INTRODUCTION

Dietary ingredients have a profound effect on the composition of the gut microflora, which in turn, regulates the physiology of metazoans (Fraune and Bosch, 2010). As such, nutritional components of the diet are of critical importance not only for meeting the nutrient requirements of the host, but also for the microbiome (Salzman, 2011). During their coevolution, bacterial microbiota has established multiple mechanisms to influence the eukaryotic host, generally in a beneficial fashion (Kau et al., 2011). The microbiome encrypts a variety of metabolic functions that complement the physiology of their hosts (yegani and Korver, 2008; Musso et al., 2010; Maslowski and Mackay, 2011). The capacity to ferment complex polysaccharides to short-chain fatty acids by intestinal microflora has a profound effect on energy homeostasis, providing as much as ~70% of energy in ruminants and 20 to 30% for several omnivorous animals (Walter et al., 2011). In exchange, their hosts have organs that enable microbial fermentation of nondigestible foodstuff (Fuller and Brooker, 1974), revealing a symbiotic evolution over time, as indicated by concurrent phylogenetic trees (Dale and Moran, 2006; McFall-Ngai, 2007; Moran, 2007).

Over a century ago Eli Metchnikoff (1907) proposed the revolutionary idea to consume viable bacteria to promote health by modulating the intestinal microflora. The idea is more applicable now than ever because bacterial antimicrobial resistance has become a serious worldwide problem in medicine and agriculture (Mathew et al., 2007; Carter et al., 2009; Sherman et al., 2009). The impending ban of antibiotics in animal feed due to the current concern over the spread of antibiotic resistance genes make a compelling case for developing alternative prophylactics (Haghighi et al., 2005; Dominguez-Bello and Blaser, 2008; MacDonald and Bell, 2010). During the last 15 yr, our laboratories have worked toward the identification of probiotic candidates for use in poultry. Extensive research resulted in the development of FloraMax-B11 (FM), a

ABSTRACT Two independent trials were conducted in the present study to evaluate the effect of 5% glycerol supplementation combined with dietary FloraMax-B11 (FM) against Salmonella Enteritidis colonization in neonate broiler chickens. In each trial, 60 chicks were randomly assigned into 4 groups. Group 1 received a control diet. Group 2 received a control diet supplemented with 5% glycerol. Group 3 received a control diet supplemented with FM, and group 4 received a control diet supplemented with 5% glycerol and FM. At placement, chickens were challenged with Salmonella Enteritidis at 10^4 cfu/bird. In each trial, 12 chicks were humanely killed 72 h postchallenge, respectively, for Salmonella Enteritidis colonization. Supplementation of 5% glycerol or FM by themselves, showed no significant effect on Salmonella Enteritidis recovery or incidence when compared with control nontreated chicks in both trials. However, no detectable Salmonella Enteritidis was observed in the chickens that received the supplementation of 5% glycerol combined with FM in both trials. Further studies are in progress in older birds to substantiate these findings.

Key words: broiler chicken, glycerol, probiotic culture, Salmonella Enteritidis, Salmonella recovery

Research Note

Glycerol supplementation enhances the protective effect of dietary FloraMax-B11 against Salmonella Enteritidis colonization in neonate broiler chickens

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defined lactic acid bacteria base probiotic culture that has demonstrated accelerated development of normal microflora in chickens and turkeys, providing increased resistance to Salmonella spp. infections (Farnell et al., 2006; Wolfenden et al., 2007a,b; Vicente et al., 2007, 2008; Higgins et al., 2007, 2008, 2010, 2011; Menconi et al., 2011; Tellez et al., 2012). Published experimental and commercial studies have shown that these selected probiotic organisms are able to reduce idiopathic diarrhoea in commercial turkey brooding houses (Higgins et al., 2005). Large-scale commercial trials indicated that appropriate administration of this probiotic mixture to turkeys and chickens increased performance and reduced costs of production (Torres-Rodriguez et al., 2008; Higgins et al., 2007, 2008, 2010, 2011; Menconi et al., 2005; Large-scale commercial trials indicated that appropriate administration of this probiotic mixture to turkeys and chickens increased performance and reduced costs of production (Torres-Rodriguez et al., 2008; Higgins et al., 2007, 2008, 2010, 2011; Menconi et al., 2005; Vicente et al., 2007, 2008a,b; Vicente et al., 2007, 2008a,b). Therefore, the purpose of the present study was to evaluate the effect of 5% glycerol supplementation combined with dietary FM against Salmonella Enteritidis colonization in neonate broiler chickens.

MATERIALS AND METHODS

Probiotic Culture

FloraMax-B11 (Pacific Vet Group USA Inc., Fayetteville, AR) is a probiotic culture derived from poultry, consisting of 2 strains of lactic acid bacteria isolates: Lactobacillus salivarius and Pediococcus parvulus of poultry gastrointestinal origin.

Bird Source and Diets

Day-of-hatch, off-sex broiler chickens were obtained from Cobb-Vantress (Siloam Springs, AR) and were placed in isolators, in a controlled age-appropriate environment. Chicks were provided ad libitum access to water and a balanced unmedicated corn-soybean diet meeting the nutrition requirements of poultry recommended by NRC (1994). The common starter diet was a typical corn-soybean meal diet (chemical analysis of nutrients is presented in Table 1). The diet with glycerol (catalog no. BDH 1172–4LP, VWR, Philadelphia, PA) or FM was similar to the common starter diet but was supplemented with 5% glycerol, FM, or the combination of both. One bottle of the dried commercially available FM was added and mixed to the starter diets at a concentration of 10^4 cfu/g of feed. Concentrations of FM were confirmed by serial dilution and further plating on de Man, Rogosa and Sharpe (catalog no. 89226–116, VWR) for enumeration of actual cfu in the mixed diets, respectively. All animal handling procedures were in compliance with the Institutional Animal Care and Use Committee at the University of Arkansas. A small number of chicks (n = 12) for each trial were humanely killed upon arrival, and ceca-cecal tonsils (CCT) were aseptically removed, individually cultured in tetrathionate enrichment broth (Tet, catalog no. 210420, Becton Dickinson, Sparks, MD), and confirmed negative for Salmonella by plating the samples on Xylose Lysine Tergitol-4 (XLT-4, catalog no. 223410, BD Difco, Sparks, MD) selective media.

Bacterial Strain and Culture Conditions

The challenge organism used in all experiments was a poultry isolate of Salmonella enterica subspecies enterica serovar Enteritidis, bacteriophage type 13A, obtained from the USDA National Veterinary Services Laboratory, Ames, IA. This isolate was resistant to 25 μg/mL of novobiocin (NO, catalog no. N-1628, Sigma) and was selected for resistance to 20 μg/mL of nalidixic acid (NA, catalog no. N-4382, Sigma) in our laboratory. For the present studies, 100 μL of Salmonella enterica strain, also known as S. enterica serovar Enteritidis, was used as the challenge organism.

Table 1. Composition of the starter diet for broiler chickens (kg)

<table>
<thead>
<tr>
<th>Item</th>
<th>Glycerol free</th>
<th>Glycerol 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn</td>
<td>551.5</td>
<td>510.7</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>372.57</td>
<td>372.57</td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>35.57</td>
<td>26.41</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>15.99</td>
<td>15.99</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>14.57</td>
<td>14.57</td>
</tr>
<tr>
<td>Salt</td>
<td>3.57</td>
<td>3.57</td>
</tr>
<tr>
<td>Vit. B12</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td>20,000,000 IU</td>
<td>6,000,000 IU</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>75,000 IU</td>
<td>10,000 IU</td>
</tr>
<tr>
<td>Vitamin K3</td>
<td>10 g</td>
<td>10 g</td>
</tr>
<tr>
<td>thiamine</td>
<td>5 g</td>
<td>5 g</td>
</tr>
<tr>
<td>riboflavin</td>
<td>8 g</td>
<td>8 g</td>
</tr>
<tr>
<td>pantothenic acid</td>
<td>60 g</td>
<td>60 g</td>
</tr>
<tr>
<td>pyridoxine</td>
<td>5 g</td>
<td>5 g</td>
</tr>
<tr>
<td>folic acid</td>
<td>2 g</td>
<td>2 g</td>
</tr>
<tr>
<td>biotin</td>
<td>0.2 g</td>
<td>0.2 g</td>
</tr>
<tr>
<td>cyanocobalamin</td>
<td>10 g</td>
<td>10 g</td>
</tr>
<tr>
<td>and ascorbic acid</td>
<td>200 g</td>
<td>200 g</td>
</tr>
</tbody>
</table>

1Glycerol supply, 4,200 kcal/kg.
2Vitamin premix supplied the following per kilogram: vitamin A, 20,000,000 IU; vitamin D3, 6,000,000 IU; vitamin E, 75,000 IU; vitamin K3, 9 g; thiamine, 3 g; riboflavin, 8 g; pantothenic acid, 18 g; niacin, 60 g; pyridoxine, 5 g; folic acid, 2 g; biotin, 0.2 g; cyanocobalamin, 16 mg; and ascorbic acid, 200 g.
3Mineral premix supplied the following per kilogram: manganese, 120 g; zinc, 100 g; iron, 120 g; copper, 10 to 15 g; iodine, 0.7 g; selenium, 0.4 g; and cobalt, 0.2 g.
4Ethoxyquin.
**Experimental Design**

Two independent trials were conducted. In each trial, 60 d old, off-sex broiler chicks were obtained, randomly assigned into 4 groups (n = 15 chickens), and placed in isolators with no restricted access to feed and water. Group 1 received a control diet. Group 2 received a control diet supplemented with 5% glycerol. Group 3 received a control diet supplemented with FM (10^4 cfu/g of feed), and group 4 received a control diet supplemented with 5% glycerol and FM (10^4 cfu/g of feed). At placement, chickens were challenged with *Salmonella Enteritidis* at 10^4 cfu/bird. In each trial, 12 chicks were humanely killed 72 h postchallenge for *Salmonella Enteritidis* recovery in cecal tonsils and *Salmonella* incidence (positive birds/total birds) as explained below.

**Salmonella Recovery**

In both trials, chickens were humanely killed by CO₂ asphyxiation; CCT were aseptically removed from culture and enumerated *Salmonella*. Cecal contents were homogenized and diluted with saline (1:4, wt/vol), and 10-fold dilutions were plated on BGA with NO and NA and incubated at 37°C for 24 h to enumerate total *Salmonella* cfu. Later, the cecal samples were enriched in double-strength Tet and further incubated at 37°C for 24 h. Following this, enrichment samples were plated on BGA with NO and NA and incubated at 37°C for 24 h to confirm presence/absence of typical lactose-negative colonies of *Salmonella*.

**Statistical Analysis**

In both experiments, log₁₀ *Salmonella Enteritidis* cecal contents were subjected to ANOVA as a completely randomized design, using the GLM procedure of SAS (SAS Institute Inc., Cary, NC). Significant differences among the means were determined by Duncan’s multiple-range test at P < 0.05. The enrichment data were expressed as positive/total chickens (%) and the percent recovery of *Salmonella Enteritidis* was compared using the chi-squared test of independence, testing all possible combinations to determine the significance (P ≤ 0.05; Zar, 1984).

**RESULTS**

The results of the effect of 5% glycerol supplementation combined with dietary FM against *Salmonella Enteritidis* colonization in broiler chickens of trial 1 and trial 2 are shown in Table 2. The supplementation of 5% glycerol or FM by themselves showed no significant effect on *Salmonella Enteritidis* recovery or incidence when compared with chickens from the control group in both trials. However, in both trials, following 72 h postchallenge, no detectable *Salmonella Enteritidis* was observed in the recovery or enrichment samples of the chickens that received supplementation of 5% glycerol combined with FM (Table 2).

**DISCUSSION**

Interactions between dietary composition and the microbiome have a profound impact on both vertebrates and invertebrates (Dethlefsen et al., 2007; Neish, 2009; Sharma et al., 2010). Glycerol is an important intermediate in several metabolic pathways of metazoans (Brisson et al., 2001). Approximately 10% of fat used for biodiesel production results in glycerol (Dasari et al., 2010). The results of the effect of 5% glycerol supplementation combined with dietary FM against *Salmonella Enteritidis* colonization in broiler chickens are shown in Table 2.

**Table 2. Effect of 5% glycerol supplementation combined with dietary FloraMax-B11 (FM) against *Salmonella Enteritidis* colonization in broiler chickens**

<table>
<thead>
<tr>
<th>Item</th>
<th>Log₁₀ <em>Salmonella Enteritidis</em> cecal contents</th>
<th>Ceca-cecal tonsils</th>
<th>Log₁₀ <em>Salmonella Enteritidis</em> cecal contents</th>
<th>Ceca-cecal tonsils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>1.54 ± 0.46^a</td>
<td>6/12 (50)^a</td>
<td>1.74 ± 0.80^a</td>
<td>7/12 (58.3)^a</td>
</tr>
<tr>
<td>Control diet plus 5% glycerol</td>
<td>1.25 ± 0.58^a</td>
<td>5/12 (41.6)^a</td>
<td>1.33 ± 0.65^a</td>
<td>6/12 (50)^a</td>
</tr>
<tr>
<td>Control diet plus FM</td>
<td>1.19 ± 0.78^a</td>
<td>5/12 (50)^a</td>
<td>1.24 ± 0.62^a</td>
<td>5/12 (50)^a</td>
</tr>
<tr>
<td>Control diet plus 5% glycerol and FM</td>
<td>0.0 ± 0.0^b</td>
<td>0/0 (0.0)^b</td>
<td>0.0 ± 0.0^b</td>
<td>0/0 (0.0)^b</td>
</tr>
</tbody>
</table>

^a,bDifferent superscripts within columns indicate significant differences, P < 0.05; n = 12/group.

^1At placement, all chickens were challenged with *Salmonella Enteritidis* at 10^4 cfu/bird. Chicks were humanely killed 72 h postchallenge. Ceca-cecal tonsils (CCT) were cultured to enumerate log₁₀ *Salmonella Enteritidis*/g of ceca content, and the data are expressed as mean ± SEM. The CCT enrichment data are expressed as positive/total chickens for each tissue sampled (%).
A live microbial food supplement that benefits the host (MacDonald and Bell, 2010). A probiotic is defined as enteric pathogens (Dominguez-Bello and Blaser, 2008; promoters, and in select cases, for control of specific as potential alternatives to antibiotics for use as growth scientific interest. In agriculture, probiotics and direct-fed as prebiotics. Probiosis, although not a new concept, has ented, beneficial bacteria and likewise the live microbial product will not succeed if the environment into which
it is introduced is unfavorable (Hamilton-Miller, 2004; Parracho et al., 2007). The concept of symbiotic has been proposed recently to characterize foods with both prebiotic and probiotic properties as health-enhancing functional foods (FAO/WHO, 2001).

*Lactobacillus salivarius* and *Pediococcus parvulus* of poultry gastrointestinal origin present in FM have been identified by 16S rRNA sequence analyses (Menconi et al., 2014). Tolerance and resistance to acidic pH, high osmotic concentration of NaCl, and bile salts of these isolates may have contributed to the efficacy of these isolates in the current study. Both strains also have in vitro antibacterial activity against *Salmonella enterica* serovar Enteritidis, *Escherichia coli* (O157:H7), and *Campylobacter jejuni* (Menconi et al., 2014). In addition, previous research from our laboratory indicates very rapid induction of specific host-gene expression pathways, which are associated with reductions in enteric colonization with *Salmonella* (Higgins et al., 2011). The aforementioned characteristics may contribute to the efficacy previously reported in laboratory and field conditions (Tellez et al., 2012). Although many mechanisms of action have been proposed for the observed efficacy, precise modalities have not been completely described for this highly effective culture.

Presently, FM has become one of the most extensive used and defined LAB probiotics in poultry (Farnell et al., 2006; Wolfenden et al., 2007a,b; Vicente et al., 2007, 2008; Higgins et al., 2007, 2008, 2010, 2011; Menconi et al., 2011; Tellez et al., 2012, 2013). The manufacturer recommends for FM to be administered in the drinking water. This is the first study of in-feed application (mash diet) of FM in a mash diet to control *Salmonella* colonization. Total bacterial counts of LAB in FM in both diets, with or without glycerol supplementation, were stable up to 14 d (data not shown). By themselves, neither glycerol nor FM were able to have a significant reduction on SE. However, in the present study, a synergistic effect on dietary supplementation of 5% glycerol combined with FM in reducing the amount and incidence of *Salmonella* from neonate broiler chickens was observed (Table 2). The preliminary results obtained from this study are relevant to the poultry industry because they could address both economic and food safety concerns faced by this industry. Due to the significant physiological, immunological, and microbiological changes during the first 10 d of age of birds, further studies are in progress to explore more time points of evaluations in older birds, and for longer period of time, that are required to substantiate these findings.

REFERENCES


