**Bacillus subtilis (DSM17299) significantly reduces Salmonella in broilers**

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**ABSTRACT** Salmonella continues to be a major public health burden worldwide. Poultry are known to be one of the main reservoirs for this zoonotic pathogen. It has previously been shown that a single dose of *Bacillus subtilis* reduces fecal shedding of *Salmonella enterica* serovar Enteritidis, whereas no effect on long-term colonization of the cecum has been observed. Here we report experiments that were undertaken to test the efficacy of a conventional diet supplemented with a probiotic (*B. subtilis* DSM17299) on 1) *Salmonella* colonization in the intestinal tract of broiler chickens, and 2) fecal shedding of *Salmonella* under production-like conditions. The trial birds fed the *B. subtilis* diet showed a significant 58% reduction in *Salmonella*-positive drag swabs compared with control birds, which had 100% presence of *Salmonella*. Feeding *B. subtilis* significantly reduced the average *Salmonella* load of cecum samples of the chickens, by 3 log units. This reduction in *Salmonella* colonization might not only positively affect broilers on the live production side by reducing the risk of infection between birds, but could also aid on the processing side by decreasing the amount of *Salmonella* entering the facility and improving food safety. Furthermore, numerical, but not statistically significant, improvements in feed conversion rate and BW gain at d 42 were observed in the *B. subtilis*-treated group compared with control birds.

**Key words:** Bacillus subtilis, chicken, probiotic, Salmonella reduction

2011 Poultry Science 90:1690–1694
doi: 10.3382/ps.2010-01056

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Received August 9, 2010.
Accepted April 9, 2011.
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**INTRODUCTION**

In both developed and developing countries, *Salmonella* is a leading cause of bacterial food-borne disease (Carter et al., 2009), and in the United States, it causes an estimated 1.3 million human food-borne illnesses and more than 500 deaths each year (Callaway et al., 2008). *Salmonella* has been isolated from all food animals and can cause morbidity and mortality in swine, cattle, sheep, and poultry (Callaway et al., 2008).

*Salmonella* are pathogens but also have the ability to live in animals and poultry as transient members of the intestinal microbial population without causing disease. Often, colonization of *Salmonella* does not affect poultry BW gain or performance; thus, asymptomatic infection can increase the likelihood of zoonotic transmission to humans through the food chain (Carter et al., 2009).

Antibiotic growth promoters (AGP) are known to reduce *Salmonella* colonization (Dibner and Richards, 2005), but there has been increasing debate regarding the use of AGP for farm animals and poultry worldwide (Barza, 2002; Bywater, 2005). Since AGP was banned in the European Union, the problem with *Salmonella* has been increasing, and 24% of broilers raised were found positive for colonization (EFSA, 2007). In the United States, only 10.9 to 16.3% of the broilers were found positive between 1998 and 2006 (USDA 2007).

In recent years, concerns about antimicrobial resistance have grown, but the main concerns have been focused specifically on resistance within the food supply (Barza, 2002; Cui et al., 2005). The recognition of multidrug-resistant *Salmonella* has prompted major concerns about the safety of the food supply directly, as a source of multidrug-resistant *Salmonella*, and indirectly, as a reservoir of antimicrobial genetic elements that can be exchanged between intestinal bacteria (Callaway et al., 2008).

Chicks can become infected vertically (from adults via the egg to the chick) or horizontally (from the environment, pests, or feed; Cox et al., 2000; Rodriguez et al., 2006). Currently, 2 types of *Salmonella* vaccines exist, an attenuated live vaccine and an inactivated vaccine. These vaccines are often administered to both breeder and layer flocks, but their effectiveness depends...
on the targeted serovar, host species, and whether reduction rather than eradication is the objective (Doyle and Erickson, 2006). However, vaccines do not eliminate initial colonization of the mucosal surfaces, particularly in the young bird (La Ragione and Woodward, 2003). Therefore, focusing on cleaning up animals and poultry may temporarily reduce the incidence of Salmonella, but intestinal populations of other Salmonella serotypes will increase to fill the vacuum (Callaway et al., 2008). Hence, it is critical to include other strategies (e.g., probiotics) to provide other intestinal bacteria a selective advantage to occupy the niche vacated by Salmonella spp., thereby preventing the entry of a new (or old) pathogen into animal and poultry populations and the food supply (Callaway et al., 2008).

Studies have demonstrated that Bacillus spp. and Bacillus subtilis spores may be successful competitive exclusion agents (La Ragione and Woodward, 2003). Bacillus subtilis modulates the intestinal microbiota and favors the growth of lactic acid bacteria with putative health-conferring properties (Knarreborg et al., 2008). A spore monoculture has the advantage of being readily produced, having a long storage life, and, in the case of B. subtilis, being avirulent (La Ragione and Woodward, 2003). Bacillus subtilis DSM17299 (GalliPiro, Chr. Hansen A/S, Hoersholm, Denmark) has been approved as a feed additive in the European Union and is a commercially available product that improves broiler performance [feed conversion ratio (FCR) and BW gain] to the same extent as an AGP (Lund et al., 2005; Knarreborg et al., 2008). Furthermore, B. subtilis may be the key in helping to reduce the bacterial load of Salmonella in chickens. The objective of this trial was therefore to test the ability of B. subtilis to reduce fecal Salmonella shedding and the intestinal Salmonella load of broilers under production-like conditions.

**MATERIALS AND METHODS**

**Salmonella Heidelberg Broiler Model**

A total of 900 one-day-old Cobb × Cobb male broiler chickens were divided into 15 pens, each with 60 birds, and allocated to 3 different treatments: 1) an uninfected control group fed a standard diet; 2) a positive control group infected with Salmonella enterica serovar Heidelberg and fed a standard diet; and 3) a group infected with Salmonella Heidelberg and fed a standard diet supplemented with B. subtilis (8 × 10^5 cfu/g of feed). The experiment was carried out with 7 replicates (pens) per treatment except for the noninfected control group, for which only 1 pen was used; see Table 1). Each pen was approximately 1.5 × 3.0 m in size, and all pens had 10 cm of built-up litter previously used with other birds, with a coating of fresh pine shavings on top. There was ambient humidity and 24-h lighting throughout the trial. The trial was conducted at Southern Poultry Research Inc. (Athens, GA).

Infection was performed on d 1, with 30 of the 60 chicks per pen (positive control group and B. subtilis group) being orally administered 0.1 mL of naladixic acid-resistant (25 μg/mL) Salmonella Heidelberg (5 × 10^7 cfu/mL) by gavage. The chicks challenged with Salmonella Heidelberg were wing banded and collectively referred to as the seeders.

**Diet**

Nonmedicated commercial-type corn- and soybean meal-based chicken rations (crumble/pellet) were fed ad libitum in the whole trial period. Diets were representative of local commercial formulations, and calculated analyses met or exceeded NRC (1994) standards. The diets did not contain any coccidiostat or other AGP (see Table 2). The B. subtilis DSM17299 diet was made containing 8 × 10^5 Bacillus spores/g of feed. Three representative samples were collected from each diet and analyzed for spore content.

**Environmental Sampling**

The environmental sampling was performed by collecting drag swabs from all pens on d 0, 14, and 42, respectively. A sterile gauze pad, soaked in 15 mL of double-strength skim milk, was dragged over the surface of the pen floor for approximately 1 min/pen and transported to the laboratory on ice for further analysis (Kinde et al., 2004). For the isolation and identification of Salmonella, a 1-μL loop of tetrathionate brilliant green broth (cat. no. 86352, Sigma-Aldrich, St. Louis, MO) was streaked onto xylose lysine tergitol 4 (cat. no. 76721, Sigma-Aldrich) and brilliant green novobiocin (cat. no. 55181, Sigma-Aldrich) agar plates containing naladixic acid and incubated at 37°C overnight. The H2S-positive colonies isolated were then placed onto triple-sugar iron slants (cat. no. 44940, Sigma-Aldrich) and incubated at 37°C overnight. Suspect Salmonella colonies were confirmed thereafter with poly O Salmonella-specific antiserum (cat. no. DF2264-47-2, Fisher Scientific Inc., Pittsburgh, PA).

**Determination of Cecal Bacterial Load**

On d 42, ten birds per pen (5 seeders and 5 non-seeders) were randomly selected for determination of

<table>
<thead>
<tr>
<th>Table 1. Treatment groups1</th>
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</thead>
<tbody>
<tr>
<td><strong>Birds per treatment (no.)</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Positive control</td>
</tr>
<tr>
<td>Bacillus subtilis (8 × 10^5 cfu/g of feed)</td>
</tr>
</tbody>
</table>

1The diets did not contain any coccidiostat or other antimicrobial growth promoter.
the Salmonella load in the cecum by quantitative real-time PCR. The birds were killed by cervical dislocation and weighed. Both ceca from each bird were aseptically collected, cut in half, placed into a sterile bag, and weighed. Phosphate-buffered saline was added to the bags, and the bags were placed in a stomacher for 1 min. Deoxyribonucleic acid was extracted from theecal preparations by using a commercial QIAmp DNA Stool Mini Kit (cat no. 51504, Qiagen, Hilden, Germany). Real-time PCR of the DNA extractions was carried out according to the manufacturer’s instructions with a TaqMan S. enterica Detection Kit (cat. no. 436832, Applied Biosystems, Foster City, CA), which demonstrates 100% specificity for more than 50 strains of S. enterica, including Salmonella Heidelberg and Salmonella Enteriditis. A standard curve was prepared by spiking ceca (intestinal) samples with a 4-fold serial dilution containing a known concentration of Salmonella Enteriditis, followed by DNA extraction. An estimation of the level of Salmonella Heidelberg in the samples was interpolated from the standard curve with spiked intestinal samples (7500 System SDS Software, Applied Biosystems). Samples with no measurable Salmonella load were recorded as 1 × 10^2 cfu/g (i.e., the detection limit of the assay).

Measurement of FCR and Statistics

At d 21 and 42 of the study, birds and feed were weighed by pen, and BW gain and FCR were calculated. Production performance data were analyzed in a completely randomized design with 3 treatments and 7 blocks using the GLM procedures of SAS (SAS Institute Inc., Cary, NC). Frequencies of Salmonella-positive drag swabs were compared using a chi-squared test. The purpose of the study was to evaluate the effect on fecal shedding and cecal load of Salmonella Heidelberg in broilers fed diets containing B. subtilis as compared with a standard diet. The inclusion of B. subtilis was successful in reducing the cecal average bacterial load of Salmonella from 1.7 × 10^8 cfu/g in the positive control to 1.1 × 10^3 cfu/g in the B. subtilis-treated group (P < 0.05; Table 3). Birds fed the B. subtilis diet (42% Salmonella positive) had a 58% reduction in Salmonella-positive drag swabs compared with the positive control birds (100% Salmonella positive). The litter examined for Salmonella before bird placement (d 0) was all negative for Salmonella. Additionally, numerical improvements in FCR (1.784 and 1.799 g/g, respectively) were seen in the B. subtilis-fed group compared with the standard diet (1.800; Table 3).

Table 2. Diet formulations

<table>
<thead>
<tr>
<th>Ingredient (%)</th>
<th>Starter, d 0 to 21</th>
<th>Grower, d 22 to 35</th>
<th>Finisher, d 36 to 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow corn, ground</td>
<td>56.12</td>
<td>60.80</td>
<td>68.00</td>
</tr>
<tr>
<td>Soybean meal, 48% CP</td>
<td>37.50</td>
<td>32.61</td>
<td>26.22</td>
</tr>
<tr>
<td>Poultry fat</td>
<td>3.00</td>
<td>3.43</td>
<td>2.99</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>1.75</td>
<td>1.56</td>
<td>1.32</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.80</td>
<td>0.78</td>
<td>0.62</td>
</tr>
<tr>
<td>Salt</td>
<td>0.30</td>
<td>0.32</td>
<td>0.35</td>
</tr>
<tr>
<td>Vitamin premix</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.20</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>Trace mineral premix</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Calculated content

<table>
<thead>
<tr>
<th>ME (kcal/kg)</th>
<th>Protein (%)</th>
<th>Lysine (%)</th>
<th>Methionine (%)</th>
<th>Methionine + cysteine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.096</td>
<td>22.3</td>
<td>1.18</td>
<td>0.53</td>
<td>0.89</td>
</tr>
<tr>
<td>3.140</td>
<td>20.6</td>
<td>1.01</td>
<td>0.48</td>
<td>0.76</td>
</tr>
<tr>
<td>3.191</td>
<td>18.1</td>
<td>0.85</td>
<td>0.45</td>
<td>0.70</td>
</tr>
</tbody>
</table>

1The vitamin mix provided the following (per kg of diet): thiamine mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; d-calcium pantothenate, 12 mg; vitamin B12 (cobalamin), 120 μg; pyridoxine hydrochloride, 4.7 mg; d-biotin, 0.11 mg; folic acid, 5.5 mg; menadione sodium bisulfite complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 27.5 μg; trans-retinyl acetate, 1,892 μg; all-rac-α-tocopheryl acetate, 1,892 μg; ethoxyquin, 125 mg.

2The trace mineral mix provided the following (per kg of diet): manganese (MnSO4·H2O), 60 mg; iron (FeSO4·7H2O), 30 mg; zinc (ZnO), 50 mg; copper (CuSO4·5H2O), 5 mg; iodine (ethylene diamine dihydroiodide), 0.15 mg; selenium (NaSeO3), 0.3 mg.

Table 3. Salmo nella in environmental swab testing by culture and bacterial load in cecal samples by quantitative real-time PCR on d 42

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Drag swabs</th>
<th>Cecal samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>1.06 × 10^2</td>
</tr>
<tr>
<td>Positive control</td>
<td>100</td>
<td>1.72 × 10^6</td>
</tr>
<tr>
<td>Bacillus subtilis (8 × 10^5 cfu/g of feed)</td>
<td>42</td>
<td>1.10 × 10^8</td>
</tr>
</tbody>
</table>

a,bMeans within columns with different superscript letters differ significantly (P ≤ 0.05).

1Salmonella-positive drag swab samples.

2Salmonella load in cecal samples.
respectively) and BW gain (2.212 and 2.182 kg, respectively) were seen at d 42 in the B. subtilis-treated group compared with the positive control group. The difference was not statistically significant (Table 4).

**DISCUSSION**

Previous studies demonstrated that B. subtilis spores can act in a competitive exclusion manner to alter the microbiota population. Dosing 1-d-old chicks with a single dose of B. subtilis (1 × 10^9 cfu/g) before challenge with Salmonella Enteritidis showed a tendency toward a transient decrease in cecal recovery of Salmonella Enteritidis. Furthermore, reduced fecal shedding was observed in B. subtilis-treated chickens as compared with untreated chickens (La Raggio and Woodward, 2003). Baltzley et al. (2010) reported that feeding B. subtilis LSSAO1 to broilers reduced the presence of Salmonella in drag swabs by 13 to 35% in the B. subtilis-treated group compared with the presence in control broilers. Our findings from feeding B. subtilis DSM17299 to broiler chickens showed a 3-log reduction in Salmonella counts in ceca compared with positive control birds on d 42. We report a 58% reduction in Salmonella-positive drag swabs in broilers fed the B. subtilis DSM17299 diet compared with the positive control birds, which had a 100% presence of Salmonella. The increased efficiency of DSM17299 could be caused by better performance of this strain, or it could be because the treatment in our study is on a daily basis and not a prechallenge dose of Bacillus spores.

*Bacillus subtilis* DSM17299 is known to favor the growth of lactic acid-producing bacteria (Knarreborg et al., 2008) and may lower the intestinal pH. Acidification of the intestinal environment was found to inhibit the growth of detrimental bacteria (e.g., *Salmonella*; Van Immerseel et al., 2006), which corresponds to the result in the present study. Our findings regarding production parameters showed only a numerical improvement in FCR and BW gain, whereas a statistically significant difference was reported previously (Knarreborg et al., 2008). The difference might be caused by the lower number of birds in this study (900 vs. 3,000) and by the fact that the small, unchallenged control group in our study gave small statistical power for this comparison. However, we observed the same numerical improvement in the FCR as described previously for *B. subtilis* DSM17299 as well as for AGP (Lund et al., 2005; Knarreborg et al., 2008).

In conclusion, this study showed that *B. subtilis* DSM17299 significantly reduced the *Salmonella* load in the intestinal tract of the chickens as well as in the surrounding environment, thereby potentially reducing the risk of infection between the birds and decreasing the amount of *Salmonella* entering the slaughterhouse, thus potentially improving food safety. The findings in this experiment are promising in regard to *Salmonella* reduction in broiler production. However, the total number of publications is limited, and more work is needed, such as on the effect of *B. subtilis* in adult poultry. Even better data on the *Salmonella* bacterial load as well as on the FCR could probably be achieved if more virulent or symptomatic strains were used.

**REFERENCES**


