Inhibition of *Salmonella* Typhimurium by medium chain fatty acids in an in vitro simulation of the porcine caecum

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Abstract

To lower the contamination of pork meat with *Salmonella*, feed additives such as medium chain fatty acids (MCFA's) can be applied at the primary production level. An in vitro continuous culture system, simulating the porcine caecum, was developed for investigating the effect of MCFAs on the pig intestinal microbial community. The system was monitored by plating on selective media, 16S rDNA PCR denaturing gradient gel electrophoresis (PCR-DGGE) and HPLC analysis of fermentation products. In a simulation of the porcine caecum without MCFA treatment, with *Salmonella* Typhimurium added after stabilization of the microbial community, the strain could establish itself at a stable population size of about 5 log cfu/ml. The effect of selected MCFAs was observed from all monitored parameters and depended on chain length and concentration applied. At a dose of 15 mM, caproate and caprinate did not show any pronounced effect, while a clear *Salmonella* inhibiting effect (3 log units reduction) was found for caprylate. Doubling the caprylate dose did not result in enhanced *Salmonella* inhibition.

Introduction

In Belgium, 49.4% of the human salmonellosis cases were identified as *Salmonella* Typhimurium (STM) in 2006. STM is the most common serovar isolated from pigs. In pig stables, complex Salmonella contamination cycles occur, in which Salmonella-free animals are contaminated through direct or indirect contact with faeces of infected animals. Therefore, if the gastrointestinal tract (GIT) of the pig can be made an unfavorable environment for Salmonella growth e.g. through the use of feed additives, this will result in decreased pathogen shedding, which at its turn will reduce carcass contamination. This will attribute to reduce the prevalence of human salmonellosis cases due to consumption of pork meat.

MCFA's show some promising beneficial effects for use as these feed additives have been postulated to modulate the porcine gut bacterial ecosystem, resulting in healthier gut mucosa and improved zootechnical parameters of piglets (Dierick et al., 2002a, 2002b, 2003). For caprylate, an inhibitory effect on Salmonella was obtained in standard bacteriological media (Skrivanova et al., 2004). Although MCFA preparations are commercially available and are used in practice, their effect on the gut bacterial ecosystem remains largely unknown.

In this study, the effect of selected MCFA's on the GIT microbial community was evaluated, with special emphasis on their inhibitory activity against STM. For this purpose, an in vitro model of the porcine caecum based on continuous culture was developed and validated.

Material and methods

The fiber- and mucin containing incubation medium for porcine caecum simulation was as described by Dierick et al. (2002a), with minor modifications. The medium was acidified to pH 2 and kept at 4°C and under constant agitation to prevent precipitation of the fiber. Caecal contents were used as inoculum; directly after slaughter, caecal contents from 12 pigs (originating from 7 different farms) were pooled, homogenized and divided in aliquots of 15 ml to which 15% glycerol was added and frozen at -80°C. Equipment for continuous culture consisted of a BioFlo110 unit (New Brunswick Scientific) with a 1.3 l fermentor vessel. Fresh medium was added via a peristaltic
pump at a constant rate of about 1.8 ml/min and spent culture liquid was wasted at the same rate to maintain a constant working volume of 500 ml (i.e. 4.6 h retention time). Temperature was kept at 37°C and pH at 6.2. The culture was constantly agitated at 300 rpm. Anaerobic conditions were maintained by flushing the headspace of the vessel with 20 ml/min of 20% CO2+80% N2. After inoculation with pooled caecal contents (15 ml), the fermentor was operated in batch mode for 24 h. Continuous culture was then started by switching on the peristaltic pumps. Ten ml of an overnight grown STM strain in Müller-Hinton broth was added after the switch to continuous culture.

The strain was originally isolated from pig colon (Botteldoorn et al., 2003) and belongs to phage type DT104. After several days, MCFAs (caproate, caprylate, caprinate) were added to the medium at a final concentration of 15 and 30 mM. Bacterial populations were monitored by plating on general and selective media: Reinforced Clostridial Medium, Tryptone Soy Agar, de Man, Rogosa & Sharpe medium, MTPY (Rada and Petr, 2000), Slanetz and Bartley medium, MacConkey agar and Xylose-Lysine-Desoxycholate agar (all from Oxoid) for enumeration of total anaerobes, total aerobes, lactic acid bacteria, bifidobacteria, streptococci, coliforms and Salmonella, respectively. Cultivation-independent community analysis was done by PCR-DGGE, as described by Boon et al. (2000). Organic acids formed during fermentation were quantified by HPLC analysis using an Aminex HPX-87H column (BioRad) as described previously (Van Coillie et al., in press). MCFAs were purchased from Sigma as sodium salts.

Results

In a simulation of the porcine caecum without MCFA addition (control experiment), with the STM strain added after stabilization of the microbial community, the strain could establish itself at a stable population size of about 5 log cfu/ml for at least 13 days. Before addition of the STM strain, no black colonies on XLD agar were observed. The effect of different MCFAs was first evaluated at a final concentration of 15 mM in the in vitro caecum model, after an ecological equilibrium was obtained. In one experiment, the STM strain was added immediately after switching to continuous culture. After seven days of operation, the incubation medium stock was replaced by a stock containing 15 mM caproate and this treatment was maintained until the end of the experiment. No clear effects on the microbial counts or produced organic acids were observed. From day 6 on, Salmonella counts remain relatively steady at around 104-105 cfu/ml. When this experiment was performed with caprinate, results were similar. However, for reasons of solubility, a lower dose than 15 mM was evaluated. Gradual replacement of the regular medium in the fermentor vessel by caprylate-containing medium resulted in a rapid decrease of the Salmonella population from 6.8 to 2.2 log cfu/ml (Fig. 1).
Effect of 15 mM caprylate on the *in vitro* porcine caecum simulation. Bacterial populations as determined by plating. The start of continuous culture, addition and withdrawal of caprylate are indicated. When after 13 days of operation, the feed was switched back to medium without caprylate, no concomitant increase of the Salmonella population was observed. Propionic and butyric acid production decreased after addition of caprylate, while lactic acid production increased. The effect on formic acid production was less clear. Acetic acid concentrations seemed to gradually increase in the course of the caecum simulations. When caprylate treatment was stopped, propionic, butyric and lactic acid production returned to their initial levels. Cultivation-independent microbial community analysis via PCR-DGGE for this experiment is shown in Fig. 2. At day 1 (directly after the start of continuous culture) equilibrium is obviously not reached yet, as shown by a pattern quite distinct from all other. From day 3 on, DGGE profiles are all quite similar, indicating a relatively stable ecosystem. During caprylate treatment, one band seems to be more prominent in the DGGE pattern. A treatment with 30 mM caprylate resulted in very similar findings as obtained with 15 mM caprylate.

![Fig. 1](image1.png)

**Fig. 1.** Effect of 15 mM caprylate on the *in vitro* porcine caecum simulation. Bacterial populations as determined by plating. The start of continuous culture, addition and withdrawal of caprylate are indicated.

![Fig. 2](image2.png)

**Fig. 2.** PCR-DGGE patterns of the microbial populations in the experiment described in Fig. 1. The arrow indicates the position of a band that seems to be correlated with the presence of caprylate in the incubation medium.

**Discussion**

The use of an *in vitro* model facilitates the study of the GIT microbial community and circumvents animal to animal variation encountered in slaughter trials. Existing continuous culture GIT models are often operated on particle free medium with a chemical composition that is quite distinct from actual chymus. In our model, we tried to mimic the physiological conditions in the porcine caecum as closely as possible through the use of a cellulose (non-digestible fiber) and mucin (glycoproteins isolated from the porcine stomach)-containing incubation medium. The caecum was specifically selected, because a pronounced proliferation of several bacterial groups occurs in this compartment of the GIT. The rationale is that, if Salmonella outgrowth can be suppressed here, this would result in reduced excretion via the faeces.

The original pooled caecal contents inoculum evolved in the *in vitro* model within 2-3 days after the onset of continuous culture into a relatively stable microbial ecosystem with stable fermentation characteristics, as observed from plating results, PCR-DGGE analysis and organic acid production. When operated under normal conditions (i.e. after stabilization and without MCFA added), average population densities of total anaerobic bacteria, lactic acid bacteria, coliforms and streptococci were 8.7, 8.6, 5.8 and 7.6 log cfu/ml, respectively. Organic acid concentrations were 0.7, 11, 94, 14 and 26 mM for lactic, formic, acetic, propionic and butyric acid, respectively. Bacterial counts as well as organic acid concentrations in our model corresponded well with literature data available for the porcine caecum (Mikkelsen et al., 2004). Since Salmonella was not detected prior to the deliberate inoculation with the STM strain, the *in vitro* simulation can be used to specifically monitor the behavior of the inoculated strain via enumeration on XLD agar plates.
The effect of MCFA on Salmonella and other microbial populations was investigated in the in vitro caecum model. At a dose of 15 mM, caproate and caprinate did not show any pronounced effect, while a clear Salmonella inhibiting effect was found for caprylate. Earlier, Skrivanova et al. (2004) found caprylic acid to be the only Salmonella-inhibiting compound among the 15 fatty acids tested in liquid cultures. Doubling the caprylate dose did not result in enhanced Salmonella inhibition in our experiments. The observed effect does not seem to be Salmonella-specific, since the coliform counts showed a similar inhibition in response to caprylate.

Conclusion

Our study indicates that caprylate addition to the medium at a dose of 15 mM, corresponding to about 1% in a feed, has potential for reducing Salmonella excretion in pig faeces.

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