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

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ABSTRACT

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

Key words: Salmonella Typhimurium – pig – SCFA – MCFA – butyric acid
1. INTRODUCTION

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

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

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

2.1. Bacterial strains

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

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

2.2. Minimal inhibitory concentrations (MIC) of fatty acids

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

2.3. Construction of the transcriptional fusions
The pCS26 plasmid was used for the construction of transcriptional fusions between the promoter region of \textit{fimA} and the \textit{luxCDABE} operon as described before for the \textit{hilA} promoter region (Van Immerseel et al., 2004). In short, the predicted promoter sequence of \textit{fimA} was amplified by PCR and cloned into the pCS26 plasmid. Primers used for amplifying the promoter sequence of \textit{fimA} were NNNNCTCGAGTGGCTATGGTTACCGTAATC (forward primer) and NNNNGGATCCAGGCTGCATTAACCAGTTTACC (reverse primer). Both the pCS26 plasmid and the amplification product containing the promoter sequence were digested and ligated. The ligation mixture was used for electroporation of \textit{Salmonella} Typhimurium strain 112910a and kanamycin-resistant colonies (selection marker of pCS26) were tested for the promoter-plasmid junction by PCR. The sequence of the promoter-plasmid junction was confirmed by DNA sequencing.

\textbf{2.4. Measurement of \textit{hilA} and \textit{fimA} expression}

Virulence expression was measured using the \textit{luxCDABE} operon in a growing bacterial culture. Since the amount of emitted light depends on the number of plasmids carrying the \textit{luxCDABE} operon (and therefore the number of bacteria present in the well) on one hand and on the actual magnitude of virulence expression on the other hand, it is crucial to use concentrations of fatty acids that do not interfere with bacterial growth. These concentrations were defined earlier in \textit{Salmonella} Enteritidis (Van Immerseel et al., 2004). The absence of antimicrobial effects of the concentrations used in the virulence expression assays on \textit{Salmonella} Typhimurium strain 112910a was evaluated as described before (Van Immerseel et al., 2004). Tested concentration for formic acid (C$_1$),
acetic acid (C\textsubscript{2}), propionic acid (C\textsubscript{3}) and butyric acid (C\textsubscript{4}) was 10 mM and for caproic (C\textsubscript{6}), caprylic (C\textsubscript{8}), or capric acid (C\textsubscript{10}) 2 mM was tested at pH 6.

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

\textbf{2.5. Invasion assays}

The porcine intestinal epithelial IPI-2I cell line was used (Kaeffer \textit{et al.}, 1993). Cells were seeded in 24 well plates at a density of approximately 10\textsuperscript{5} cells per well and were allowed to grow to confluency for 48 h. Bacteria were grown for 6 h in LB medium, after which the suspension was diluted 1:1,000 in Dulbecco's modified Eagle's medium with 10% fetal calf serum, supplemented with 10 mM of the individual SCFA or 2 mM of the individual MCFA. After 4 h of incubation at 37°C, the suspensions were centrifuged and resuspended in Dulbecco's modified Eagle's medium with 10% fetal calf serum. The number of CFU/ml was determined by plating six 20-µl samples of a dilution series of the suspensions on brilliant green agar (BGA) plates, after which the plates were incubated.
for 20 h at 37°C. The suspensions were kept at 4°C until they were used in the assay. The bacterial suspensions were diluted to a density of $5 \times 10^6$ CFU/ml. From these diluted suspensions, 200 µl was transferred to the cells. To synchronize the infection, the inoculated multiwell plates were centrifuged at 365 x g for 5 min. After 25 min incubation at 37 °C under 5% CO$_2$, the wells were washed and fresh medium supplemented with 50 µg/ml gentamicin (Gibco, Life Technologies, Paisley, Scotland) was added. After an additional 60 min incubation at 37 °C under 5% CO$_2$, the wells were washed three times. A previously described non-invasive isogenic deletion mutant in hilA was used as a control for invasion (Boyen et al., 2006a).

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

### 2.6. In vivo trial with supplementation of coated fatty acids

Based on the data obtained from the in vitro trials, an in vivo experiment using coated butyric acid and caprylic acid was performed. Six-week-old piglets (commercial closed line based on Landrace) were obtained from a serologically negative breeding herd and were negative for *Salmonella* at faecal sampling. They arrived at the facility 14 days before they were inoculated and were divided at random into four groups: three groups of 6 inoculated pigs and one negative control group of 3 pigs. Throughout the experiment,
group 1 and the control group received unsupplemented feed, group 2 feed supplemented with coated butyrate (Greencab, Sanluc International, 2 g/kg feed) and group 3 received feed supplemented with coated caprylic acid (Sanluc International, 3.1 g/kg feed), according to the suppliers recommendations. The piglets were housed in pairs in separate isolation units at 25 °C under natural day-night rhythm with ad libitum access to feed and water. Twelve days after the piglets were given the different feeds, the animals were orally inoculated with approximately $7 \times 10^7$ CFU of *Salmonella Typhimurium* in 1 ml Hank’s Balanced Salt Solution (HBSS). The inocula for the oral infection models were prepared as described previously (Boyen et al., 2006b).

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

On day 4 pi, all piglets of each *Salmonella* inoculated group and 3 control pigs were euthanized. Samples of tonsils, liver, spleen, mesenterial lymph nodes, ileocaecal lymph nodes, colonic lymph nodes, jejunum, ileum, caecum, colon and contents of jejunum, ileum, caecum and colon were taken for bacteriological analysis and were stored at -70 °C until use. The samples were thawed and weighed, 10 % (w/v) suspensions were made in buffered peptone water (BPW; Oxoid, Basingstoke, UK) after which the material was homogenized with a stomacher. The homogenized samples were examined for the presence of the *Salmonella* strain by plating tenfold dilutions on BGA with addition of 20 µg ml$^{-1}$ nalidixic acid (BGA$_{nal}$). If negative at direct plating, the samples were pre-enriched overnight in BPW at 37 °C, enriched overnight at 37 °C in tetrathionate broth and then plated on BGA$_{nal}$. Samples that were negative after direct
plating but positive after enrichment, were presumed to contain 83 CFU g\(^{-1}\) (detection limit for direct plating). Samples that remained negative were presumed to have 0 CFU g\(^{-1}\).

The data were analysed using a linear mixed effect regression model with animal as random factor using S-Plus 7.0. Differences with a P value \(\leq 0.05\) were considered as significant. Differences with a P value \(\leq 0.1\) were considered as a trend.

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

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

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

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

3.1. Minimal inhibitory concentrations (MIC) of fatty acids

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

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

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

*Salmonella* Typhimurium grown in LB-broth containing sub-MIC concentrations of butyric acid, propionic acid, caproic or caprylic acid showed a significantly (p ≤ 0.05) lower expression of hilA compared to *Salmonella* Typhimurium grown in LB-broth without acid supplementation. The expression of fimA was significantly (p ≤ 0.05) lower when *Salmonella* Typhimurium was grown in LB-broth containing caproic or caprylic acid compared to *Salmonella* Typhimurium grown in LB-broth without acid supplementation (Figure 1). The expression of fimA was significantly (p ≤ 0.05) higher when *Salmonella* Typhimurium was grown in LB-broth containing acetic acid.
3.3. Sub-MIC concentrations of fatty acids influence invasive capacities of *Salmonella Typhimurium*

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

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

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

The *Salmonella Typhimurium* colonization of the internal organs was determined on day 4 pi. Supplementation of coated butyric acid and coated caprylic acid resulted in a similar colonization of the tonsils, spleen and liver compared to the control group. In both groups the gut samples were colonized to a lower extent, however, the group receiving coated butyric acid showed a trend \( p = 0.095 \) towards lower colonization of the intestines and the associated lymph nodes, while this was not statistically significant in the group receiving coated caprylic acid \( p = 0.495 \) (Figure 4).
Neither uncoated butyric acid nor uncoated caproic acid influenced the *Salmonella* excretion until 3 days pi compared to the control group. Supplementation of uncoated butyric acid and uncoated caproic acid did not reduce the *Salmonella* colonization of the internal organs (data not shown).
4. DISCUSSION

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

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

SCFA and MCFA were shown to have an indirect effect on *Salmonella* pathogenicity. Even nonbacteriostatic concentrations as low as 2 mM for caproic or caprylic acid and 10 mM for butyric and propionic acid considerably decreased virulence gene expression and
epithelial cell invasion by *Salmonella* Typhimurium. This means that an increase of only a few mM butyric acid in the gut contents could result in reaching the threshold concentration for activation of the indirect effect of the SCFA. Since it has been shown that invasion is important for intestinal colonization and induction of inflammation in pigs (Boyen et al., 2006c; Volf et al., 2007), one could expect that any measure that interferes with this invasion step will decrease the bacterial load in the gut.

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

It has been shown recently that at pH 7 butyric acid is of nearly no influence on *hilA* expression (Papezova et al. 2007). In weaned piglets, the pH of caecum and colon, and to a lesser extent the ileum, is readily below 7 (Castillo et al., 2007). This means that theoretically, butyric acid should be able to exert its influence on *hilA* expression at these sites. Reduced colonization of the distal parts of the intestinal tract may in turn
correlate with the reduced faecal shedding. Under field conditions, a lot of animals are negative for *Salmonella*, while carrier pigs are colonized by small numbers of *Salmonella*, often only detectable after enrichment (Malorny and Hoorfar, 2005). In such conditions, reduction of faecal shedding by one or two logs may have considerable epidemiological consequences on the *Salmonella* status of the uninfected animals in the herd. The obvious effects of these products could have been statistically more significant if larger numbers of animals were used. However, this was practically impossible due to strict biosafety requirements and animal ethics regulations.

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

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Tables and figures

Figure 1: Relative gene expression (average +/- SD) of fimA and hilA of Salmonella Typhimurium strain 112910a grown in LB broth with sub-MIC concentrations of different fatty acids relative to Salmonella Typhimurium strain 112910a grown in unsupplemented LB broth at 37°C for 20 hours, using hilA-luxCDABE or fimA-luxCDABE transcriptional fusions. One or 2 asterisks refer to significantly lower or respectively higher gene expression relative to that of the Salmonella Typhimurium strain 112910a grown in unsupplemented LB broth (p ≤ 0.05).
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 ≤ 0.05).
Figure 3: The mean (+/- SD) log(10) cfu gram⁻¹ faeces of piglets fed on a diet supplemented with either coated butyric or caprylic acid or unsupplemented after oral inoculation with $10^7$ cfu of *Salmonella* Typhimurium strain 112910a.
Fig. 4: The mean (+/− 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.

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