased Treg percentage and IL-10 mRNA content and decreased IL-1 mRNA content in the cecal tonsil to produce a net an-
ti inflammatory milieu. The immunomodulatory effect of CitriStim supplementation was a local effect rather than a systemic effect, as CitriStim supplementation did not alter any of the immune parameters studied in the spleen. Further studies with CitriStim supplementation in a pathogen challenge model need to be conducted to identify the immunomodulatory effects of CitriStim supplementation. Earlier studies with mannan-oligosaccharides decreased Salmonella colonization in chicks (Fernandez et al., 2002) and in pigs challenged with Salmonella (Burkey et al., 2004).

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REFERENCES