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1 **Evaluation of the inclusion of a mixture of organic acids or lactulose into the feed**
2 **of pigs experimentally challenged with *Salmonella* Typhimurium**

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23

23 **Abstract**

24 A mixture of organic acids and lactulose for preventing or reducing colonization
25 of the gut by *Salmonella* Typhimurium was evaluated in pigs. A total of 63 4-week-old
26 commercial piglets were randomly distributed into three different experimental dietary
27 groups: a plain diet without additives (PD) and the same diet supplemented with either
28 0.4 % (w/v) formic acid and 0.4 % lactic acid (w/v) (AC) or 1 % (w/v) lactulose (LC).
29 After 7 days of adaptation, two-thirds of the pigs (14 from each diet) were challenged
30 with a 2 mL oral dose of 10^8 CFU/mL of *Salmonella* Typhimurium, leaving the
31 remaining animals unchallenged (UC). After 4 and 10 days post-challenge, pigs were
32 euthanized and the ileum and caecum content were aseptically sampled to (a) quantify
33 lactic, formic, and short-chain fatty acids (SCFA), (b) quantify bacterial populations and
34 *Salmonella* by fluorescence *in situ* hybridization and (c) qualitatively analyse bacterial
35 populations through denaturing gradient gel electrophoresis (DGGE). Modification of
36 fermentation products and counts of some of the bacterial groups analysed in the
37 challenged pigs receiving the treatments AC and LC were minimal. Treatments only
38 influenced the bacterial diversity after 10 days post-challenge, with AC generating a
39 lower number of DGGE bands than UC ($P < 0.05$). Neither the inclusion of a mixture of
40 0.4 % (w/v) formic and 0.4 % (w/v) lactic acids nor of 1 % (w/v) lactulose in the feed
41 influenced numbers of *Salmonella* in the ileum and caecum of experimentally
42 challenged pigs.

43 1. Introduction

44 In 2007 salmonellosis still remained the second most commonly reported
45 zoonotic disease in the EU with a rate of 31.1 cases per population of 100,000. As in
46 previous years, *S. Enteritidis* and *S. Typhimurium* were the most frequently reported
47 serovars (81% of all known serovars in human cases). *Salmonella* was most often found
48 in fresh poultry (*S. Enteritidis*) and pig meat (*S. Typhimurium*) (European Food safety
49 Authority, 2007). In the case of the pig meat, the route of infection requires
50 asymptomatic infected pigs from the farm, which serve as a source of contamination of
51 carcasses in the slaughter chain (Widders *et al.*, 1996).

52 In order to reduce the incidence of *Salmonella* in pigs on farms, dietary
53 interventions are being considered. One of these is the inclusion of organic acids (OA)
54 in the feed of the pigs. Various studies have shown the potential of some OA to reduce
55 the prevalence of the pathogen in the pigs when added to water (van der Wolf *et al.*,
56 1999) or feed (Jorgensen *et al.*, 1999; Creus *et al.*, 2007). The use of prebiotic
57 compounds has also been proposed as a possible strategy to reduce *Salmonella*
58 prevalence in pigs (Wiemer *et al.*, 1999). Lactulose (a synthetic disaccharide) has been
59 shown to elicit a prebiotic effect (increased counts of bifidobacteria and lactobacilli) in
60 pigs (Konstantinov *et al.*, 2004) and has been proposed as feed additive (1 %) to prevent
61 salmonellosis in fattening pigs (Wiemer, 1999). *In vitro* experiments have also shown
62 potential inhibitory effects of lactulose on *Salmonella Typhimurium* (Martín-Peláez *et*
63 *al.*, 2008).

64 The aim of the present study was to investigate the potential of a mixture of
65 either lactic and formic acids or lactulose to prevent or reduce the colonization of the
66 gut by *Salmonella Typhimurium* in pigs.

67

68 2. Materials and methods

69 2.1. Strain

70 The *Salmonella* Typhimurium phagetype U302 strain used in the study was
71 obtained from the stock NI-3 belonging to the Veterinary Laboratory on Infectious
72 diseases (UAB) which had been originally isolated from a clinical case of porcine
73 salmonellosis and was shown to be resistant to the antibiotics: ampicillin,
74 chloramphenicol and gentamicin. The bacteria were grown on tryptic soy agar (TSA,
75 Oxoid, UK) for 24 h at 37°C. Afterwards three tubes containing brain-heart infusion
76 broth (BHI, Oxoid, UK) (3 mL/tube) were inoculated with one colony from the TSA
77 plate (1 colony/tube) and incubated for 24 h at 37°C. The day of the challenge, the
78 contents of the three tubes were mixed, diluted with PBS (1:20, v/v) and used for
79 challenge. The placebo was prepared by mixing 2.5 mL of BHI broth plus 47.5 mL of
80 PBS (Oxoid, UK). Doses (2 mL) consisted of either the placebo or the inoculum
81 dispensed in 2.5 mL syringes. One millilitre of inoculum and 1 millilitre of placebo
82 were kept for cultivation and counting. The challenge dose was 1×10^8 CFU.

83 All procedures involving the treatment, housing, husbandry and euthanasia of
84 animals received prior approval from the Animal Protocol Review Committee of the
85 Universitat Autònoma de Barcelona (Spain) and of the Veterinary Laboratories Agency
86 (United Kingdom), and were carried out according to European Union Guidelines (The
87 Council of the European Communities, 1986).

88

89 2.2. Animals and diets

90 A total of 63 4-week-old Large White male piglets (7.5 ± 0.2 kg body weight)
91 were used for the study. Animals were weaned at 3 weeks of age and obtained from a
92 high-health status farm, free from all major pathogens of swine including *Salmonella*.

93 Sows ($n=24$) and piglets were tested serologically (Svanovir *Salmonella* Ab ELISA,
94 Svanova, Uppsala, Sweden) and bacteriologically (faeces) for *Salmonella* prior to
95 inclusion in the experimental infections. All animals were negative using both tests.

96 Diets consisted of either the plain diet (Table 1) without additives (PD), the plain
97 diet with 0.4 % (w/v) formic acid and 0.4 % (w/v) lactic acid added (AC) or the plain
98 diet with 1 % (w/v) lactulose added as syrup (15 mg/kg) (LC).

99

100 2.3. Experimental design

101 Pigs were transported from the weaning farm to the biosafety level 3 facilities at
102 Centre de Recerca en Sanitat Animal (CRESA), ear-tagged and distributed into three
103 separate boxes (1, 2 and 3) with three compartments each, one for each diet (AC, LC
104 and PD; 7 animals per compartment). Animals were evenly distributed to get a similar
105 mean live weight between compartments. Each compartment was physically separated
106 from the others by means of a solid partition. Once distributed, the animals were
107 provided with feed and water *ad libitum* for 18 days. After 7 days of adaptation, each
108 pig from boxes 1 and 2 was challenged with an oral dose of 2ml of inoculum as
109 previously described, whereas each pig from box 3 (UC) was dosed with 2 mL of a
110 placebo. In order to ensure that stomach contained feed before challenge, the pigs were
111 not be fed from 20:00 h until next day early in the morning, approximately 1 h before
112 challenge. All animals were clinically examined from days 1 to 10 post-challenge and
113 body temperature was recorded. Faeces (about 5 grams) were collected directly from the
114 rectum daily from day 0 to day 4 post-challenge and then on days 7 and 10 post-
115 challenge. On days 4 and 10 post-challenge, three randomly selected piglets from each
116 subgroup were sedated with azaperone (2 mg/kg, intra-muscular) and then euthanized
117 by an intravenous overdose of sodium thiopental (200 mg/kg live weight). Mesenteric

118 lymph nodes were also taken at that time for bacteriological analysis. The rest of
119 animals (one per compartment) were also euthanized during the 11 PC day but they
120 were not sampled. They were included in the study in prevention of occasional deaths.

121 At *post-mortem* ileal and caecal contents were aseptically sampled for analysis
122 of lactic acid and SCFA by adding 2 mL of digesta to 2 mL of a preservative solution
123 (0.0074 M HgCl₂, 0.344 M H₃PO₄, 0.0172 M C₆H₁₂O₂ in distilled water), for FISH
124 analysis by adding 0.5 mL of digesta to 1.5 mL of 4 % (w/v) paraformaldehyde and for
125 DGGE analysis by adding 0.25 mL of digesta to a tube containing 1.75 mL of buffer
126 ASL (QIAmp® DNA Stool Mini Kit, Qiagen Ltd, West Sussex, UK).

127

128 2.4. Analytical procedures

129 SCFA concentrations in caecal samples were analysed by gas-liquid
130 chromatography using the method of Richardson et al. (1989) modified by Jensen et al.
131 (1995).

132 Faecal samples were examined microbiologically both qualitatively and semi-
133 quantitatively and lymph nodes samples only qualitatively. For the qualitative analysis,
134 1 gr of faeces (or 1 gr of minced tissue) was re-suspended on 10 mL of buffered peptone
135 water (BPW, Oxoid, UK) and incubated overnight at 37°C. Then 100 µl of the BPW
136 were transferred to 10 mL of Rappaport-Vassiliadis broth (RVB, Oxoid, UK) and
137 incubated for 48h at 41.5°C. Finally, 10 µl of RVB were streaked onto xylose-lysine-
138 tergitol 4 (XLT4, Oxoid, UK) plates and incubated for 24 h at 37°C. Suspected
139 *Salmonella* colonies were further identified by means of the API 20E system. For the
140 semi-quantitative analysis, once the samples had been re-suspended in BPW and before
141 any incubation, 100 µl of BPW were taken and serially diluted from 10⁻¹ to 10⁻⁵ in
142 0.85% sterile saline. One hundred microlitres of the 10⁻² and 10⁻⁵ dilutions were

143 streaked onto XLT4 plates to which chloramphenicol and gentamicin had been added.
144 *Salmonella* colonies were counted after 24 h of incubation at 37°C. Randomly chosen
145 positive isolates were identified by means of the API20E system (Bio-Mérieux,
146 Barcelona, Spain), serotyped and the antimicrobial susceptibility pattern was
147 determined in an antibiogram to confirm that they corresponded to the original strain
148 used for the challenge. With this scheme, all animals had a double evaluation for each
149 day: positive/negative and, if positive, a score was given as following: 0, for negative
150 animals; 1, for positive animals but with no colonies at 10^{-2} ($<10^3$ CFU/gr); 2 for
151 animals with less than 100 colonies at 10^{-2} ($<10^5$ CFU/gr); 3 for more than 100 colonies
152 at 10^{-2} but less than 10 at 10^{-5} (10^6 - 10^7 CFU/gr); 4, with >10 CFU at 10^{-5} ($>10^7$
153 CFU/gr).

154 Processing of samples for FISH analysis, and enumeration of bacteria present in
155 500- μ L ileal and caecal samples were carried out as described by Martín-Peláez et al.
156 (2008). Table 2 gives details of the probes used in this study; all probes were Cy3-
157 labelled and synthesized by Sigma Aldrich.

158 DNA from caecal samples was extracted using the QIAmp® DNA Stool Mini
159 Kit following the manufacturer's instructions. The recommended lysis temperature was
160 increased to 90°C, and afterwards an incubation step with lysozyme (1 mg/mL of 50000
161 U/mg protein; Sigma) was added (10 mg/mL, 37°C for 30 min) in order to improve
162 lysis of bacterial cells, according to Castillo et al. (2006). DNA quality was checked by
163 running 5 μ L aliquots of the samples on 0.8 % (w/v) ultraPURE agarose (GibcoBRL)
164 gels containing ethidium bromide (0.4 mg/mL), and visualizing the bands using a UV
165 light. The quantity of DNA in each sample was determined using a NanoDrop ND-1000
166 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Fragments (230
167 bp) derived from the variable V3 region of the 16S rRNA gene were amplified from

168 samples by using the universal primers P2 (5'-ATTACCGCGGCTGCTGG-3') and P3
 169 (5'-
 170 CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG
 171 GCAGCAG-3') of Muyzer et al. (1993) according to Waldram et al. (2009). Primers
 172 were synthesized by Sigma-Genosys, UK.

173 DGGE was carried out as described by Waldram et al. (2009) but with minor
 174 modifications: the V20-HCDC DGGE system (BDH) was used with gels comprising
 175 35–60 % gradients and run at 100 V. Gels were silver-stained according to the method
 176 of Sanguinetti et al. (1994). Gels were scanned using CanonScan (Canon, Surrey, UK),
 177 saved as TIFF files (600 dpi) and imported into TotalLab TL120 v2006f (NonLinear
 178 Dynamics Ltd, UK) for analyses. Matching calculations were performed based on
 179 Jaccard similarity coefficient (matches by the presence/absence of bands). Dendrograms
 180 were constructed by using Unweighted Pair-Group Method with Arithmetic mean
 181 (UPGMA) analysis. Finally, Shannon index, a measure of diversity, was calculated
 182 following the methodology proposed by Magurran (1988).

183

184 2.5. Statistical analysis

185 Data from FISH and SCFA were analyzed by ANOVA using the GLM
 186 procedure of SAS (v. 9.1., SAS Inst. Inc., Cary, NC, USA). Firstly, results for the UC
 187 pigs receiving the three experimental diets (PD, AC, LC) were run separately from the
 188 challenged pigs, and by day of sacrifice, according to the following model:

$$189 \quad Y_i = \mu + \alpha_i + \varepsilon_i$$

190 Where Y_i was the dependent variable, μ was the overall mean, α_i was the effect of the
 191 diet and $\varepsilon \sim N(0, \sigma^2_\varepsilon)$ represented the unexplained random error. As experimental diets
 192 did not show consistent effects on UC animals, in a second analysis all the UC animals

193 were consider as one single experimental treatment (UC) and analysed together with the
194 challenged animals received the three diets (PD, AC, LC).

195 Treatment means or LS means were assessed by multiple comparison methods
196 using Tukey's correction. Statistical significance was accepted at $P<0.05$ and
197 differences among means with $0.05<P<0.10$ were accepted as representing tendencies
198 to differences.

199

200 **3. Results**

201 Challenge of the pigs resulted in mild clinical signs of salmonellosis with slight
202 fever seen from day 1 until day 4 post-challenge and slight diarrhoea (loose or liquid
203 faeces) from day 2 until day 7 post-challenge, normalizing in the following days. Faecal
204 shedding of *Salmonella* and positive cultures from mesenteric lymph nodes were found
205 in all pigs. The quantitative analysis of faeces (considering all inoculated pigs) showed
206 that the highest *Salmonella*-score was detected by day 3 post-challenge ($P<0.05$) with a
207 mean value of 1.9 (pigs with a score higher than 2: 8/14 in groups AC and PD and 7/14
208 in group LC) and a mean bacterial load of $10^{4.36} \pm 10^{0.72}$ CFU/gr. Although no
209 significant differences were seen among treatments ($P = 0.15$), it is interesting to point
210 out that maximum *Salmonella* scores ($4=$ more than 10^7 CFU/gr) were only seen in
211 groups AC (1 pig, day 3 post-challenge) and PD (3 pigs day 3 post-challenge).

212 Concentrations of lactic and formic acid and of total SCFA were not consistently
213 modified by the experimental diets in the UC pigs. Total SCFA in ileum were
214 significantly lower ($P<0.05$) in AC and LC diets compared to PD at day 4 post-
215 challenge (9.09, 9.11 and 17.41 for AC, LC and PD, respectively, SEM: 1.352) and at
216 day 10 post-challenge, AC diets showed higher levels than PD ($P=0.004$) (12.16, 4.93,
217 5.25 for AC, LC and PD, respectively, SEM: 1.055). Regarding SCFA concentrations in

218 the caecum, no differences were observed at say 4 post-challenge or 10 post-challenge.
219 With regards to molar percentages of each SCFA, no significant differences were found
220 except for BCFA in the ileum at day 4 post-challenge, with higher proportions
221 ($P=0.013$) for the AC diet (1.29, 0.30, 0.00 % for AC, LC and PD, respectively, SEM:
222 0.260).

223 Table 3 shows the results obtained when data from UC and challenged pigs were
224 included in the same statistical model as one experimental treatment. After 4 days post-
225 challenge treatments tended to influence the amounts of formic acid and SCFA in the
226 ileum of pigs but these differences disappeared after 10 days post-challenge. Treatments
227 AC and LC generated the same amount of total SCFA as UC, but PD generated less
228 than UC ($P=0.046$). Profiles of the different SCFA were not influenced by treatment. In
229 the caecum, treatments only exerted an effect after 4 days post-challenge. Treatment LC
230 generated lower amounts of SCFA than UC ($P=0.032$). Formic and lactic acids and the
231 proportions of the different SCFA were not modified by the treatments.

232 Changes promoted by the different experimental treatments in some of the most
233 relevant microbial groups were examined by FISH analysis of ileal and caecal samples.
234 No significant differences related to diets were found in UC pigs. Table 4 shows the
235 results obtained when data from UC and challenged pigs were included in the same
236 statistical model. As expected, challenge of the pigs with *Salmonella* resulted in Sal303
237 counts in both the ileum and caecum at 4 and 10 days post-challenge. No *Salmonella*
238 (Sal303) was detected in the UC group or in any challenge group prior to challenge. No
239 differences in Sal303 counts were found in the challenged pigs with any of the diets.

240 The treatments influenced the levels of different bacterial populations in ileal
241 (Rbro730/Rfla729) and caecal (Bac303, Bif164, Erec482) samples. Only Lab158 and
242 Prop853 were not affected at any time by the treatment in either segment of the GIT

243 analysed. In the ileum, differences were found only after 10 days post-challenge. AC
244 and PD showed lower Bac303 counts compared to UC ($P = 0.075$, $P = 0.083$
245 respectively) whereas LC showed lower Rbro730/Rfla729 counts compared to UC
246 ($P < 0.001$).

247 Regarding microbial communities in the caecum, some changes promoted by the
248 experimental treatments were seen at 4 days post-challenge but not at 10 days post-
249 challenge. At 4 days post-challenge, LC increased Bac303 counts compared to UC ($P =$
250 0.022). At 4 days post-challenge, LC decreased Erec482 counts ($P = 0.017$), and
251 increased Bif164 counts ($P = 0.009$) compared to UC. Treatment LC increased the
252 counts of Bif164 counts compared to AC ($P = 0.008$)

253 Changes in the caecal microbial ecosystem promoted by the experimental
254 treatments were analysed by DGGE. Figure 1 shows the box plots for the number of
255 DGGE bands. No significant differences were found in species richness at day 4 post-
256 challenge (AC, 28.3; LC, 28.0; PD, 22.6; UC, 26.3; $P = 0.150$). At day 10 post-challenge,
257 AC generated significantly fewer bands than UC ($P < 0.05$) (20.67, 22.83, 22.50 and 25.0
258 for AC, LC, PD and UC, respectively). Results from Shannon index did not show any
259 differences among treatments at day 4 post-challenge (AC, 5.28; LC, 5.27; PD, 4.92,
260 UC, 5.58) nor at day 10 post-challenge (AC, 5.01; LC, 5.14; PD, 5.10, UC, 5.58). The
261 similarity indices of the DGGE profiles at days 4 and 10 post-challenge are shown in
262 Figure 2. The high complexity and inter-individual differences in the DGGE profiles
263 were such that the cluster analysis did not show any obvious grouping of profiles.

264

265 **4. Discussion**

266 The aim of this study was to investigate if the inclusion of a mixture of OA
267 (lactic and formic acid) or lactulose in feed was able to reduce gut colonization by

268 *Salmonella* Typhimurium in experimentally challenged pigs. The mixture of OA
269 assayed had shown positive results against *Salmonella* in growing-finishing pigs (Creus
270 et al., 2007) and lactulose had shown promising results against *Salmonella*
271 Typhimurium compared to other prebiotics *in vitro* (Martín-Peláez et al., 2008).

272 The experimental infection aimed to induce mild diarrhoea, in order to be able to
273 be overcome by the natural defences of the animal, defences which would be reinforced
274 by the use of feeding strategies as those tested in our study. Besides the moderate level
275 of the infection, no diet-related differences in numbers of *Salmonella* (probe Sal303)
276 were found in ileal or caecal samples although in the group of inoculated pigs receiving
277 the diet without additives a higher number of pigs excreting more than 10^7
278 *Salmonella*/gr faeces was found.

279 Regarding the use of OA against *Salmonella*, one theoretical mechanism by
280 which they are thought to work is by increasing the concentration of OA in the stomach;
281 this would reduce the population of enterobacteria, thereby creating a biological barrier
282 against enterobacteria colonizing the lower parts of the GIT (Canibe et al., 2005;
283 Hansen et al., 2007). In this study, pH and OA in the stomach were not measured;
284 therefore, no conclusions can be made regarding this potential effect, but if any
285 differences in the concentration of lactic or formic acid were promoted, they were not
286 detected in the ileum. A decrease in species richness in the caecum, as determined by
287 DGGE, was seen with the use of OA, possibly due to the inhibition of bacterial
288 populations not analysed in this study.

289 Despite the absence of inhibition of *Salmonella* Typhimurium by the OA used in
290 this study, other authors have found significant effects on *Salmonella* when using the
291 same mixture. Creus et al. (2007) using a commercial diet supplemented with 0.4 %
292 lactic acid and 0.4 % formic acid during the last 8–9 weeks in fattening pigs observed

293 lower levels of *Salmonella* seroprevalence compared to a control diet and also decreases
294 in the presence of *Salmonella* in faeces and mesenteric lymph nodes. Similarly, Do
295 Santos et al. (2007), using the same mixture for 5 weeks in fattening pigs, found lower
296 numbers of seropositive pigs compared to a commercial control diet. The lack of effect
297 found in our study compared to the results of these field studies could be explained by
298 several reasons; for instance, differences in the age of the pigs (market age pigs vs
299 piglets) and the length of the treatment (5–9 weeks vs 2 weeks). Young pigs are known
300 to be unable to acidify their stomach contents as efficiently as adult pigs, due to the low
301 capacity to synthesize HCl by gastric parietal cells (Cranwell, 1985), leading to
302 insufficient killing of microbes. In this sense, inclusion of OA in the diet would help to
303 compensate the insufficient acidification of the stomach. However, some authors have
304 suggested that inclusion of acids in post-weaning diets could have a contrary effect.
305 Bosi et al. (2006) found that supplementation of weaned pigs with calcium formate (1.2
306 %) decreased the number of HCl-secreting parietal cells and increased somatostatin-
307 producing cells (somatostatin has a suppressive action on HCl secretion). Thus, addition
308 of formate to the feed of weaned pigs could exert a negative feedback on gastric acid
309 secretion. An additional reason that could explain the lack of effects of the OA may be
310 the differences in the way the animals are exposed to the pathogen in the natural
311 environment compared to an experimental infection. Natural infections may arise
312 through continuous exposure to low doses of *Salmonella* which is probably protected by
313 being bound in faecal, soil or other environmental materials. This situation is difficult to
314 reproduce in experimentally infected animals, which are exposed to a single high dose
315 of *Salmonella*. In that situation, *Salmonella* probably has more chance to overwhelm the
316 gastric barrier fortified by the use of in-feed acidifiers. Further studies in which naïve

317 animals are mixed with deliberately inoculated animals would be useful in interrogating
318 the power of OA to protect against naturally acquired infection.

319 The inclusion of lactulose in the feed did not influence *Salmonella* Typhimurium
320 survival compared to PD. This lack of effect could be related to a lack of stimulation of
321 fermentative activity in the ileum or caecum, as indicated by the scarce variations found
322 in the amounts and patterns of SCFA produced. This absence of increases in the
323 fermentative activity could be related to the rapid fermentability of lactulose found by
324 other authors (Kamphues et al., 2007), implying that lactulose would be almost
325 completely fermented before reaching the large intestine. In addition, a lactulose-
326 splitting capacity of the pig's small intestine mucosa has been indentified (Harju, 1986).
327 It is possible that the dose of lactulose used in this study (1 %, w/v), although
328 recommended by other authors to prevent *Escherichia coli* infection (Sutton and
329 Patterson, 1996) or salmonellosis in fattening pigs (Wiemer, 1999), was insufficient.
330 Another study involving fattening pigs used higher doses of lactulose (2.8 %, w/v) and
331 did not demonstrate any protective effect of lactulose against *Salmonella*, with the
332 authors attributing this to an insufficient dose used, based on the absence of significant
333 changes in total SCFA and lactic acid along the gut (Kamphues et al., 2007).

334 The inclusion of lactulose did not reduce the invasive capacity of *Salmonella*,
335 since all pigs were *Salmonella*-positive in mesenteric lymph nodes. It is known that an
336 increase in a specific SCFA, such as butyric acid, can downregulate the expression of
337 virulence genes of *Salmonella* (Cherrington et al., 1991). Gantois et al. (2006)
338 speculated that modifications of the gut microbiota to produce more butyric acid could
339 reduce invasive infections by *Salmonella* serovars in farm animals. Previous studies *in*
340 *vitro* have shown increases in the percentage of butyric acid formed with the
341 fermentation of lactulose (Martín-Peláez et al., 2008). However, in the present study,

342 LC did not increase the proportion of butyric acid detected; therefore, an inhibitory
343 effect elicited by butyric acid on *Salmonella* virulence would not be expected.

344 **5. Conclusions**

345 Under our study conditions, neither the inclusion of a mixture of 0.4 % formic
346 and 0.4 % lactic acids nor of 1 % lactulose in the feed influenced survival of *Salmonella*
347 Typhimurium in the ileum and caecum of pigs challenged by direct oral challenge with
348 high doses of *Salmonella*. More studies with higher doses and/or under different
349 conditions of infection would be needed to fully explore the potential of these
350 compounds.

351

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358

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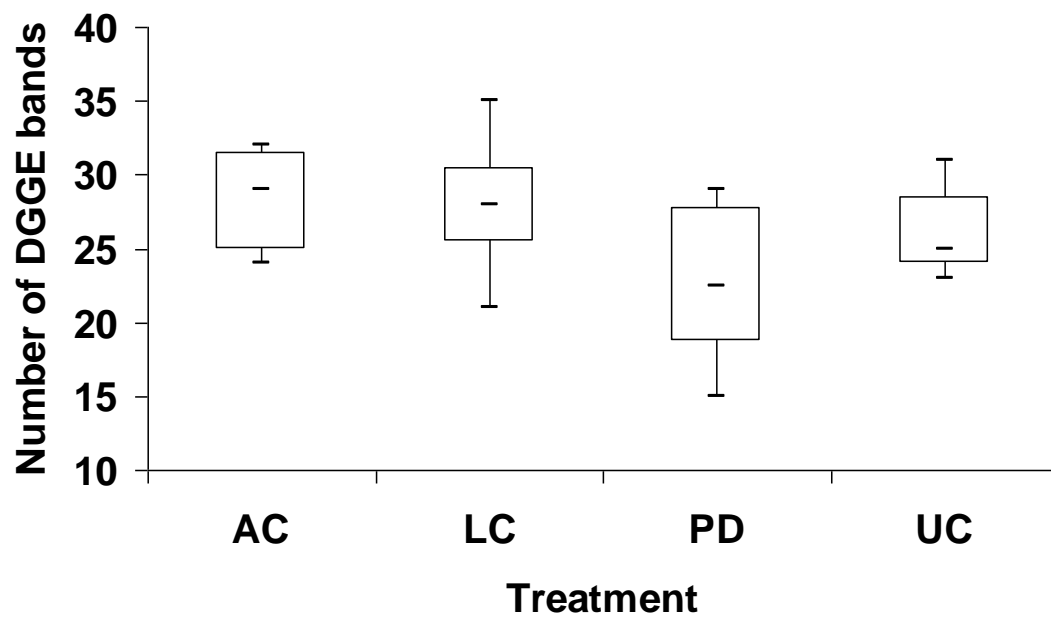
Fig.1. Box plot of the number of DGGE bands detected in caecal digesta of pigs sacrificed at (a) 4 and (b) 10 days post-challenge. AC, *Salmonella*-challenged pigs consuming plain diet with 0.4 % formic acid and 0.4 % lactic acid added; LC, *Salmonella*-challenged pigs consuming plain diet plus 1 % lactulose added; PD, *Salmonella*-challenged pigs consuming plain diet without additives; UC, unchallenged pigs.

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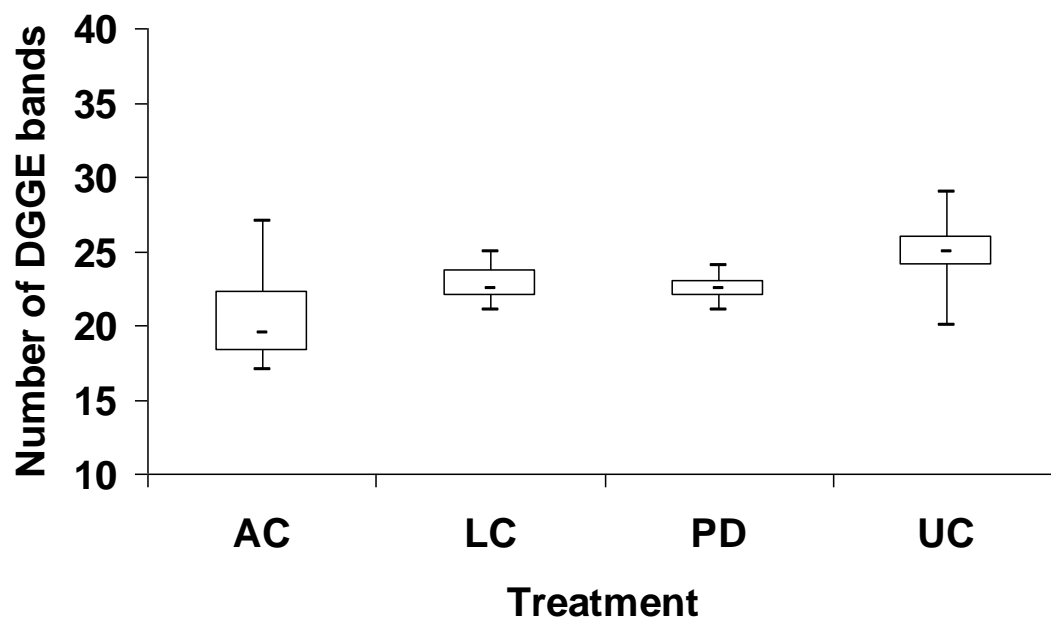
Fig.2. Dendrograms generated from DGGE profiles obtained from caecal digesta of pigs sacrificed at (a) 4 and (b) 10 days post-challenge. Scale bar, per cent similarity of profiles. AC, *Salmonella*-challenged pigs consuming plain diet with 0.4 % formic acid and 0.4 % lactic acid added; LC, *Salmonella*-challenged pigs consuming plain diet with 1 % lactulose added; PD, *Salmonella*-challenged pigs consuming plain diet without additives; UC, unchallenged pigs.

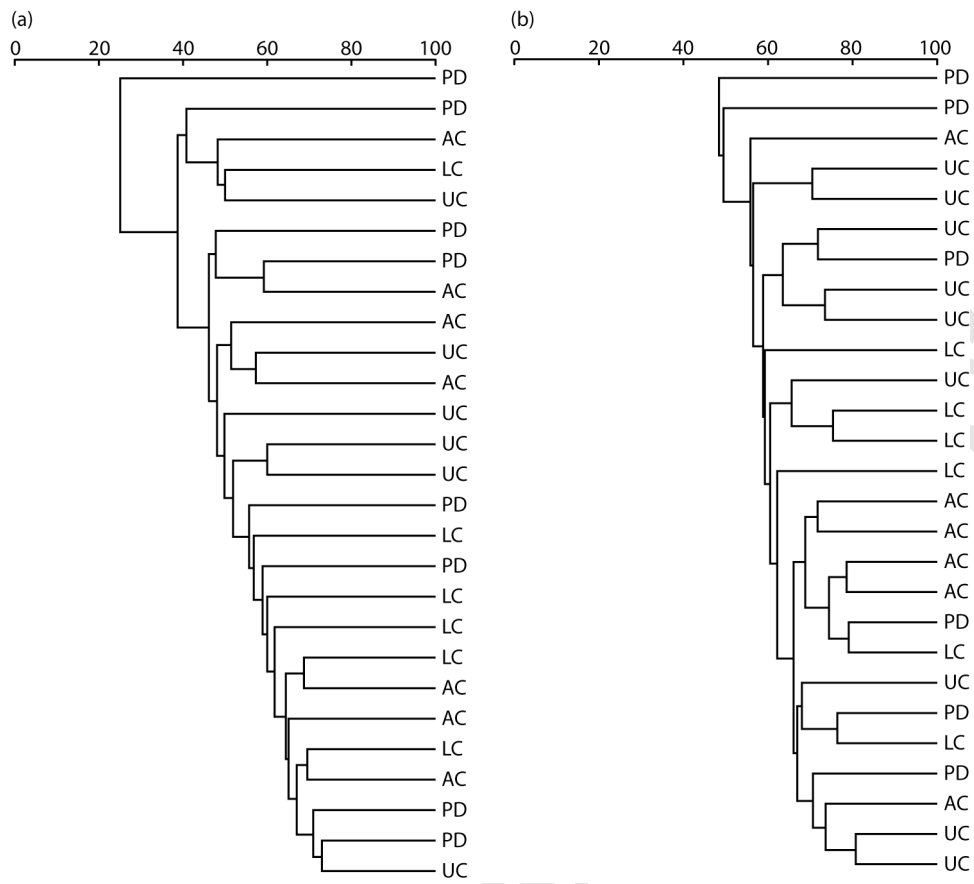
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a)



b)





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Table 1
Details for the composition of the plain diet

Ingredient	g/kg
Corn flakes	360.5
Wheat flakes	240.3
Full fat extruded soybeans	40.0
Soya protein concentrate ^a	60.0
Wheat gluten	60.0
Potato protein	30.0
Fat-filled sweet whey	30.0
Sweet whey	150.0
Calcium phosphate (dicalcium) (18 %)	16.0
L-Lysine HCl 99 %	5.0
DL-Methionine 99 %	0.3
L-Threonine 98 %	0.7
L-Tryptophan 98 %	0.2
Choline hydrochloride 50 %	2.0
Vitamin and mineral pre-mix ^b and additives	5.0
Chemical analysis	
Gross energy	176.8 kJ
Dry matter	920.3
Ashes	47.2
Crude protein	196.8
Crude fat	37.2
Acid detergent fibre	18.2
Neutral detergent fibre	55.5

^aSoya HP-300. Hamlet protein A/S (Spain).

^bProvided the following per kilogram of diet: vitamin A, 10000 IU; vitamin D3, 2000 IU; vitamin E, 15 mg; vitamin B1, 1.3 mg; vitamin B2, 3.5 mg; vitamin B12, 0.025; vitamin B6, 1.5 mg; calcium pantothenate, 10 mg; nicotinic acid, 15 mg; biotin, 0.1 mg; folic acid, 0.6 mg; vitamin K3, 2 mg; Fe, 80 mg; Cu, 6 mg; Co, 0.75 mg; Zn, 185 mg; Mn, 60 mg; I, 0.75 mg; Se, 0.10; etoquin, 0.15 mg.

Table 2

Probes used for FISH analysis of bacterial populations in ileal and caecal samples

Short name	Accession no. ^a	Full name ^b	Target species	Sequence (5' to 3')	Reference
Bif164	pB-00037	S-G-Bif-0164-a-A-18	Most <i>Bifidobacterium</i> spp. and <i>Parascardovia denticolens</i>	CATCCGGCATTACCACCC	Langendijk et al., 1995
Bac303	pB-00031	S-*-Bacto-0303-a-A-17	Most <i>Bacteroides sensu stricto</i> and <i>Prevotella</i> spp.; all <i>Parabacteroides</i> ; <i>Barnesiella viscericola</i> and <i>Odoribacter splanchnicus</i>	CCAATGTGGGGGACCTT	Manz et al., 1996
Erec482	pB-00963	S-*-Erec-0482-a-A-19	Most members of <i>Clostridium</i> cluster XIVa; <i>Syntrophococcus sucromutans</i> , [<i>Bacteroides</i>] <i>galacturonicus</i> and [<i>Bacteroides</i>] <i>xylanolyticus</i> , <i>Lachnospira pectinschiza</i> and <i>Clostridium saccharolyticum</i>	GCTTCTTAGTCARGTACCG	Franks et al., 1998
Lab158	ND	S-G-Lab-0158-a-A-20	Most <i>Lactobacillus</i> , <i>Leuconostoc</i> and <i>Weissella</i> spp.; <i>Lactococcus lactis</i> ; all <i>Vagococcus</i> , <i>Enterococcus</i> , <i>Melisococcus</i> , <i>Tetragenococcus</i> , <i>Catelicoccus</i> , <i>Pediococcus</i> and <i>Paralactobacillus</i> spp.	GGTATTAGCAYCTGTTTCCA	Harmsen et al., 1999
Prop853	ND	ND	<i>Clostridium</i> cluster IX	ATTGCGTAACTCCGGCAC	Walker et al., 2005
Rbro730 ^c	pB-00558	S-*-Rbro-730-a-A-18	<i>Ruminococcus bromii</i> -like; <i>Clostridium sporosphaeroides</i> and <i>Clostridium leptum</i>	TAAAGCCCAGYAGGCCGC	Harmsen et al., 2002
Rfla729 ^c	pB-00557	S-*-Rfla-729-a-A-18	<i>Ruminococcus albus</i> and <i>Ruminococcus flavefaciens</i>	AAAGCCCAGTAAGCCGCC	Harmsen et al., 2002
Sal303	ND	L-S-Sal-1713-a-A-18	Different serovars of <i>Salmonella</i> spp.	AATCACTTCACCTACGTG	Nordentoft et al., 1997

^aND, No information relating to these probes has been deposited in probeBase (<http://www.microbial-ecology.net/probebase>).

^bProbe designation according to Alm et al. (1996). This information was retrieved from probeBase.

^cThese probes were used together in equimolar concentrations (both at 50 ng/μL). Formamide (20 %) was included in the hybridization buffer.

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Table 3

Concentrations and molar ratios of various fatty acids found in ileal and caecal samples of pigs challenged or unchallenged with *Salmonella* Typhimurium at 4 and 10 days post-challenge.

Treatment*	Concentration (mM) on Day 4 post-challenge			Molar ratio of total SCFA (%) on Day 4 post-challenge					Concentration (mM) on Day 10 post-challenge			Molar ratio of total SCFA (%) on Day 10 post-challenge				
	Lactic	Formic	Total SCFA†	Acetic	Propionic	Butyric	Valeric	BCFA	Lactic	Formic	Total SCFA†	Acetic	Propionic	Butyric	Valeric	BCFA
Ileum																
AC	18.60	10.47	8.45	77.04	7.03	13.96	1.33		27.99	12.02	8.75	79.57	9.18	8.93	0.97	1.35
LC	9.03	12.17	8.94	81.00	1.69	15.52	1.22		8.27	11.68	9.09	84.35	1.82	12.3	0.91	0.63
PD	9.49	6.86	6.24	85.62	2.22	9.86	1.98		23.82	11.26	8.34	83.76	5.14	9.07	1.15	0.87
UC	13.26	17.20	11.87	83.43	1.60	13.31	1.10		14.41	10.07	7.45	80.43	2.68	13.08	1.61	2.19
RSD	9.776	7.422	3.720	6.290	5.008	6.165	0.891		25.55	7.132	4.538	6.734	7.360	4.472	0.834	1.120
<i>P-treatment</i>	0.326	0.092	0.063	0.135	0.200	0.431	0.308		0.532	0.953	0.904	0.150	0.314	0.221	0.361	0.064
Caecum																
AC	8.85	3.44	73.75 ^{ab}	60.22	28.60	7.75	1.76	1.67	7.11	2.66	110.33	56.94	31.23	8.60	2.32	0.92
LC	12.36	3.78	70.61 ^b	57.63	31.30	7.74	1.67	1.66	16.64	5.16	98.12	54.84	31.90	9.23	2.90	1.14
PD	3.41	1.37	80.85 ^{ab}	59.36	28.81	7.82	2.47	1.55	19.15	3.93	85.35	59.73	26.40	10.14	2.91	0.82
UC	9.28	4.11	109.82 ^a	60.35	27.29	9.52	1.89	0.95	10.59	3.52	128.82	57.07	28.96	10.75	2.40	0.82
RSD	9.779	3.043	24.34	4.461	3.531	1.984	1.123	0.789	16.99	3.416	32.71	6.845	7.915	2.885	1.328	0.428
<i>P-treatment</i>	0.472	0.386	0.020	0.677	0.235	0.236	0.615	0.248	0.419	0.660	0.105	0.641	0.624	0.523	0.778	0.519

Values are given as means and residual standard deviation (RSD) (AC, LC, PD, n=6; UC, n=9).

*AC, challenged pigs consuming plain diet with 0.4 % formic acid and 0.4 % lactic acid added; LC, challenged pigs consuming plain diet with 1 % lactulose added; PD, challenged pigs consuming plain diet without additives; UC, unchallenged pigs.

†Sum of acetic, propionic, butyric, valeric and BCFA.

^{a,b} Different superscripts within the same column indicate significant differences within the values ($P<0.05$).

Table 4

Bacterial populations, as determined by FISH analysis, present in ileal and caecal samples of pigs challenged or unchallenged with *Salmonella* Typhimurium at 4 and 10 days post-challenge

Treatment*	Log ₁₀ bacteria/mL sample on day 4 post-challenge							Log ₁₀ bacteria/mL sample on day 10 post-challenge						
	Bac303	Bif	Erec	Lab	Prop	Rbro	Sal303	Bac303	Bif	Erec	Lab	Prop	Rbro	Sal303
Ileum														
AC	5.57	5.37	5.58	6.80	7.46	4.88	5.91	5.37 ^b	5.48	5.33	6.57	6.86	4.93 ^{ab}	5.53
LC	5.73	5.15	5.69	6.62	7.04	4.88	5.73	5.46 ^{ab}	5.55	5.09	6.76	6.99	4.61 ^b	5.52
PD	5.62	5.27	5.63	6.41	7.15	5.04	5.57	5.31 ^b	5.49	4.77	6.65	6.60	5.07 ^a	5.59
UC	5.11	5.10	5.67	7.01	7.01	4.88	bd [†]	5.77 ^a	5.67	5.34	6.54	6.90	5.19 ^a	bd [†]
RSD	0.482	0.270	0.309	0.520	0.377	0.465	0.289	0.303	0.259	0.409	0.474	0.537	0.184	0.107
<i>P-treatment</i>	0.097	0.271	0.946	0.183	0.154	0.912	0.167	0.035	0.468	0.120	0.835	0.713	<0.001	0.601
Cecum														
AC	8.24 ^{ab}	5.35 ^b	8.21 ^{ab}	7.15	8.05	7.04	7.92	8.11	5.19	7.94	7.18	7.82	7.21	7.73
LC	8.32 ^a	6.11 ^a	8.13 ^b	6.90	8.15	7.09	7.92	8.00	5.16	7.92	6.91	7.79	6.78	7.70
PD	8.20 ^{ab}	5.87 ^{ab}	8.32 ^{ab}	6.96	8.14	7.09	8.01	8.07	5.66	8.00	6.96	7.72	6.99	7.81
UC	8.02 ^b	5.43 ^b	8.40 ^a	7.45	8.09	7.09	bd [†]	8.06	5.68	8.06	6.91	7.82	7.11	bd [†]
RSD	0.181	0.369	0.156	0.502	0.142	0.233	0.131	0.173	0.482	0.181	0.504	0.250	0.300	0.101
<i>P-treatment</i>	0.025	0.003	0.018	0.174	0.588	0.967	0.421	0.755	0.101	0.523	0.732	0.923	0.095	0.302

Values are given as LS means and residual standard deviations (RSD) (AC, LC, PD, n=6; UC, n=9).

*AC, challenged pigs consuming plain diet with 0.4 % formic acid and 0.4 % lactic acid added; LC, challenged pigs consuming plain diet with 1 % lactulose added; PD, challenged pigs consuming plain diet without additives; UC, unchallenged pigs.

†bd, below detection limit (<4.13 Log₁₀ bacteria/mL sample).

^{a,b} Different superscripts within the same column indicate significant differences within the values ($P<0.05$).