Short communication

Efficacy of *Clostridium butyricum* as probiotic feed additive against experimental *Salmonella Typhimurium* infection in pigs

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d Glossary

**ARTICLE INFO**

**Keywords:**
*Salmonella Typhimurium* (S. Typhimurium) infections in pigs constitute a risk for human salmonellosis. The use of probiotics may be a promising tool to reduce *Salmonella* infections in pigs. The present study investigated the efficacy of *Clostridium butyricum* (*C. butyricum*), at two different dosages, as probiotic feed additive against *S. typhimurium* infection in experimentally challenged pigs.

After weaning, 35 *Salmonella*-negative pigs were randomly divided into 4 groups; Negative control: no feed additive (*n* = 5), Positive control: no feed additive (*n* = 10), CB-H: \( \pm 2 \times 10^6 \) CFU *C. butyricum/g* feed (*n* = 10), CB-L: \( \pm 5 \times 10^5 \) CFU *C. butyricum/g* feed (*n* = 10). Pigs were fed *ad libitum* with the experimental feed, including the probiotic feed additive according to the group, from arrival (day -7) until euthanasia (day 42). One week after arrival (day 0), pigs in the positive control group, CB-H and CB-L were orally inoculated with \( 2 \times 10^8 \) CFU/mL nalidixic acid resistant *S. typhimurium* strain 112910a (1 mL/pig). Fecal excretion, serological response, intestinal carriage and prevalence of *S. typhimurium* positive ileocecal lymph nodes were evaluated.

Under the present conditions, the probiotic feed additive *C. butyricum* did not significantly reduce fecal excretion, serological response, intestinal carriage and prevalence of *S. typhimurium* in the ileocecal lymph nodes in experimentally challenged pigs. Further research is needed to investigate the results under field conditions and to detect possible additional effects of the application of the probiotic for a longer time period.

1. Introduction

Worldwide, pig herds are frequently colonized with *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S. typhimurium*). *Salmonella* infections in pigs constitute a risk for human salmonellosis, which is the second most reported foodborne zoonosis in Europe for several years (EFSA, 2015b,a,2016,2017). Since most infections in pigs are subclinical, the shedding pattern is intermittent and carriers occur commonly, it is difficult to control infections at farm level (Funk et al., 2001; Pires et al., 2013). Current on-farm control measures are mainly focused on biosecurity, cleaning and disinfection, all-in all-out management, acidification of feed and drinking water and vaccination (Wales et al., 2011; Andres and Davies, 2015; Callegari et al., 2015; Hill et al., 2016). Since the ban on antimicrobial growth promoters in the EU and the concern about the development of antibiotic resistance, probiotics have been an important field of interest to reduce intestinal infections, including *Salmonella* infections in pigs (Roselli et al., 2005; Cho et al., 2011; Liu et al., 2018).

In this study, the gram-positive, spore-forming bacterium *Clostridium butyricum* (*C. butyricum*) is used as probiotic feed additive. To our knowledge, no data are available on the effects of *C. butyricum* as a feed additive on *Salmonella* infections in pigs, whereas it has been shown to significantly reduce the amount of *Salmonella* in the cecal contents of broilers (Yang et al., 2012). The aim of the present study was to evaluate the efficacy of *C. butyricum*, at two different dosages, as probiotic feed additive against *S. typhimurium* infection in experimentally challenged pigs.

2. Materials & methods

*Salmonella*-negative pigs were purchased from a farm which was,
based on previous bacteriology and serology results, considered to wear Salmonella-negative pigs. At the day of weaning (28 days of age), 35 pigs were randomly selected and transported in a clean and disinfected trailer to the animal research facilities of Sciensano (Brussels, Belgium).

Upon arrival, the pigs were randomly assigned to four different groups:

1. Negative control: no feed additive, no challenge \((n = 5)\)
2. Positive control: no feed additive, \(S. \text{typhimurium}\) challenge \((n = 10)\)
3. CB-H: \(\pm 2 \times 10^6\) CFU \(C. \text{butyricum}/g\) feed, \(S. \text{typhimurium}\) challenge \((n = 10)\)
4. CB-L: \(\pm 5 \times 10^5\) CFU \(C. \text{butyricum}/g\) feed, \(S. \text{typhimurium}\) challenge \((n = 10)\)

The pigs were housed in 4 separate, similar stables equipped with a separate air flow system and a hygienic barrier with stable-specific overalls and boots. Before onset of the trial, the pens and all feed mixes were confirmed to be Salmonella-negative by bacteriological analysis.

The experimental feed, including the probiotic feed additive according to the group, was supplied at libitum from arrival (day −7) until euthanasia (day 42). The feed was manufactured and mixed by ABZ Diervoeding (Nijkerk, NL) and was, except for the probiotic feed additive, the same for all groups.

During the trial, the pigs were checked daily, clinical symptoms (e.g., diarrhea, loss of appetite) were recorded and no antibiotics were administered. One week after arrival (day 0), all pigs in the positive control group, CB-H and CB-L were orally inoculated with \(2 \times 10^7\) CFU/mL nalidixic acid resistant \(S. \text{typhimurium}\) strain 112910a (1 mL/pig), which was previously isolated from a pig without clinical signs of salmonellosis (Boyen et al., 2009). Individual fecal samples were collected at day −2, 7, 21 and 42, blood samples were collected at day −2, 21 and 42. At necropsy, intestinal contents samples (cecum and colon) and ileocecal lymph nodes were collected. All pigs were weighed individually at arrival and at euthanasia. Individual daily weight gain was calculated by dividing the total weight gain by the number of trial days.

The experimental design was approved by the Ethical Committee of CODA-CERVA and WIV (currently Sciensano, approval number: 20170620-01).

2.1. Bacteriological analysis

All fecal samples, intestinal contents samples and ileocecal lymph nodes were tested for the presence of \(Salmonella\) based on ISO 6579:2002/Amd1:2007 Annex D. In short, 1:10 dilutions in bupeptone water (BPW) were incubated and after that, 100 µL of each BPW-solution was spotted in three drops on a Modified semi-solid Rappaport-Vassiliadis (MSRV) agar plate. MSRV-plates were incubated \(24\) h, or \(48\) h if no typical migration zone was present after \(24\) h. From all positive MSRV-plates, a loopful from the edge of a typical migration zone was streaked onto a xylose lysine deoxycholate agar plate (XLD) and a RAPID \(Salmonella\) agar. Final confirmation of suspect colonies was done by a \(S. \text{typhimurium}\) specific qPCR (Maurischat et al., 2015).

To determine CFU/g sample, the BPW-solutions were directly plated on MacConkey agar supplemented with nalidixic acid (100 µg/mL). These plates were incubated for 18–24 h, after which lactose negative colonies were counted and CFU/g was calculated (detection limit: \(\pm 10^2\) CFU/g).

2.2. Serological analysis

After coagulation, blood samples were centrifuged for 5 minutes at 3000 g to collect serum. All serum samples were analyzed for the presence of \(Salmonella\)-specific antibodies with a commercial ELISA kit based on lipopolysaccharide (LPS) O-antigens of serogroup B, C1 and D (HerdChek Swine \(Salmonella\)*, IDEXX Laboratories). Sample-to-positive ratios (S/P-ratios) were assessed using the cut-off value \(S/P \geq 0.25\) as positive.

2.3. Statistical analysis

The number of bacteriologically and serologically positive samples from the inoculated groups at the different sampling moments was compared with Fisher exact tests. The average S/P-ratios of the inoculated groups at the different sampling moments were, after a natural logarithmic transformation, compared with an ANOVA test. The start weight, end weight and daily weight gain of all groups were compared with an ANOVA test.

All statistical analyses were performed in SPSS Statistics (version 25). \(P\)-values \(\leq 0.05\) were considered statistically significant.

3. Results

3.1. Clinical symptoms

No clinical signs of salmonellosis were detected during the trial.

3.2. Bacteriological analysis

Before inoculation, all fecal samples tested negative for \(Salmonella\). The negative control group remained \(Salmonella\) negative throughout the study. The fecal and intestinal contents samples from all groups were negative after direct plating, indicating that fecal excretion and intestinal carriage levels were below \(10^2\) CFU/g. In total, 10 lymph nodes were positive after direct plating (range: \(2 \times 10^2\)–\(10^4\) CFU/g, median: \(4 \times 10^2\) CFU/g).

Table 1 Percentage fecal samples bacteriological \(S. \text{typhimurium}\) positive (after enrichment), percentage serological positive samples and the average S/P-ratios and standard deviations (SD) per group\(^a\), at the different sampling moments.\(^b\)

<table>
<thead>
<tr>
<th>Group</th>
<th>Samples collected at day ..</th>
<th>2 (%)</th>
<th>7 (%)</th>
<th>21 (%)</th>
<th>42 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Feces % positive</td>
<td>Serum % positive</td>
<td>Average S/P-ratio (SD)</td>
<td>Feces % positive</td>
<td>Serum % positive</td>
</tr>
<tr>
<td>Negative control</td>
<td>0</td>
<td>0</td>
<td>0.06 (0.07)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Positive control</td>
<td>0</td>
<td>0</td>
<td>0.03 (0.05)</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>CB-H</td>
<td>0</td>
<td>10</td>
<td>0.02 (0.10)</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>CB-L</td>
<td>0</td>
<td>0</td>
<td>0.01 (0.05)</td>
<td>30</td>
<td>25</td>
</tr>
</tbody>
</table>

No significant differences were detected between the different inoculated group.

* Negative control: no feed additive (\(n = 5\)); Positive control: no feed additive (\(n = 10\)); CB-H: \(\pm 2 \times 10^6\) CFU \(C. \text{butyricum}/g\) feed (\(n = 10\)); CB-L: \(\pm 5 \times 10^5\) CFU \(C. \text{butyricum}/g\) feed (\(n = 10\)).

\(^b\) Day -2 = 2 days before inoculation; Day 7 = 7 days after inoculation; Day 21 = 21 days after inoculation; Day 42 = 42 days after inoculation.

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The percentage fecal samples positive after enrichment in each group at the different sampling moments is shown in Table 1. The percentage positive samples at necropsy is shown in Fig. 1. When combining the bacteriological analysis results of fecal samples, intestinal contents samples and ileocecal lymph nodes, 100%, 100% and 90% of the pigs in the positive control group, CB-H and CB-L, respectively, tested positive at least once during the trial.

No significant differences were detected between the different inoculated groups for the number of positive fecal samples at the different sampling moments, the number of positive lymph nodes after direct culture and after enrichment, and the number of positive colon contents samples. The number of positive cecum contents samples was significantly higher in the group receiving ± 2 × 10^6 CFU C. butyricum/g feed (CB-H), compared to the positive control group (p = 0.033).

3.3. Serological analysis

One of the serum samples tested positive (S/P-ratio: 0.29) before the inoculation, presumably due to maternal immunity (the pig tested serologically negative at day 21). The negative control group was serologically negative for Salmonella throughout the study. The percentage serologically positive samples and the average S/P-ratios at the different sampling moments per group are shown in Table 1. At day 42, 80%, 90% and 80% of the pigs in the positive control group, CB-H and CB-L, respectively, tested serologically positive.

No significant differences were detected between the different inoculated groups for the percentage of pigs serologically positive and the average S/P-ratios at the different sampling moments.

3.4. Daily weight gain

The average daily weight gain of the pigs in the negative control group, positive control group, CB-H and CB-L were 0.416, 0.477, 0.447 and 0.462 kg, respectively. No significant differences were detected between the different groups.

4. Discussion

In the current trial, the probiotic feed additive C. butyricum, at two different dosages, did not significantly reduce the fecal excretion, serological response, intestinal carriage and prevalence of S. typhimurium in the ileocecal lymph nodes in experimentally challenged pigs.

Based on the results before inoculation and the negative control group, it can be concluded that the purchased pigs were Salmonella-negative. The inoculation was successful, resulting in a subclinical infection with intermittent fecal excretion of S. typhimurium, as can be concluded based on the results of the positive control group. The induced infection seemed to be mild, resulting in relatively little room for improvement. In addition, the used dosage, time frame and duration of application of the probiotic additive, could have influenced the results of the trial. Also, individual differences in the response to the additive (including different breeds and ages of the pigs), differences in husbandry practices (Simon et al., 2001; Liao and Nyachoti, 2017) and diet dependent effects have been proposed (Merrifield et al., 2013; Liu et al., 2018) to explain inconsistent results.

Yang et al., reported a dose dependent response of broiler chickens receiving C. butyricum as a feed additive (Yang et al., 2012). In the current trial, no significant differences were detected between groups CB-H and CB-L, the first group receiving four times the dose of C. butyricum compared to the last group, ± 2 × 10^6 CFU/g feed and ± 5 × 10^5 CFU/g feed, respectively.

In previous research, the percentage positive cecum and colon contents samples (Farrow et al., 2012) and the number of S. typhimurium (Wood and Rose, 1992) in the cecum and colon were similar after oral experimental infection of pigs. In the current trial, the percentage positive colon contents samples was higher than the percentage positive cecum contents samples in all inoculated groups. No clear explanation for this difference could be found. Therefore, the biological relevance of the increased prevalence of S. typhimurium in the cecum contents in the group receiving ± 2 × 10^6 CFU C. butyricum/g feed (CB-H), compared to the positive control group is uncertain.

Several papers report positive effects of probiotics containing C. butyricum on the performance of pigs (Meng et al., 2010; Balasubramanian et al., 2018). In the current experimental study, the probiotic feed additive, nor the experimental S. typhimurium infection, influenced the performance of the pigs. Performance was however not a major parameter, due to large biological variation in the growth of pigs, larger groups of animals are needed to reliably assess possible effects on performance.

Although the experimental infection was successful, care should be taken to extrapolate the results to field situations. Under field conditions many other factors, e.g. infection with multiple Salmonella serotypes, co-infections with other intestinal pathogens, suboptimal nutrition, housing and management and long-term effects of intervention measures, may play a role and determine the outcome of Salmonella infections and control measures. Also, the effects of probiotic feed additives may be influenced by farm- and time-specific factors (Taras et al., 2007). Therefore, it is not easy to predict their effects in field situations, based on sole experimental trials.

5. Conclusion

Under the present conditions, the probiotic feed additive C.
butyricum, at two different dosages, did not significantly reduce fecal excretion, serological response, intestinal carriage, and prevalence of S. typhimurium in the ileocecal lymph nodes in an experimental model of subclinical salmonellosis in pigs.

Acknowledgments

We thank the farmer and veterinarian of the origin farm of the pigs for facilitating this trial. Also, we gratefully appreciate the technicians and veterinarians of the animal research facilities and the students for their assistance during inoculation, sampling and the care taking of the pigs, as well as the lab personnel for analyzing all the samples.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interests statement

The authors have no competing interests to declare.

References


