Controlling *Salmonella* infection in weanling pigs through water delivery of direct-fed microbials or organic acids. Part I: Effects on growth performance, microbial populations, and immune status\textsuperscript{1}

M. C. Walsh,*\textsuperscript{2} M. H. Rostagno,† G. E. Gardiner,‡\textsuperscript{3} A. L. Sutton,*
B. T. Richert,* and J. S. Radcliffe*\textsuperscript{4}

*Department of Animal Science, Purdue University, West Lafayette, IN 47907;
†Livestock Behavior Research Unit, USDA-ARS, West Lafayette, IN 47907;
and ‡Teagasc, Moorepark Food Research Centre, Fermoy, County Cork, Ireland

**ABSTRACT:** Pigs (n = 88) weaned at 19 ± 2 d of age were used in a 14-d study to evaluate the effects of water-delivered direct-fed microbials (DFM) or organic acids on growth, immune status, *Salmonella* infection and shedding, and intestinal microbial populations after intranasal inoculation of *Salmonella Typhimurium* (10\textsuperscript{10} cfu/pig). Pigs were challenged with *Salmonella* 6 d after commencement of water treatments. Treatments were 1) control diet; 2) control diet + DFM (*Enterococcus faecium*, *Bacillus subtilis*, and *Bacillus licheniformis*) in drinking water at 10\textsuperscript{9} cfu/L for each strain of bacteria; 3) control diet + an organic acid-based blend (predominantly propionic, acetic, and benzoic acid) in drinking water at 2.58 mL/L; and 4) control diet + 55 mg/kg of carbadox. Serum samples were taken on d 6, 8, 10, and 14 for determination of tumor necrosis factor α (TNFα) concentrations. Fecal samples were taken on d 0, 5, 7, and 11 for determination of *Salmonella* shedding and enumeration of coliforms. Pigs were euthanized on d 6, 8, 10, and 14. Intestinal and cecal tissue and digesta and mesenteric lymph nodes were sampled and analyzed for *Salmonella*. Duodenal, jejunal, and ileal mucosal scrapings were sampled for measurement of mucosal TNFα concentrations. Water delivery of DFM prevented a decline in ADG on d 2 to 6 postchallenge compared with the negative control (\(P < 0.05\)). Coliform counts tended to be greater (\(P = 0.09\)) in the cecum of the DFM treatment group on d 2 postinfection compared with the negative control and acid treatment groups. However, *Salmonella* prevalence in the feces, gastrointestinal tract, or lymph nodes was not affected by water delivery of acids or DFM. Serum and mucosal TNFα concentrations were not affected by treatment throughout the study with the exception of ileal concentrations on d 4 postchallenge, which were greater in the negative control group compared with all other treatments (\(P < 0.05\)). The in-feed antibiotic was the only treatment that reduced *Salmonella* prevalence and this was localized to the cecum on d 8 postinfection. In conclusion, the DFM and organic acid treatments used in this study offered little or no benefits to pigs infected with *Salmonella* and should not be considered under the constraints of this study as viable alternatives to in-feed antibiotics in a pathogen challenge situation.

**Key words:** direct-fed microbial, immune status, organic acid, pig, *Salmonella*

\textsuperscript{1}Financial support provided in part by the National Pork Board, Des Moines, IA.
\textsuperscript{2}Current address: Teagasc, Pig Development Department, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, County Cork, Ireland.
\textsuperscript{3}Current address: Department of Chemical and Life Sciences, Waterford Institute of Technology, Waterford, Ireland.
\textsuperscript{4}Corresponding author: jradclif@purdue.edu

©2012 American Society of Animal Science. All rights reserved.

doi:10.2527/jas.2010-3598

**INTRODUCTION**

*Salmonella* is the second leading cause of bacterial foodborne illness in the United States, with the majority of these infections associated with contaminated animal products (Foley et al., 2008). Pathogens such as *Salmonella* and *Escherichia coli* are most successful at colonizing the gastrointestinal tract during times of stress, when nutrition and immunity are suboptimum, as is the case in the newly weaned pig (Aumaître et al., 1995). Timely interventions may interrupt lifetime carriage of *Salmonella* and improve the health and growth of the weaned pig.
On-farm *Salmonella* control strategies have traditionally focused on increased biosecurity, all-in all-out management practices, feed particle size, and antibiotic treatment (Wilcock and Schwartz, 1992; Fedorka-Cray et al., 1997a; Hedemann et al., 2005). Research has found that the enteric antibiotic, carboxad, is particularly effective in weaned pigs challenged with *Salmonella* Typhimurium (Burkey et al., 2004). Recently, interventions, such as inclusion of organic acids and direct-fed microbials (DFM) in feed and drinking water, have been the focus of much research as an alternative to subtherapeutic antibiotics. Numerous studies have demonstrated the potential of some organic acids in feed (Jørgensen et al., 1999; Creus et al., 2007) or added to drinking water (van der Wolf et al., 1999) to reduce the prevalence of *Salmonella* in pigs. Likewise, certain DFM such as strains of *Bacillus* have reduced *Salmonella* invasion of gastrointestinal epithelial cells in vitro (Aperce et al., 2010) and *Enterococcus faecium*, in a 14-d study to evaluate the potential of DFM or *Salmonella* challenge in weanling pigs on growth performance, *Salmonella*-positive animals allow for the elimination of *Salmonella* in pigs (Lodemann et al., 2006). The objective of this study was to evaluate the potential of water-delivered additives as substitutes for antibiotics after a *Salmonella* challenge in weaning pigs on growth performance, microbial communities, and immune response. This investigation was part of a larger *Salmonella* challenge study, and the results on jejunal ion transport and intestinal histology are reported in a companion paper (M. C. Walsh, M. H. Rostagno, G. E. Gardiner, A. L. Sutton, B. T. Richert, and J. S. Radcliffe, unpublished data).

## MATERIALS AND METHODS

All procedures were approved by the Purdue University Animal Care and Use Committee and conducted in a Biosafety Level (BSL)-2 facility at the USDA-ARS, Livestock Behavior Research Unit at Purdue University.

### Experimental Diets and Animal Research

Eighty-eight crossbred pigs (equal barrows and gilts) were weaned at an average of 19 ± 2 d of age and used in a 14-d study to evaluate the potential of DFM or organic acids to replace antibiotics after a *Salmonella enterica* serovar Typhimurium challenge. Pigs were randomly assigned to 1 of 4 treatments based on litter origin, sex, and initial BW (average = 6.2 ± 2.1 kg). Treatments included 1) control diet (negative control); 2) control diet + *Ent. faecium*, *Bacillus subtilis*, *Bacillus licheniformis* DFM (Bioplus DP, Chr. Hansen, Horsholm, Denmark) in drinking water at 10^7 cfu/L for each strain of bacteria; 3) control diet + an organic acid-based blend (KEM SAN, predominantly propionic, acetic, and benzoic acid; Kemin Americas, Des Moines, IA) in drinking water at 2.58 mL/L; and 4) control diet + 55 mg/kg of carboxad (antibiotic; Pfizer, Exton, PA). Treatments were administered continuously for 14 d (from d 0 to 14 postweaning). The composition of the control and antibiotic diets are shown in Table 1.

There were 4 pigs/pen and 6 pens per treatment with the exception of treatments 1 and 4, which had 5 pens each. All pigs had unlimited access to feed and water through a 5-hole self feeder and a single nipple waterer in each pen. The DFM and organic acid treatments were added to separate water lines, with administered amounts regulated by a water-driven chemical dilution pump (Dosatron, Clearwater, FL). Pigs were housed in floor pens and pens of the same treatment were arranged together. To limit cross contamination between treatments, solid dividers were erected between pens of pigs on different treatments. All diets were formulated to meet or exceed the estimated nutrient requirements for pigs (NRC, 1998).

### Sample Collection

All pigs were screened for the presence of *Salmonella* in fecal samples before weaning and at weaning (d 0) to allow for the elimination of *Salmonella*-positive animals from the study. Before *Salmonella* challenge, 1 pig/pen (n = 22) was randomly selected and euthanized by as-

### Table 1. Composition of basal nursery pig diets, as-is basis

<table>
<thead>
<tr>
<th>Item</th>
<th>Control diet</th>
<th>Antibiotic diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ground corn</td>
<td>40.70</td>
<td>40.42</td>
</tr>
<tr>
<td>Whey</td>
<td>25.00</td>
<td>25.00</td>
</tr>
<tr>
<td>Soybean meal, 48% CP</td>
<td>17.20</td>
<td>17.23</td>
</tr>
<tr>
<td>Plasma protein</td>
<td>6.00</td>
<td>6.00</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Fishmeal</td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.71</td>
<td>0.71</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>0.59</td>
<td>0.59</td>
</tr>
<tr>
<td>Vitamin premix1</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Carbadox</td>
<td>—</td>
<td>0.25</td>
</tr>
<tr>
<td>Salt</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Trace mineral premix2</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>Lys-HCl</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Se premix3</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>DL-Met</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>Calculated composition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME, kcal/kg</td>
<td>3,473</td>
<td>3,464</td>
</tr>
<tr>
<td>CP, %</td>
<td>24.00</td>
<td>24.00</td>
</tr>
<tr>
<td>Lys, %</td>
<td>1.55</td>
<td>1.55</td>
</tr>
<tr>
<td>Met, %</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>Met + Cys, %</td>
<td>0.89</td>
<td>0.89</td>
</tr>
<tr>
<td>Thr, %</td>
<td>1.08</td>
<td>1.08</td>
</tr>
<tr>
<td>Ca, %</td>
<td>0.90</td>
<td>0.90</td>
</tr>
<tr>
<td>P, %</td>
<td>0.74</td>
<td>0.74</td>
</tr>
</tbody>
</table>

1Vitamin premix provided the following per kilogram of diet: vitamin A, 6,064 IU; vitamin D₃, 606 IU; vitamin E, 44.1 IU; menadione, 2 mg; vitamin B₁₂, 35 μg; riboflavin, 7.1 mg; β-pantothenic acid, 22 mg; niacin, 33 mg.
2Trace mineral premix provided the following per kilogram of diet: Fe, 121.2 mg as iron sulfate; Zn, 121.2 mg as copper sulfate; Mn, 15.0 mg as magnesium sulfate; Cu, 11.2 mg as copper sulfate; I, 0.46 mg as potassium iodate.
3Se premix provided 0.3 mg of Se as sodium selenite per kilogram of diet.
phyxiation with CO2 on d 6 postweaning. All remaining pigs were given an intranasal dose of 1010 cfu of *Salmonella* Typhimurium (Strain χ4232, nalidixic acid resistant) per pig. The inoculum was prepared as described by Hurd et al. (2001). On d 2, 4, and 8 postchallenge, 1 pig per pen (n = 22 pigs/d) was also euthanized as described before. Samples of distal ileal (15 cm distal to the ileo-cecal junction) and cecal tissues and digesta and mesenteric lymph nodes (MLN) were collected from all pigs for *Salmonella* detection. Cecal and ileal digesta were also used for the enumeration of coliform. Pigs were individually weighed, and pen feed disappearance was recorded on d 0, 5, 8, 10, and 14 postweaning for calculation of ADG, ADFI, and G:F. Cecal samples were collected from all pigs on d 5, 7, and 11 postweaning for the detection of *Salmonella*. Enumeration of enterococci was performed on fecal samples taken on d 5 (2 pigs/pen) and on cecal digesta of pigs (n = 22) euthanized on d 14. Rectal temperatures were recorded on d 5, 7, 8, 10, and 14 postweaning to monitor changes in body temperature.

**Water Analysis**

Water samples were taken on d 0 and 13 for pH recording and on d 0, 7, and 14 for enumeration of *Ent. faecium* and *Bacillus* populations. Enumeration of *Ent. faecium* in water was conducted by serial dilution of water samples in buffered peptone water followed by spread plating on sterile mitus salivarius agar (Sigma-Aldrich, St. Louis, MO) containing 0.001% potassium tellurite (VWR, Batavia, IL). Plates were incubated for 2 to 3 days aerobically at 37°C. *Bacillus* spores were enumerated by heating the water sample to 80°C in a water bath for 10 min followed by cooling in cold water. Water samples were then serially diluted in buffered peptone water and spread plated on trypticase soy agar with 5% sheep blood (TSA II, Krackelar Scientific, Albany, NY). Plates were incubated aerobically overnight at 37°C.

**Tissue Collection and Preparation**

Venous blood samples were obtained (Lawhorn, 1988) from 1 pig/pen before euthanasia on d 6, 8, 10, and 14 postweaning. Blood (10 mL) was collected from the anterior vena cava in serum separation Vacutainer blood collection tubes (Becton Dickinson, Franklin Lakes, NJ). Blood samples were allowed to clot at room temperature for 2 h followed by centrifugation at 2,000 × g for 20 min at room temperature. Serum was removed and frozen at −80°C for subsequent cytokine analysis. Duodenal, jejunal, and ileal mucosal scrapings were taken using a microscope slide as described by Adeola and King (2006). Mucosal tissue was placed in a protein extraction reagent (Complet, Aphaarma, Gaithersburg, MD) with added protease inhibitor cocktail (Sigma-Aldrich) and homogenized. Supernatant was removed after centrifugation at 27,000 × g for 15 min at room temperature and stored at −80°C for subsequent cytokine analysis.

**Microbiology**

All tissue, digesta, and fecal samples were processed for isolation of the challenge *Salmonella* strain under aerobic conditions as follows: selective enrichment was performed in tetrathionate broth for 20 to 24 h at 37°C (Neogen Corporation, Lansing, MI) and then in Rappaport-Vassiliadis broth (Neogen) containing novobiocin for 20 to 24 h at 42°C (20 μg/mL; Sigma-Aldrich). Samples were then streaked onto XLT-4 agar (Neogen) containing nalidixic acid (50 μg/mL; Sigma-Aldrich) and incubated for 20 to 24 h at 37°C. Any putative *Salmonella* colonies were streaked onto Rambach agar (VWR) and incubated for 20 to 24 h at 37°C to confirm identity (Pignato et al., 1995). All samples taken for *Salmonella* screening before challenge were cultured similar to the postchallenge samples but without the use of the selective antibiotic nalidixic acid. Coliform were enumerated on MacConkey agar (Neogen) incubated at 37°C for 24 h under aerobic conditions. Samples taken for enterococci enumeration were serially diluted 10-fold in buffered peptone water, inoculated onto kanamycin azide esculin agar plates (KAA agar; Fisher, San Diego, CA) using the spread plating method and incubated aerobically overnight at 37°C.

**Cytokine Analysis**

Concentrations of tumor necrosis factor α (TNFα) were determined in the supernatant of duodenal, jejunal, and ileal mucosal scrapings and blood serum using porcine-specific ELISA kits (R&D Systems, Minneapolis, MN). The protein content of mucosal scrapings was determined using a protein assay kit (BCA, Pierce, Rockford, IL), which has a working range of 20 to 2,000 μg/mL. Cytokine concentration in mucosal tissue was expressed as mass per gram of tissue protein. The dynamic range of the porcine-specific TNFα ELISA kit was 23.4 to 1,500 pg/mL with a sensitivity of 3.7 pg/mL. For serum, the inter- and intraassay CV were 8.6 and 6.1%, respectively.

**Statistical Analysis**

The prevalence of *Salmonella* in tissues, digesta, and feces was analyzed using Fisher’s exact test with PROC FREQ (SAS Inst. Inc., Cary, NC). Growth performance data and enterococci counts were analyzed using the GLM procedure of SAS, and treatment means were compared using Tukey’s Studentized range test. For all other experimental variables, data were presented as least squares means ± SEM and analyzed using the MIXED procedure of SAS as a repeated measures analysis with Tukey-Kramer adjustments for multiple comparisons. The slice option of the MIXED procedure of SAS was used to separate main effects within different
time periods. For all response criteria, the pen served as the experimental unit. Microbiology data were log-transformed to ensure data were normally distributed. The level of significance for all tests was \( P < 0.05 \). Trends were reported up to \( P = 0.10 \).

**RESULTS**

**Water Analysis**

Water from the organic acid treatment line was the only sample that was acidic, and all other water samples were found to have a pH around neutral (pH 7; data not shown). *Enterococcus* and *Bacillus* spp. were detected in water samples taken from the DFM water line but were not found in any other water samples taken (data not shown).

**Growth Performance**

Prechallenge (d 0 to 5 postweaning) and during the immediate postchallenge period (d 5 to 8 postweaning), there were no differences in ADG, ADFI, G:F, or BW among any of the 4 treatments (Table 2). By d 2 to 4 postchallenge (d 8 to 10 postweaning), pigs receiving drinking water treated with DFM had greater \( P = 0.02 \) ADG than negative control pigs, but were not different from pigs on other treatments. There was no effect of treatment on d 10 BW, d 8 to 10 ADFI, or G:F. On d 10 to 14 postweaning, ADG, ADFI, G:F, and d 14 BW were not different between any of the 4 treatments.

**Body Temperature**

Rectal temperatures taken before *Salmonella* challenge (d 5 postweaning) and on d 1, 2, 4, and 8 postchallenge to determine the effect of *Salmonella* infection on body temperature and to investigate the ability of treatments to ameliorate any temperature increases indicated that there was a tendency \( P = 0.07 \) for a treatment \( \times \) time interaction (Figure 1). On d 8 postchallenge, pigs receiving DFM in drinking water tended to have a higher body temperature than pigs drinking acidified water or the negative control pigs, but their body temperature was not different from the pigs receiving in-feed antibiotics.

---

**Table 2. Effects of antibiotic or antibiotic alternatives on weanling pig growth performance after a *Salmonella* Typhimurium challenge**

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment (^2)</th>
<th>MSE (^3)</th>
<th>( P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pens/treatment</td>
<td>DFM</td>
<td>Acid</td>
<td>Carbadox</td>
</tr>
<tr>
<td>Initial BW, kg</td>
<td>6.29</td>
<td>6.22</td>
<td>6.26</td>
</tr>
<tr>
<td>d 0 to 5</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Pigs/pen</td>
<td>150</td>
<td>136</td>
<td>136</td>
</tr>
<tr>
<td>ADFI, g</td>
<td>222</td>
<td>204</td>
<td>222</td>
</tr>
<tr>
<td>G:F</td>
<td>0.67</td>
<td>0.65</td>
<td>0.61</td>
</tr>
<tr>
<td>d 5 BW, kg</td>
<td>7.14</td>
<td>6.93</td>
<td>6.94</td>
</tr>
<tr>
<td>d 5 to 8</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>ADFI, g</td>
<td>313</td>
<td>272</td>
<td>304</td>
</tr>
<tr>
<td>G:F</td>
<td>0.46</td>
<td>0.65</td>
<td>0.54</td>
</tr>
<tr>
<td>d 8 BW, kg</td>
<td>7.47</td>
<td>7.44</td>
<td>7.41</td>
</tr>
<tr>
<td>d 8 to 10</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>ADFI, g</td>
<td>304</td>
<td>241</td>
<td>272</td>
</tr>
<tr>
<td>G:F</td>
<td>0.39</td>
<td>0.30</td>
<td>0.18</td>
</tr>
<tr>
<td>d 10 BW, kg</td>
<td>7.60</td>
<td>7.34</td>
<td>7.63</td>
</tr>
<tr>
<td>d 10 to 14</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ADFI, g</td>
<td>381</td>
<td>340</td>
<td>427</td>
</tr>
<tr>
<td>G:F</td>
<td>0.50</td>
<td>0.68</td>
<td>0.67</td>
</tr>
<tr>
<td>d 14 BW, kg</td>
<td>8.41</td>
<td>8.28</td>
<td>8.73</td>
</tr>
</tbody>
</table>

\(^{a,b}\)Within a row, means without a common superscript differ \( P < 0.05 \).

\(^1\)Pigs were intranasally inoculated with *Salmonella* Typhimurium (10\(^{10}\) cfu/pig) on d 6 postweaning.

\(^2\)DFM (direct-fed microbials): *Enterococcus faecium*, *Bacillus subtilis*, and *Bacillus licheniformis* (Chr. Hansen, Horsholm, Denmark); acid: predominantly propionic, acetic, and benzoic acids (Kemin Americas, Des Moines, IA); carbadox: 55 mg/kg of carbadox (Pfizer, Exton, PA); and neg. ctrl.: negative control (i.e., no additive).

\(^3\)MSE: mean squared error.


**Effects on Intestinal Salmonella, Coliforms, and Enterococci**

Before intranasal *Salmonella* challenge, *Salmonella* was not detected in the feces, ileal digesta, or cecal tissue of any of the pigs in the study (Figure 2). However, prechallenge negligible *Salmonella* contamination was detected in the MLN, ileal tissue, and cecal tissue of a small proportion of pigs. There were no differences among treatments in the prevalence of *Salmonella* in feces on d 1 or 5 postchallenge (Figure 3). However, on d 5 postchallenge, *Salmonella* was no longer detectable in the feces of pigs receiving the DFM but was detectable in pigs on other treatments. On d 2 and 4 postchallenge, *Salmonella* was present in cecal digesta of 100% of pigs on all treatments (Table 3). By d 8 postchallenge, the administration of in-feed antibiotic resulted in a 60% reduction (*P* = 0.01) in *Salmonella* prevalence in the cecal digesta, whereas it remained at 100% for pigs on all other treatments. The prevalence of *Salmonella* increased to almost 100% in the MLN, ileal and cecal tissue, and the ileal digesta of pigs on all treatments on d 2 postchallenge (Table 3). The control measure used in this study did not affect prevalence in the MLN, ileal and cecal tissue, or the ileal digesta on d 2, 4, or 8 postchallenge.

There was no effect of treatment on coliform counts in the ileal digesta (Table 4). However, there was an effect (*P* = 0.001) of time on ileal coliform counts. Decreased coliform counts were observed on d 4 postchallenge (7.1 ± 0.1 vs. 5.9 ± 0.1 log\(_{10}\) cfu/g; *P* < 0.001), but by d 8 postchallenge, counts had returned to previous levels. On d 2 postchallenge, there tended to be greater (*P* = 0.09) counts of coliforms in the cecal digesta of pigs receiving DFM in drinking water compared with pigs receiving acidified drinking water or negative control pigs. There tended to be an effect (*P* = 0.10) of time on cecal coliform counts. Similar to the ileum, coliform counts in the cecum decreased on d 4 postchallenge but increased again by d 8 postchallenge.

Enterococci were enumerated in feces prechallenge and in cecal digesta on d 8 postchallenge. Pigs receiving DFM in drinking water tended to have greater counts in feces than pigs receiving in-feed antibiotic before challenge (7.02 vs. 5.78 log\(_{10}\) cfu/g, SEM = 0.47; *P* = 0.07), but their counts were not different from pigs administered the negative control or acidified water treatment (6.06 and 5.96 log\(_{10}\) cfu/g, respectively, SEM =...
0.47). On d 8 postchallenge, there was no difference in cecal enterococci counts between treatments (data not shown).

**Cytokine Responses**

Concentrations of TNFα were measured in serum and in duodenal, jejunal, and ileal mucosa (Table 5). There was no effect of treatment on TNFα concentrations in serum. However, there was an effect of time \((P = 0.04)\), whereby serum TNFα increased up to d 4 postchallenge \((132.8 \pm 9.0 \text{ pg/mL})\) but serum concentrations decreased by d 8 postchallenge \((124.6 \pm 12.6 \text{ pg/mL})\). There was no effect of treatment on TNFα concentration in duodenal mucosa at any time during the study; however, there was a tendency for an effect of time \((P = 0.08)\). The TNFα concentration in duodenal mucosa decreased initially on d 2 postchallenge and then in-

**Figure 3.** Effects of antibiotic or antibiotic alternatives on *Salmonella* presence (% of pigs) in feces (all pigs were sampled on d 0, 1, and 5 postchallenge) of weanling pigs after a *Salmonella Typhimurium* challenge. Pigs were intranasally inoculated with *Salmonella Typhimurium* \((10^{10} \text{ cfu/pig})\) on d 6 postweaning. DFM = direct-fed microbials (*Enterococcus faecium*, *Bacillus subtilis*, and *Bacillus licheniformis*; Chr. Hansen, Horsholm, Denmark); acid: predominantly propionic, acetic, and benzoic acids (Kemin Americas, Des Moines, IA); carbadox: 55 mg/kg of carbadox (Pfizer, Exton, PA); neg. ctrl.: negative control diet (i.e., no additive).

**Table 3.** Effects of antibiotic or antibiotic alternatives on *Salmonella* presence (% of pigs) in mesenteric lymph nodes, cecal and ileal digesta, and cecal and ileal tissues of weanling pigs after a *Salmonella Typhimurium* challenge\(^{1,2}\)

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>DFM</th>
<th>Acid</th>
<th>Carbadox</th>
<th>Neg. ctrl.</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesenteric lymph nodes, %</td>
<td></td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>2 d</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>4 d</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>80</td>
<td>0.454</td>
<td></td>
</tr>
<tr>
<td>8 d</td>
<td>83.3</td>
<td>83.3</td>
<td>60</td>
<td>100</td>
<td>0.528</td>
<td></td>
</tr>
<tr>
<td>Cecal digesta, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 d</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>4 d</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>8 d</td>
<td>100</td>
<td>100</td>
<td>40</td>
<td>100</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>Cecal tissue, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 d</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>4 d</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>8 d</td>
<td>83.3</td>
<td>66.7</td>
<td>60</td>
<td>80</td>
<td>0.470</td>
<td></td>
</tr>
<tr>
<td>Ileal digesta, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 d</td>
<td>100</td>
<td>100</td>
<td>80</td>
<td>80</td>
<td>0.325</td>
<td></td>
</tr>
<tr>
<td>4 d</td>
<td>100</td>
<td>100</td>
<td>75</td>
<td>100</td>
<td>0.444</td>
<td></td>
</tr>
<tr>
<td>8 d</td>
<td>50</td>
<td>100</td>
<td>80</td>
<td>80</td>
<td>0.377</td>
<td></td>
</tr>
<tr>
<td>Ileal tissue, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 d</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>4 d</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>80</td>
<td>0.844</td>
<td></td>
</tr>
<tr>
<td>8 d</td>
<td>83.3</td>
<td>83.3</td>
<td>60</td>
<td>80</td>
<td>0.829</td>
<td></td>
</tr>
</tbody>
</table>

\(^{1}\)Pigs were intranasally inoculated with *Salmonella Typhimurium* \((10^{10} \text{ cfu/pig})\) on d 6 postweaning, and the presence of *Salmonella* was determined 2, 4, and 8 d postchallenge.

\(^{2}\)DFM (direct-fed microbials): *Enterococcus faecium*, *Bacillus subtilis*, and *Bacillus licheniformis* (Chr. Hansen, Horsholm, Denmark); acid: predominantly propionic, acetic, and benzoic acids (Kemin Americas, Des Moines, IA); carbadox: 55 mg/kg of carbadox (Pfizer, Exton, PA); and neg. ctrl.: negative control diet (i.e., no additive).
increased on d 4 postchallenge before decreasing to below prechallenge levels on d 8 postchallenge. There was no effect of treatment or time on TNFα concentrations in jejunal mucosa. On d 4 postchallenge, TNFα concentrations in ileal mucosa were greater \((P = 0.02)\) in pigs receiving the negative control treatment compared with all other treatments. There was an effect of time on TNFα concentrations in ileal mucosa \((P = 0.003)\). Prechallenge concentrations of TNFα in the ileum were greater than postchallenge concentrations \((70.0 \pm 9.8\) vs. \(47.3 \pm 9.8, 59.3 \pm 9.8, \) and \(17.8 \pm 10.1\) pg/g of protein for d 0, 2, 4, and 8, respectively).

**DISCUSSION**

Whereas every attempt was made to include *Salmonella*-free pigs only in this study, 14% of the pigs euthanized on d 0 (before *Salmonella* challenge) were found to have *Salmonella* in either the digesta or tissues. The 3 pigs that were identified as *Salmonella* positive on d 0 were not shedding *Salmonella* in feces during the screening process and were the progeny of a single sow. In this instance, the findings indicate that the sow or farrowing crate was the source of the *Salmonella* infection. Although this indicates that a small proportion of the pigs had previously been exposed to *Salmonella*, it is not possible to comment on the *Salmonella* status of all pigs before deliberate infection. However, none of the pigs were shedding *Salmonella* in the feces and any *Salmonella* detected before challenge was not the challenge strain because it was not resistant to the selective antibiotic used (nalidixic acid). Furthermore, the large dose of *Salmonella* used to challenge the pigs would have ensured that the challenge strain would most likely have become dominant over any existing *Salmonella* and the use of nalidixic acid for selective isolation of the challenge strain ensured that any pre-existing *Salmonella* strains were not recovered. *Salmonella* was not enumerated in the current study, so it is therefore not possible to determine if *Salmonella* counts were decreased by the treatments administered. Therefore, discussion is limited to the ability of the treatments to decrease *Salmonella* prevalence within the herd.

The potential for cross-contamination of treatments, particularly DFM between different groups of pigs, can be a major concern in this type of challenge study. Every attempt was made to minimize the risk of cross-contamination by erecting solid dividers between pens of pigs on different treatments. Ideally, the potential for cross-contamination between treatments should be eliminated by housing treatments in different rooms. However, it was thought that this would add additional unnecessary variation to the data collected. The commercial DFM used in this study was not engineered to be antibiotic resistant or was not readily identifiable by any other marker. Therefore, tracking of the specific strains of DFM in pigs was hindered. However, cecal enumeration of enterococci d 8 postchallenge did not demonstrate any differences among treatment groups.

The results of this study demonstrated that addition of a *Bacillus/Enterococcus* combination to the drinking water of weaned pigs challenged with \(10^{10}\) cfu of *Salmonella* Typhimurium had no effect on prevalence of the pathogen in organs or digesta compared with other treatments. *Salmonella* was no longer being shed in feces of pigs fed the DFM treatment by 5 d postchallenge. However, all of these pigs were still harboring *Salmonella* in the cecum 8 d after the challenge, indi-
Table 5. Effects of antibiotic or antibiotic alternatives on tumor necrosis factor α (TNFα) concentration in serum and small intestinal mucosa of weanling pigs after a *Salmonella Typhimurium* challenge 1,2,3

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum, pg/mL</td>
<td></td>
<td>Treatment</td>
</tr>
<tr>
<td>Pens/treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>122.0 ± 17.1</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>113.1 ± 17.1</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>118.8 ± 17.1</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>125.2 ± 11.0</td>
</tr>
<tr>
<td>Duodenum, pg/g of protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>28.6 ± 6.6</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>16.3 ± 6.6</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>25.0 ± 7.3</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>6.0 ± 6.6</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>18.9 ± 3.2</td>
</tr>
<tr>
<td>Jejunum, pg/g of protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>18.6 ± 6.6</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>11.3 ± 6.0</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>2.6 ± 6.6</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>6.3 ± 6.0</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>9.7 ± 3.1</td>
</tr>
<tr>
<td>Ileum, pg/g of protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>49.5 ± 18.6</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>27.5 ± 18.6</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>37.9 ± 18.6</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>22.1 ± 18.6</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>34.2 ± 10.4</td>
</tr>
</tbody>
</table>

1 Within a row, means without a common superscript differ (*P* < 0.05).
2 TNFα concentration in serum is reported as picograms per milliliter of blood and in duodenal, jejunal, and ileal mucosal tissue is reported as picograms per gram of protein (adjusted to an average protein yield of 0.066 g in duodenal mucosa, 0.049 g in jejunal mucosa, and 0.050 g in ileal mucosa).
3 Pigs were intranasally inoculated with *Salmonella Typhimurium* (10¹⁰ cfu/pig) on d 6 postweaning, and TNFα concentrations were determined 2, 4, and 8 d postchallenge.

4 DFM (direct-fed microbials): *Enterococcus faecium*, *Bacillus subtilis*, and *Bacillus licheniformis* (Chr. Hansen, Horsholm, Denmark); acid: predominantly propionic, acetic, and benzoic acids (Kemin Americas, Des Moines, IA); carbadox: 55 mg/kg of carbadox (Pfizer, Exton, PA); neg. ctrl.: negative control diet (i.e., no additive).

dicating that the DFM treatment did not rid the pigs of the pathogen. Although *Salmonella* was not quantified in this study, results from a previous challenge trial found that the amount of *Salmonella* excreted in feces increased considerably in pigs treated with an *Ent. faecium* probiotic (Szabó et al., 2009). However, in nonchallenged pigs, *Ent. faecium* has been found to reduce the pathogenic bacterial load (Pollmann et al., 2005; Lodemann et al., 2006). *Bacillus subtilis* and *B. licheniformis* have been shown in vitro to reduce the invasion of *Salmonella* into swine intestinal epithelial cells (Aperce et al., 2010), although this has not been confirmed in vivo.

Research in pigs has shown a clear positive correlation between the prevalence of *Salmonella*-positive pigs and the density of coliform bacteria in the gastrointestinal tract (Jørgensen et al., 1999). On d 2 postchallenge, cecal counts of coliforms tended to be greater in pigs on the DFM treatment than those on the negative control or acid treatments. However, it is worth noting that the coliform counts on d 2 postchallenge in the DFM-treated pigs were not different from prechallenge counts, indicating the increased coliform counts were not likely associated with increased *Salmonella* colonization in cecum of these pigs.

Body temperature was measured as an indication of clinical infection, and although it increased postinfection, as expected, it was not affected by DFM. This is in agreement with previous work (Szabó et al., 2009). The *Enterococcus/Bacillus* DFM combination successfully prevented the decline in ADG experienced by all other groups of pigs on d 8 to 10 of the study (d 2 to 4 postinfection). Whereas Szabó et al. (2009) showed that *Ent. faecium* administered alone under *Salmonella*-challenge conditions did not beneficially alter the growth of weaned pigs, *B. subtilis* and *B. licheniformis* have been found to improve the growth performance of healthy pigs under field conditions (Alexopoulos et al., 2004). Indeed, whereas the growth response to dietary probiotics is often variable (Jost and Bracher, 1999), a report examining the effects of *B. subtilis* and *B. licheniformis* inclusion in pig diets found that in 30 out of 31 studies, a positive growth response was observed in weanling pigs (Kremer, 2006).
Salmonella infection in pigs is characterized in part by the upregulation of inflammatory cytokines TNFα, IL-1, and IL-8 (Utter et al., 2007). In vitro studies examining the interaction of B. subtilis and L. acidophilus with Salmonella Typhimurium in swine intestinal cells found a reduction in the secretion of the neutrophil chemoattractant IL-8 in response to the probiotics (Aperce et al., 2010). In contrast, Ent. faecium resulted in increased IgM and IgA production in pigs challenged with Salmonella Typhimurium (Szabo et al., 2009).

In the present study, the Enterococcus/Bacillus combination used had no effect on TNFα concentrations in the serum or intestinal mucosa in S. Typhimurium-challenged pigs with the exception of the ileum where concentrations were less than in the negative control pigs, but only on d 4 postchallenge. Therefore, it is unlikely that this probiotic combination conferred any immunomodulatory benefits to the pigs during the Salmonella infection either via exacerbation of the immune response to aid rapid pathogen clearance or by reducing the overall amplitude of the response. Potentially, some of the pigs in this study were preexposed to Salmonella before the Salmonella Typhimurium challenge. However, this exposure was either spread evenly across treatment groups or the effect was minor because no statistically significant differences were detected between treatments in the concentration of TNFα in serum or the gut before Salmonella Typhimurium challenge.

Organic acids have for some time played a key role in the control of Salmonella in poultry (Van Immerseel et al., 2006) and to a lesser extent in pigs (Creus et al., 2007; Gebru et al., 2010). Similar to probiotics, not all organic acids are equal in their ability to combat infection and enhance growth when administered to pigs via feed or water. The type and concentration of acid used determines their efficacy. The efficacy of acid at inhibiting bacterial growth is dependent on its pKa value, which is the pH that 50% of the acid is dissociated (Partanen and Mroz, 1999). Acetic, propionic, and benzoic acid used in this study have pKa values ranging from 4.2 to 4.9 and so will remain in the undissociated state when the luminal pH is less than the pKa value. Organic acid in an undissociated form can freely diffuse through microbial semi-permeable membranes into the cytosol and inhibit bacterial growth (Lueck, 1980). The acids used in this study are likely to be most effectively in the upper part of the intestine where the pH is lower and may not reach the main site of infection of Salmonella, which is the ileum. The short-chain fatty acid butyrate specifically downregulates expression of invasion genes in Salmonella spp. at decreased concentrations, and propionate has been found to decrease the ability of Salmonella spp. to invade epithelial cells in vitro (Van Immerseel et al., 2006). The administration of acidified drinking water (van der Wolf et al., 2001) and in-feed organic acids (do Santos et al., 2007) on commercial pig farms has been shown to reduce the prevalence of pigs that were seropositive for Salmonella. Likewise, feeding a microencapsulated mixture of organic acids (20% citric and fumaric acids and 10% malic and phosphoric acids; Gebru et al., 2010) or 2.8% lactic acid alone (Tanaka et al., 2010) to Salmonella-challenged pigs resulted in decreased fecal shedding of Salmonella compared with the control group. In contrast, Martin-Pelaez et al. (2010) found the inclusion of a mixture of 0.4% formic and 0.4% lactic acid in feed to have no effect on Salmonella counts in the ileum or cecum of challenged pigs. Similarly, water acidification with a mixture of propionate, acetate, and butyrate had no effect on the proportion of pigs shedding Salmonella after deliberate infection in the present study. However, we are unable to comment on Salmonella counts in these pigs. Whereas the combination of malic, fumaric, citric, and phosphoric acid administered as a microencapsulated form in feed has been shown to enhance the growth performance of Salmonella-challenged pigs (Gebru et al., 2010), our study found that, under similar conditions, water delivery of a combination of acetic, propionic, and butyric acids did not enhance growth. Micro-encapsulation prevents the absorption of acids in the upper gastrointestinal tract (Van Immerseel et al., 2006), thereby enabling the acids to reach the site of Salmonella colonization lower in the gastrointestinal tract, which may explain the differences in growth response observed. In the current study, the body temperature of Salmonella-infect ed pigs returned to preinfection levels in pigs drinking acidified water by d 2 postinfection, similar to the negative control pigs. However, the increase in body temperature observed was less and for a shorter duration than in a similar study, in which pigs were inoculated with a smaller dose (10^6 cfu) of Salmonella (Gebru et al., 2010). The immune response to water acidification in Salmonella-challenged pigs was minimal and of little consequence in this study, with a transient decrease in the ileal TNFα concentration observed compared with the negative control pigs on d 4 postinfection but not on d 0, 2, or 8 postinfection.

The subtherapeutic use of antibiotics has long been shown to be an effective means of reducing fecal Salmonella shedding as well as clinical signs of infection in swine, calves, and chickens (Girard et al., 1976). A recent review by Denagamage et al. (2010) summarizing the association between subtherapeutic use of antibiotics and Salmonella Typhimurium in pigs found that out of 14 studies challenged with Salmonella Typhimurium, the most commonly used antibiotic was the tetracycline group. Antibiotics from the tetracycline group that inhibit protein synthesis are effective against both gram-positive and gram-negative bacteria and are often used in the treatment of respiratory infections (del Castillo et al., 1998). However, the antibiotic carbadox has also been shown to be effective when used as a positive control for the comparison of other feed additives in Salmonella-challenged pigs (Burkey et al., 2004). A possible limitation of the current study was the failure of the pigs to respond to the carbadox treatment after the Salmonella challenge. Ebner and
Mathew (2000) found that almost 50% of 
_Salmonella_
 isolated from pigs 2 d postinfection had developed resistance to carbadox. One possible explanation for the poor growth response to carbadox in the current study may be due to the development of resistance to this antibiotic by the 
_Salmonella_
 strain used to infect the pigs. Whereas in-feed carbadox had no effect on the growth performance of 
_Salmonella_
-infected pigs in this study, the prevalence of pigs with 
_Salmonella_
 in the cecal digesta 8 d postinfection was reduced by 60% compared with all other treatments. The inconsistent response to subtherapeutic antibiotics highlighted here is one of the main difficulties experienced when using feed additives as a control treatment and is a particular limitation of this pig study.

The cecum is one of the main sites where 
_Salmonella_
 is harbored in the body (Mainar-Jaime et al., 2008) and one of the reservoirs from which asymptotically infected animals continue to shed 
_Salmonella_
 intermittently throughout their lives. By reducing 
_Salmonella_
 prevalence in the cecum, carbadox may help to reduce the proportion of 
_Salmonella_
-infected pigs that continue to be carriers and intermittent shedders of the infection through to slaughter.

In conclusion, under our study conditions neither water delivery of a 
Bacillus/Enterococcus combination nor an organic acid mixture were able to beneficially influence the prevalence of 
_Salmonella_
 in the gastro-intestinal tract or lymph nodes of 
_Salmonella_
-infected pigs. In-feed enteric antibiotic was the only treatment that successfully decreased cecal carriage of 
_Salmonella_, although the growth response to the antibiotic was less than anticipated. These findings may indicate resistance of the 
_Salmonella_
 strain to the antibiotic used in this study. However, there were some positive performance findings. Pigs receiving the 
Bacillus/Enterococcus combination in drinking water did not experience the decrease in ADG observed in all other treatments groups 2 d postinfection. Overall, the probiotic and organic acid treatments used in this study resulted in little or no benefits in 
_Salmonella_
-infected pigs and, therefore, under the constraints of this study, cannot be recommended as viable alternatives to in-feed antibiotics for the control of 
_Salmonella_
 in weanling pigs. However, the short time frame, during which these treatments were administered, may not have been sufficient to observe any real improvements and the pigs may have benefited from more prolonged treatment.

**LITERATURE CITED**


_Bacillus licheniformis_ and 

Aperce, C. C., T. E. Burkey, B. KuKanich, B. A. Crozier-Dodson, S. S. Dritz, and J. E. Minton. 2010. Interaction of 
_Bacillus species_ and 


Creus, E., J. F. Perez, B. Peralta, F. Bautells, and F. Mateu. 2007. Effect of acidic feed on the prevalence of 


Denagamage, T., A. O’Connor, J. Sargeant, and J. McKean. 2010. The association between sub-therapeutic antibiotics and 

do Santos, J., E. Creus, J. F. Perez, E. Mateu, and S. M. Martin-Orue. 2007. Efficacy of a micro-encapsulated or non-encapsulated blend of lactic and formic acid to reduce the prevalence of 

Ebner, P. D., and A. G. Mathew. 2000. Effects of antibiotic regimens on the fecal shedding patterns of pigs infected with 
_Salmonella_ Typhimurium. _J. Food Prot._ 63:709-714.

Fedorka-Cray, P. J., A. Hogg, J. T. Gray, K. Lorenzen, J. Velasque, and P. Von Behren. 1997a. Feed and feed trucks as sources of 
_Salmonella_ contamination in swine. _Swine Health and Production_ 5:189-193.


Hedemann, M. S., L. L. Mikkelsen, P. J. Naughton, and B. B. Jensen. 2005. Effect of feed particle size and feed processing on morphological characteristics in the small and large intestine of pigs and on adhesion of 

Hurd, H. S., J. K. Gailey, J. D. McKean, and M. H. Rostagno. 2001. Rapid infection in market-weight swine following exposure to a 

Jorgensen, L., J. Dahl, and A. Wingstrand. 1999. The effect of feeding pellets, meal and heat treatment on the 
_Salmonella_ prevalence in finishing pigs. Pages 308-312 in Proceedings of the 3rd International Symposium on the Epidemiology and Control of 
_Salmonella_ in Pork, Washington, DC.


Kremer, B. 2006. DFM products provide consistent outcomes. _Feed News and Information_ 16:73N-79N.


