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1 **Coated fatty acids alter virulence properties of *Salmonella* Typhimurium and**
2 **decrease intestinal colonization of pigs**

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22 **ABSTRACT**

23 *Salmonella* Typhimurium infections in pigs are a major source of human
24 foodborne salmonellosis. To reduce the number of infected pigs, acidification of feed or
25 drinking water is a common practice. The aim of the present study was to determine
26 whether some frequently used short- (SCFA) and medium-chain fatty acids (MCFA) are
27 able to alter virulence gene expression and to decrease *Salmonella* Typhimurium
28 colonization and shedding in pigs using well established and controlled *in vitro* and *in*
29 *vivo* assays. Minimal inhibitory concentrations (MIC) of 4 SCFA (formic acid, acetic
30 acid, propionic acid, butyric acid) and 2 MCFA (caproic and caprylic acid) were
31 determined using 54 porcine *Salmonella* Typhimurium field strains. MIC values
32 increased at increasing pH values and were 2-8 times lower for MCFA than for SCFA.
33 Expression of virulence gene *fimA* was significantly lower when bacteria were grown in
34 LB-broth supplemented with sub-MIC concentrations of caproic or caprylic acid (2mM).
35 Expression of *hilA* and invasion in porcine intestinal epithelial cells was significantly
36 lower when bacteria were grown in LB-broth containing sub-MIC concentrations of
37 butyric acid or propionic acid (10mM) and caproic or caprylic acid (2mM). When given
38 as feed supplement to pigs experimentally infected with *Salmonella* Typhimurium, coated
39 butyric acid decreased the levels of faecal shedding and intestinal colonization, but had
40 no influence on the colonization of tonsils, spleen and liver. Uncoated fatty acids,
41 however, did not influence fecal shedding, intestinal or tonsillar colonization in pigs. In
42 conclusion, supplementing feed with certain coated fatty acids, such as butyric acid, may
43 help to reduce the *Salmonella* load in pigs.

44 **Key words:** *Salmonella* Typhimurium – pig – SCFA – MCFA – butyric acid

45 1. INTRODUCTION

46

47 In Europe, *Salmonella* Typhimurium is by far the dominant serovar isolated from pigs
48 (EFSA, 2006). In most cases, *Salmonella* Typhimurium will subclinically colonize the
49 pigs, without causing obvious symptoms. These carrier pigs are a vast reservoir of
50 *Salmonella* and pose a major threat to animal and human health (Boyen et al., 2008a).

51 The battle against nontyphoidal *Salmonella* infections in pigs requires a strategic
52 implementation of different approaches across the pork production and processing chains
53 (Ojha and Kostrzynska, 2007). In addition to general hygiene and biosecurity measures,
54 the supplementation of feed with acidic compounds has been proposed as a possible tool
55 to combat *Salmonella* in pigs (Creus et al., 2007). Currently, short-chain fatty acids
56 (SCFA) and medium-chain fatty acids (MCFA) are commonly used in the poultry
57 industry for this purpose (Van Immerseel et al., 2006). Apart from their antimicrobial
58 actions at high concentrations, even low concentrations of SCFA and MCFA can
59 decrease intestinal colonization by *Salmonella* Enteritidis in poultry, mediated by their
60 influence on virulence gene expression (Van Immerseel et al., 2004; 2005).

61 It was the aim of the present study to evaluate the usefulness of SCFA and MCFA
62 in controlling *Salmonella* infections in pigs. Minimal inhibitory concentrations (MIC) of
63 4 SCFA and 2 MCFA for 54 *Salmonella* Typhimurium strains were determined. The
64 influence of sub-MIC concentrations of these acids on virulence gene expression and
65 invasive capacities of *Salmonella* Typhimurium was evaluated. Finally, the efficacy of
66 coated as well as uncoated fatty acids in reducing the early colonization of piglets
67 inoculated with *Salmonella* Typhimurium was assessed in two *in vivo* trials.

68 2. MATERIALS AND METHODS

69

70 2.1. Bacterial strains

71 *Salmonella* Typhimurium strain 112910a (DT 120/ad) was used in all *in vitro*
72 experiments and its invasive nalidixic acid resistant derivative was used in the *in vivo*
73 trial. Strain 112910a was isolated from a pig stool sample and persists in tonsils,
74 intestines and gut-associated lymphoid tissue (GALT) of experimentally infected pigs
75 during at least 28 days (Boyen et al., 2008b).

76 Fifty-four independent *Salmonella* Typhimurium strains, isolated from pigs in
77 Belgian slaughterhouses and farms, were used to perform minimal inhibitory
78 concentration assays.

79

80 2.2. Minimal inhibitory concentrations (MIC) of fatty acids

81 Minimal inhibitory concentrations (MIC) were determined for SCFA and MCFA
82 at pH 4, 5 and 6, using HCl or NaOH to obtain the different pH values. Formic acid (C₁),
83 acetic acid (C₂), propionic acid (C₃), butyric acid (C₄), caproic acid (C₆) and caprylic acid
84 (C₈) (all products from Sigma, St. Louis, Mo.) were tested after serial twofold dilutions in
85 96-well microplate in LB broth ranging from 0.0391 mM to 2560 mM. Bacteria were
86 grown for 18 h in 5 ml Luria-Bertani broth (LB) at 37°C. Five µl of this suspension was
87 inoculated in 195 µl medium in each microwell plate. These suspensions were incubated
88 for 20 h at 37°C after which bacterial growth was assessed.

89

90 2.3. Construction of the transcriptional fusions

91 The pCS26 plasmid was used for the construction of transcriptional fusions
92 between the promoter region of *fimA* and the *luxCDABE* operon as described before for
93 the *hilA* promoter region (Van Immerseel et al., 2004). In short, the predicted promoter
94 sequence of *fimA* was amplified by PCR and cloned into the pCS26 plasmid. Primers
95 used for amplifying the promoter sequence of *fimA* were
96 NNNNCTCGAGTGGCTATGGTTACCGTAATC (forward primer) and
97 NNNNGGATCCAGGCTGCATTAACCAGTTTACC (reverse primer). Both the pCS26
98 plasmid and the amplification product containing the promoter sequence were digested
99 and ligated. The ligation mixture was used for electroporation of *Salmonella*
100 Typhimurium strain 112910a and kanamycin-resistant colonies (selection marker of
101 pCS26) were tested for the promoter-plasmid junction by PCR. The sequence of the
102 promoter-plasmid junction was confirmed by DNA sequencing.

103

104 **2.4. Measurement of *hilA* and *fimA* expression**

105 Virulence expression was measured using the *luxCDABE* operon in a growing
106 bacterial culture. Since the amount of emitted light depends on the number of plasmids
107 carrying the *luxCDABE* operon (and therefore the number of bacteria present in the well)
108 on one hand and on the actual magnitude of virulence expression on the other hand, it is
109 crucial to use concentrations of fatty acids that do not interfere with bacterial growth.
110 These concentrations were defined earlier in *Salmonella* Enteritidis (Van Immerseel et
111 al., 2004). The absence of antimicrobial effects of the concentrations used in the
112 virulence expression assays on *Salmonella* Typhimurium strain 112910a was evaluated as
113 described before (Van Immerseel et al., 2004). Tested concentration for formic acid (C_1),

114 acetic acid (C₂), propionic acid (C₃) and butyric acid (C₄) was 10 mM and for caproic
115 (C₆), caprylic (C₈), or capric acid (C₁₀) 2 mM was tested at pH 6.

116 A FluoroScan Ascent fluorometer (Labsystems, Helsinki, Finland) was used to
117 quantify light production (luminescence) by *Salmonella* Typhimurium strain 112910a
118 carrying the plasmids containing the *hila-luxCDABE* or *fimA-luxCDABE* transcriptional
119 fusions. Bacterial cultures were grown in microplates in 200 µl of LB medium,
120 supplemented with SCFA or MCFA and in nonsupplemented LB medium at 37°C. Light
121 production was measured automatically every 2 min for 20 hours. Total light production
122 (area under the curve) was calculated and was used for statistical analysis. The results of
123 each experiment were divided by the calculated mean value of all results of that
124 experiment to reduce inter-experimental variations. Statistical analysis was performed by
125 an analysis of variance using SPSS version 11.5 software.

126

127 **2.5. Invasion assays**

128 The porcine intestinal epithelial IPI-2I cell line was used (Kaeffer *et al.*, 1993).
129 Cells were seeded in 24 well plates at a density of approximately 10⁵ cells per well and
130 were allowed to grow to confluency for 48 h. Bacteria were grown for 6 h in LB medium,
131 after which the suspension was diluted 1:1,000 in Dulbecco's modified Eagle's medium
132 with 10% fetal calf serum, supplemented with 10 mM of the individual SCFA or 2 mM of
133 the individual MCFA. After 4 h of incubation at 37°C, the suspensions were centrifuged
134 and resuspended in Dulbecco's modified Eagle's medium with 10% fetal calf serum. The
135 number of CFU/ml was determined by plating six 20-µl samples of a dilution series of the
136 suspensions on brilliant green agar (BGA) plates, after which the plates were incubated

137 for 20 h at 37°C. The suspensions were kept at 4°C until they were used in the assay. The
138 bacterial suspensions were diluted to a density of 5×10^6 CFU/ml. From these diluted
139 suspensions, 200 µl was transferred to the cells. To synchronize the infection, the
140 inoculated multiwell plates were centrifuged at 365 x g for 5 min. After 25 min
141 incubation at 37 °C under 5% CO₂, the wells were washed and fresh medium
142 supplemented with 50 µg/ml gentamicin (Gibco, Life Technologies, Paisley, Scotland)
143 was added. After an additional 60 min incubation at 37 °C under 5% CO₂, the wells were
144 washed three times. A previously described non-invasive isogenic deletion mutant in *hilA*
145 was used as a control for invasion (Boyen et al., 2006a).

146 To assess invasion, the cells were lysed with 0.25% deoxycholate (Sigma-Aldrich,
147 Steinheim, Germany) 90 min after inoculation and 10-fold dilutions were plated on BGA
148 plates. The results of each experiment were divided by the calculated mean value of all
149 results of that experiment to reduce inter-experimental variations. Statistical analysis was
150 performed by an analysis of variance using SPSS version 11.5 software.

151

152 **2.6. *In vivo* trial with supplementation of coated fatty acids**

153

154 Based on the data obtained from the *in vitro* trials, an *in vivo* experiment using
155 coated butyric acid and caprylic acid was performed. Six-week-old piglets (commercial
156 closed line based on Landrace) were obtained from a serologically negative breeding herd
157 and were negative for *Salmonella* at faecal sampling. They arrived at the facility 14 days
158 before they were inoculated and were divided at random into four groups: three groups of
159 6 inoculated pigs and one negative control group of 3 pigs. Throughout the experiment,

160 group 1 and the control group received unsupplemented feed, group 2 feed supplemented
161 with coated butyrate (Greencab, Sanluc International, 2 g/kg feed) and group 3 received
162 feed supplemented with coated caprylic acid (Sanluc International, 3.1 g/kg feed),
163 according to the suppliers recommendations. The piglets were housed in pairs in separate
164 isolation units at 25 °C under natural day-night rhythm with ad libitum access to feed and
165 water. Twelve days after the piglets were given the different feeds, the animals were
166 orally inoculated with approximately 7×10^7 CFU of *Salmonella* Typhimurium in 1 ml
167 Hank's Balanced Salt Solution (HBSS). The inocula for the oral infection models were
168 prepared as described previously (Boyen et al., 2006b).

169 For 3 consecutive days post-inoculation (pi), the clinical condition of the pigs was
170 monitored (anorexia, lethargy, diarrhoea) and fresh faecal samples were taken from each
171 pig for bacteriological analysis.

172 On day 4 pi, all piglets of each *Salmonella* inoculated group and 3 control pigs
173 were euthanized. Samples of tonsils, liver, spleen, mesenterial lymph nodes, ileocaecal
174 lymph nodes, colonic lymph nodes, jejunum, ileum, caecum, colon and contents of
175 jejunum, ileum, caecum and colon were taken for bacteriological analysis and were
176 stored at -70 °C until use. The samples were thawed and weighed, 10 % (w/v)
177 suspensions were made in buffered peptone water (BPW; Oxoid, Basingstoke, UK) after
178 which the material was homogenized with a stomacher. The homogenized samples were
179 examined for the presence of the *Salmonella* strain by plating tenfold dilutions on BGA
180 with addition of 20 $\mu\text{g ml}^{-1}$ nalidixic acid (BGA_{nal}). If negative at direct plating, the
181 samples were pre-enriched overnight in BPW at 37 °C, enriched overnight at 37 °C in
182 tetrathionate broth and then plated on BGA_{nal}. Samples that were negative after direct

183 plating but positive after enrichment, were presumed to contain 83 CFU g⁻¹ (detection
184 limit for direct plating). Samples that remained negative were presumed to have 0 CFU g⁻¹.
185

186 The data were analysed using a linear mixed effect regression model with animal
187 as random factor using S-Plus 7.0. Differences with a P value ≤ 0.05 were considered as
188 significant. Differences with a P value ≤ 0.1 were considered as a trend.

189 The experiments were approved by the ethical committee of the Faculty of Veterinary
190 Medicine, Ghent University (EC 2006/15).

191

192 **2.7. *In vivo* trial with supplementation of uncoated fatty acids**

193

194 An *in vivo* experiment with uncoated fatty acids was performed analogous to the
195 trial with coated fatty acids, except for the addition of the feed supplements. Group 1 and
196 the control group received unsupplemented feed, group 2 feed supplemented with
197 uncoated butyric acid (Sanluc International, 1 g/kg feed) and group 3 received feed
198 supplemented with uncoated caproic acid (Sanluc International, 1.7 g/kg feed), according
199 to the suppliers recommendations.

200 The data were analysed as described in 2.6. The experiments were approved by the
201 ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC
202 2007/003).

203 3. RESULTS

204

205 3.1. Minimal inhibitory concentrations (MIC) of fatty acids

206 The results of the determination of the MIC values of SCFA and MCFA are
207 summarized in Table 1. The MIC of all fatty acids increased at increasing pH-values of
208 the medium and were comparable between the different SCFA and MCFA respectively.
209 In comparison with the MIC of SCFA, MIC values of MCFA were 2 to 8 times lower.

210

211 3.2. Sub-MIC concentrations of fatty acids modify *hilA* and *fimA* expression of 212 *Salmonella* Typhimurium

213 Using growth curves it was demonstrated that the MCFA did not influence the growth
214 of *Salmonella* Typhimurium strain 112910a at a concentration of 2 mM, while for the
215 SCFA a concentration of 10 mM was not bacteriostatic at pH 6 (data not shown).

216 *Salmonella* Typhimurium grown in LB-broth containing sub-MIC concentrations
217 of butyric acid, propionic acid, caproic or caprylic acid showed a significantly ($p \leq 0.05$)
218 lower expression of *hilA* compared to *Salmonella* Typhimurium grown in LB-broth
219 without acid supplementation. The expression of *fimA* was significantly ($p \leq 0.05$) lower
220 when *Salmonella* Typhimurium was grown in LB-broth containing caproic or caprylic
221 acid compared to *Salmonella* Typhimurium grown in LB-broth without acid
222 supplementation (Figure 1). The expression of *fimA* was significantly ($p \leq 0.05$) higher
223 when *Salmonella* Typhimurium was grown in LB-broth containing acetic acid.

224

225 **3.3. Sub-MIC concentrations of fatty acids influence invasive capacities of**
226 ***Salmonella* Typhimurium**

227 In the gentamicin protection assay, *Salmonella* Typhimurium grown in LB-broth
228 containing sub-MIC concentrations of propionic acid, butyric acid, caprylic acid or
229 caproic acid, invaded significantly ($p \leq 0.05$) less compared to *Salmonella* Typhimurium
230 grown in LB-broth not containing acids. Formic acid, acetic acid and capric acid did not
231 decrease invasion efficiency ($p > 0.05$) at sub-MIC concentrations (Figure 2).

232

233 **3.4. Feed supplementation with coated fatty acids reduces *Salmonella* Typhimurium**
234 **excretion and colonization in pigs**

235 The group receiving coated butyric acid showed a strong trend ($p = 0.082$), of
236 decreased *Salmonella* shedding at the first 3 days after inoculation. At 2 and 3 days p.i.
237 faecal shedding was approximately 100 times lower in the group fed coated butyric acid
238 compared to the control group. Coated caprylic acid did not significantly ($p = 0.89$)
239 reduce fecal shedding of *Salmonella* Typhimurium (Figure 3).

240 The *Salmonella* Typhimurium colonization of the internal organs was determined
241 on day 4 pi. Supplementation of coated butyric acid and coated caprylic acid resulted in a
242 similar colonization of the tonsils, spleen and liver compared to the control group. In both
243 groups the gut samples were colonized to a lower extent, however, the group receiving
244 coated butyric acid showed a trend ($p = 0.095$) towards lower colonization of the
245 intestines and the associated lymph nodes, while this was not statistically significant in
246 the group receiving coated caprylic acid ($p = 0.495$) (Figure 4).

247 Neither uncoated butyric acid nor uncoated caproic acid influenced the
248 *Salmonella* excretion until 3 days pi compared to the control group. Supplementation of
249 uncoated butyric acid and uncoated caproic acid did not reduce the *Salmonella*
250 colonization of the internal organs (data not shown).

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251 **4. DISCUSSION**

252

253 SCFA and MCFA have a direct antimicrobial activity against *Salmonella*
254 Typhimurium, even at moderate concentrations. Our results demonstrate increasing MIC-
255 values as the pH-level increases for both SCFA and MCFA. This is in agreement with the
256 findings in propionic acid made by Kwon et al. (1998) and in anaerobic digester
257 conditions for several SCFA (Salsali et al., 2006). Even though the MIC values were
258 comparable between the different SCFA and MCFA respectively, subtle differences can
259 be noted. The MIC values of formic acid for example are highly dependent on pH,
260 showing a large range between the MIC values at pH 4 and pH 6. In contrast, the MIC
261 values of propionic acid are less influenced by changes in pH. When using these acids to
262 inhibit growth of *Salmonella* in feed or drinking water, the correct combination of acid
263 concentration and pH should be chosen.

264

265 Despite the relatively low MIC values at low pH, a direct antimicrobial effect of these
266 acids in the intestines is not expected. In order to achieve a direct antimicrobial effect in
267 the porcine gut to combat *Salmonella* Typhimurium, quite high concentrations of SCFA
268 (≥ 160 mM at pH 6) and MCFA (≥ 40 mM at pH 6) are needed. Depending on the used
269 feed, concentrations of butyric acid in the porcine caecum contents vary around 10
270 mmol/kg (≈ 10 mM) (Mikkelsen et al., 2004). Therefore, fatty acid concentrations
271 currently used in supplemented feed (10-30 mmol/kg feed) will not be able to increase
272 the intraluminal concentrations to antimicrobial concentrations (≥ 160 mM at pH 6).

272

273 SCFA and MCFA were shown to have an indirect effect on *Salmonella* pathogenicity.
274 Even nonbacteriostatic concentrations as low as 2 mM for caproic or caprylic acid and 10
275 mM for butyric and propionic acid considerably decreased virulence gene expression and

275 epithelial cell invasion by *Salmonella* Typhimurium. This means that an increase of only
276 a few mM butyric acid in the gut contents could result in reaching the threshold
277 concentration for activation of the indirect effect of the SCFA. Since it has been shown
278 that invasion is important for intestinal colonization and induction of inflammation in
279 pigs (Boyen et al., 2006c; Volf et al., 2007), one could expect that any measure that
280 interferes with this invasion step will decrease the bacterial load in the gut.

281 Because SCFA and MCFA are usually rapidly metabolised by the microbiota of the
282 gut and absorbed by epithelial cells along the gastro-intestinal tract (Van Immerseel et al.,
283 2006; Louis et al., 2007), the supplemented fatty acids should be protected from the
284 intestinal environment until they reach the major sites of colonization by *Salmonella*,
285 namely the ileum, caecum and colon. In this report, a trend for decreased intestinal
286 *Salmonella* load and bacterial shedding in pigs was shown using supplemented coated
287 butyric acid, but not using supplemented uncoated butyric acid, as also previously
288 described in poultry (Van Immerseel et al., 2006). Even though caprylic acid showed a
289 stronger effect on invasion *in vitro*, this positive effect was not reproduced *in vivo*. This
290 may partially be explained by the fact that the coated butyric acid supplement was an
291 extensively characterized and optimized commercial product, while the caprylic acid
292 supplement was not.

293 It has been shown recently that at pH 7 butyric acid is of nearly no influence on *hilA*
294 expression (Papezova et al. 2007). In weaned piglets, the pH of caecum and colon, and to
295 a lesser extent the ileum, is readily below 7 (Castillo et al., 2007). This means that
296 theoretically, butyric acid should be able to exert its influence on *hilA* expression at
297 these sites. Reduced colonization of the distal parts of the intestinal tract may in turn

298 correlate with the reduced faecal shedding. Under field conditions, a lot of animals are
299 negative for *Salmonella*, while carrier pigs are colonized by small numbers of
300 *Salmonella*, often only detectable after enrichment (Malorny and Hoorfar, 2005). In such
301 conditions, reduction of faecal shedding by one or two logs may have considerable
302 epidemiological consequences on the *Salmonella* status of the uninfected animals in the
303 herd. The obvious effects of these products could have been statistically more significant
304 if larger numbers of animals were used. However, this was practically impossible due to
305 strict biosafety requirements and animal ethics regulations.

306 In conclusion, to our knowledge, this is the first report demonstrating the effect of
307 SCFA and MCFA on *Salmonella* Typhimurium infections in swine under controlled and
308 well established *in vitro* and *in vivo* conditions. Certain short-chain fatty acids and
309 medium-chain fatty acids decrease virulence gene expression and inhibit invasion in
310 porcine intestinal epithelial cells. Coated butyric acid was effective in decreasing the
311 levels of shedding and colonization of internal organs when given as a feed supplement to
312 pigs.

313

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322 REFERENCES

323

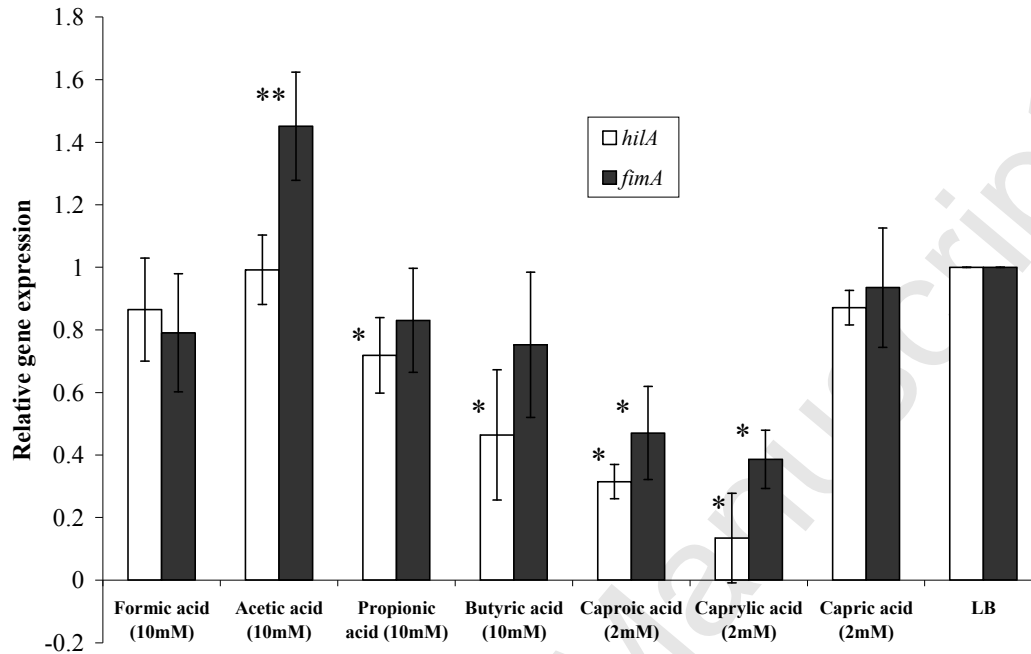
- 324 1. Althouse, C., Patterson, S., Fedorka-Cray, P., Isaacson, R.E. 2003. Type 1
325 fimbriae of *Salmonella enterica* serovar Typhimurium bind to enterocytes and
326 contribute to colonization of swine in vivo. *Infect. Immun.* 71, 6446-6452.
- 327 2. Boyen, F., Pasmans, F., Donné, E., Van Immerseel, F., Adriaensen, C.,
328 Hernalsteens, J.-P., Ducatelle, R., Haesebrouck, F., 2006a. Role of SPI-1 in the
329 interactions of *Salmonella* Typhimurium with porcine macrophages. *Vet.*
330 *Microbiol.* 113, 35-44.
- 331 3. Boyen, F., Haesebrouck, F., Maes, D., Van Immerseel, F., Ducatelle, R., Pasmans,
332 F. 2008a. Non-typhoidal *Salmonella* infections in pigs: a closer look at
333 epidemiology, pathogenesis and control. *Vet. Microbiol.* In Press.
- 334 4. Boyen, F., Pasmans, F., Donné, E., Van Immerseel, F., Morgan, C., Adriaensen,
335 C., Hernalsteens, J.-P., Wallis, T.S., Ducatelle, R., Haesebrouck, F., 2006b. The
336 fibronectin binding protein ShdA is not a prerequisite for long term fecal shedding
337 of *Salmonella* Typhimurium in pigs. *Vet. Microbiol.* 115,284-290.
- 338 5. Boyen, F., Pasmans, F., Van Immerseel, F., Donné, E., Morgan, E., Ducatelle, R.,
339 Haesebrouck, F., 2008b. Porcine *in vitro* and *in vivo* models to assess the
340 virulence of *Salmonella enterica* serovar Typhimurium for pigs. *Lab. Anim.* In
341 Press.
- 342 6. Boyen, F., Pasmans, F., Van Immerseel, F., Morgan, E., Adriaensen, C.,
343 Hernalsteens, J.-P., Decostere, A., Ducatelle, R., Haesebrouck, F., 2006c.

- 344 *Salmonella* Typhimurium SPI-1 genes promote intestinal but not tonsillar
345 colonization in pigs. *Microb. Infect.* 8, 2899-2907.
- 346 7. Carnell, S.C., Bowen, A., Morgan, E., Maskell, D.J., Wallis, T.S., Stevens, M.P.,
347 2007. Role in virulence and protective efficacy in pigs of *Salmonella enterica*
348 serovar Typhimurium secreted components identified by signature-tagged
349 mutagenesis. *Microbiology.* 153, 1940-1952.
- 350 8. Castillo, M., Martín-Orúe, S.M., Nofrarías, M., Manzanilla, E.G., Gasa, J., 2007.
351 Changes in caecal microbiota and mucosal morphology of weaned pigs. *Vet.*
352 *Microbiol.* 124, 239-247.
- 353 9. Creus, E., Pérez, J.F., Peralta, B., Baucells, F., Mateu, E., 2007. Effect of acidified
354 feed on the prevalence of *Salmonella* in market-age pigs. *Zoonoses Public Health.*
355 54, 314-319.
- 356 10. Dalton, C.B., Gregory, J., Kirk, M.D., Stafford, R.J., Givney, R., Kraa, E., Gould,
357 D., 2004. Foodborne disease outbreaks in Australia, 1995 to 2000. *Commun. Dis.*
358 *Intell.* 28, 211-224.
- 359 11. EFSA. 2006. Opinion of the Scientific Panel on Biological Hazards on the request
360 from the Commission related to “Risk assessment and mitigation options of
361 *Salmonella* in pig production”. *The EFSA Journal* 341, 1-131.
- 362 12. EFSA. 2008. The Community Summary Report on Trends and Sources of
363 Zoonoses, Zoonotic Agents, Antimicrobial resistance and Foodborne outbreaks in
364 the European Union in 2006. <http://www.efsa.europa.eu/EFSA/>
- 365 13. Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Hautefort, I., Thompson,
366 A., Hinton, J.C., Van Immerseel, F., 2006. Butyrate specifically down-regulates

- 367 *Salmonella* pathogenicity island 1 gene expression. Appl. Environ. Microbiol. 72,
368 946-949.
- 369 14. Kaeffer, B., Bottreau, E., Velge, P., Pardon, P., 1993. Epithelioid and fibroblastic
370 cell lines derived from the ileum of an adult histocompatible miniature boar (d/d
371 haplotype) and immortalized by SV40 plasmid. Eur. J. Cell. Biol. 62, 152-162.
- 372 15. Kwon, Y.M., Ricke, S.C., 1998. Survival of a *Salmonella* Typhimurium poultry
373 isolate in the presence of propionic acid under aerobic and anaerobic conditions.
374 Anaerobe. 4, 251-256.
- 375 16. Louis, P., Scott, K.P., Duncan, S.H., Flint, H.J., 2007. Understanding the effects
376 of diet on bacterial metabolism in the large intestine. J. Appl. Microbiol. 102,
377 1197-1208.
- 378 17. Malorny, B., Hoorfar, J., 2005. Toward standardization of diagnostic PCR testing
379 of fecal samples: lessons from the detection of salmonellae in pigs. J. Clin.
380 Microbiol. 43, 3033-3037.
- 381 18. Mikkelsen, L.L., Naughton, P.J., Hedemann, M.S., Jensen, B.B., 2004. Effects of
382 physical properties of feed on microbial ecology and survival of *Salmonella*
383 *enterica* serovar Typhimurium in the pig gastrointestinal tract. J. Appl. Microbiol.
384 70, 3485-3492.
- 385 19. Ojha, S., Kostrzynska, M., 2007. Approaches for reducing *Salmonella* in pork
386 production. J. Food Prot. 70, 2676-2694.
- 387 20. Papezova, K., Gregorova, D., Jonuschies, J., Rychlik, I., 2007. Ordered
388 expression of virulence genes in *Salmonella enterica* serovar typhimurium. Folia
389 Microbiol. (Praha). 52, 107-114.

- 390 21. Salsali, H.R., Parker, W.J., Sattar, S.A., 2006. Impact of concentration,
391 temperature, and pH on inactivation of *Salmonella* spp. by volatile fatty acids in
392 anaerobic digestion. *Can. J. Microbiol.* 52, 279-286.
- 393 22. Van Immerseel, F., Boyen, F., Gantois, I., Timbermont, L., Bohez, L., Pasmans,
394 F., Haesebrouck, F., Ducatelle, R., 2005. Supplementation of coated butyric acid
395 in the feed reduces colonization and shedding of *Salmonella* in poultry. *Poult Sci.*
396 84: 1851-1856.
- 397 23. Van Immerseel, F., De Buck, J., Boyen, F., Bohez, L., Pasmans, F., Volf, J.,
398 Sevcik, M., Rychlik, I., Haesebrouck, F., Ducatelle, R., 2004. Medium-chain fatty
399 acids decrease colonization and invasion through *hilA* suppression shortly after
400 infection of chickens with *Salmonella enterica* serovar Enteritidis. *Appl. Environ.*
401 *Microbiol.* 70, 3582-3587.
- 402 24. Van Immerseel, F., Russell, J.B., Flythe, M.D., Gantois, I., Timbermont, L.,
403 Pasmans, F., Haesebrouck, F., Ducatelle, R., 2006. The use of organic acids to
404 combat *Salmonella* in poultry: a mechanistic explanation of the efficacy. *Avian*
405 *Pathol.* 35: 182-188.
- 406 25. Volf, J., Boyen, F., Faldyna, M., Pavlova, B., Navratilova, J., Rychlik, I., 2007.
407 Cytokine Response of Porcine Cell Lines to *Salmonella enterica* serovar
408 Typhimurium and its *hilA* and *ssrA* mutants. *Zoonoses Public Health.* 54, 286-
409 293.
- 410

1 Tables and figures



2

3 Figure 1: Relative gene expression (average +/- SD) of *fimA* and *hilA* of
 4 *Salmonella* Typhimurium strain 112910a grown in LB broth with sub-MIC
 5 concentrations of different fatty acids relative to *Salmonella* Typhimurium strain 112910a
 6 grown in unsupplemented LB broth at 37°C for 20 hours, using *hilA-luxCDABE* or *fimA-*
 7 *luxCDABE* transcriptional fusions. One or 2 asterisks refer to significantly lower or
 8 respectively higher gene expression relative to that of the *Salmonella* Typhimurium strain
 9 112910a grown in unsupplemented LB broth ($p \leq 0.05$).

10

11

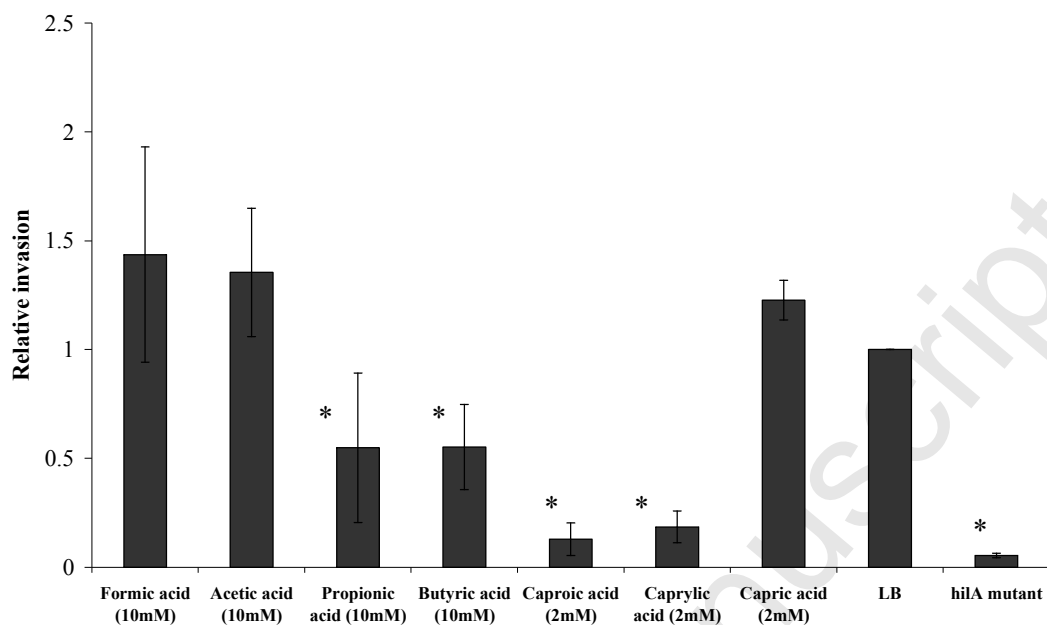


Figure 2: Relative invasion (average +/- SD) in IPI-2I cells of *Salmonella* Typhimurium grown in LB broth, supplemented with sub-MIC concentrations of different fatty acids for 4 h at 37°C in relation to *Salmonella* Typhimurium grown in unsupplemented LB broth. A *hilA* mutant strain was used as internal control. An asterisk refers to significantly lower invasion relative to the *Salmonella* Typhimurium strain 112910a grown in unsupplemented LB broth ($p \leq 0.05$).

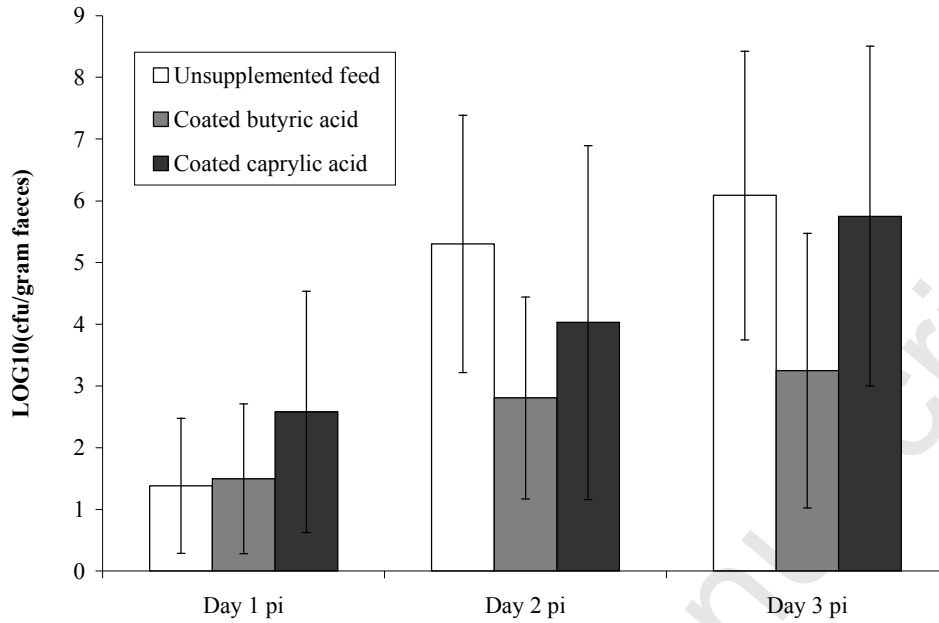


Figure 3: The mean (\pm SD) log₁₀ cfu gram⁻¹ faeces *Salmonella* of piglets fed on a diet supplemented with either coated butyric or caprylic acid or unsupplemented after oral inoculation with 10⁷ cfu of *Salmonella* Typhimurium strain 112910a.

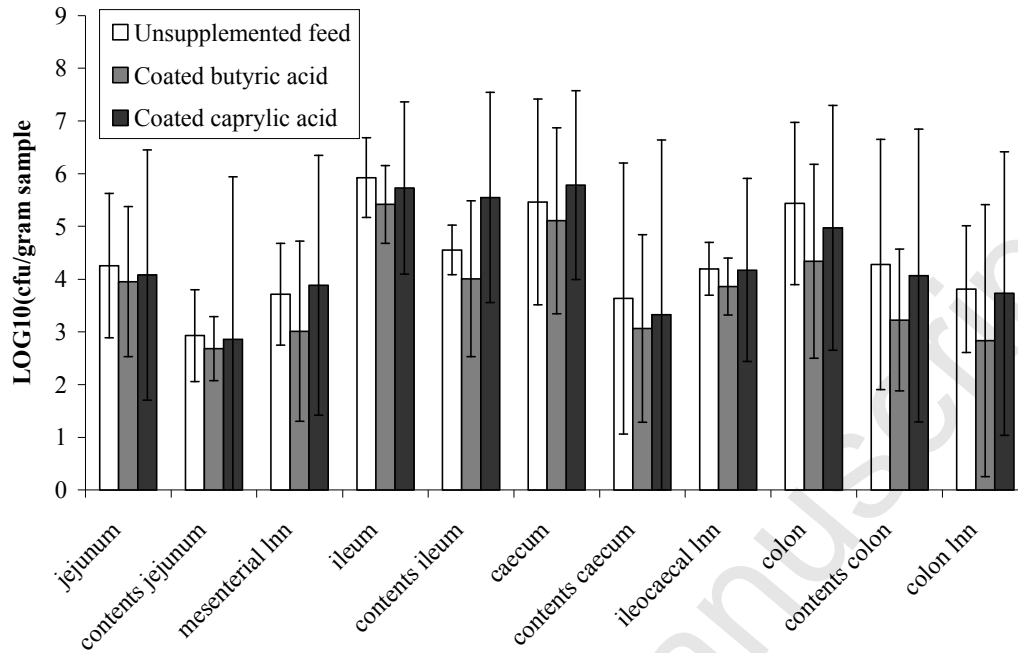


Fig. 4: The mean (\pm SD) \log_{10} cfu gram^{-1} sample *Salmonella* of piglets fed on a diet supplemented with either coated butyric or caprylic acid or unsupplemented 4 days after oral inoculation with 10^7 cfu of *Salmonella* Typhimurium strain 112910a.

Table 1: Overview of the MIC values of formic acid, acetic acid, propionic acid, butyric acid, caproic acid and caprylic acid tested at pH 4, 5 and 6, using 54 porcine *Salmonella* Typhimurium strains.

	number of strains with a MIC of (mM)										
	0.625	1.25	2.5	5	10	20	40	80	160	320	640
<u>Formic acid</u>											
pH4	-	9	45	-	-	-	-	-	-	-	-
pH5	-	-	-	-	-	-	54	-	-	-	-
pH6	-	-	-	-	-	-	-	-	-	54	-
<u>Acetic acid</u>											
pH4	-	-	-	54	-	-	-	-	-	-	-
pH5	-	-	-	-	-	3	51	-	-	-	-
pH6	-	-	-	-	-	-	-	-	1	48	5
<u>Propionic acid</u>											
pH4	-	-	-	22	32	-	-	-	-	-	-
pH5	-	-	-	-	-	20	34	-	-	-	-
pH6	-	-	-	-	-	-	-	1	49	4	-
<u>Butyric acid</u>											
pH4	1	3	1	49	-	-	-	-	-	-	-
pH5	-	-	-	-	8	46	-	-	-	-	-
pH6	-	-	-	-	-	-	-	-	53	1	-
<u>Caproic acid</u>											
pH4	-	3	51	-	-	-	-	-	-	-	-
pH5	-	-	-	-	54	-	-	-	-	-	-
pH6	-	-	-	-	-	-	7	47	-	-	-
<u>Caprylic acid</u>											
pH4	-	51	2	1	-	-	-	-	-	-	-
pH5	-	-	-	1	43	10	-	-	-	-	-
pH6	-	-	-	-	-	-	47	7	-	-	-