



Sub-lethal levels of carvacrol reduce *Salmonella* Typhimurium motility and invasion of porcine epithelial cells

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ABSTRACT

The European ban on the use of antibiotic growth promotors has increased the search for new alternatives to prevent pig intestinal microbial diseases and to stimulate growth. The addition of essential oils or components thereof, such as carvacrol, to pig feed is a promising alternative. In this report we determined the effect of sub-lethal concentrations of carvacrol on *Salmonella* Typhimurium. At concentrations where growth of *Salmonella* was not inhibited, carvacrol completely inhibited motility of the bacterium. This loss of motility was not due to the loss of the flagellum or to ATP shortage upon carvacrol treatment. Adhesion of *Salmonella* to IPEC-J2, porcine intestinal epithelial cells, was not affected by carvacrol but invasion was significantly reduced. In addition, the epithelial gene expression of porcine β -defensin 2, an innate immune response to *Salmonella* infection, was reduced when *Salmonella* was exposed to carvacrol. This indicates that invasion but not adhesion of *Salmonella* triggers the porcine β -defensin 2 expression of porcine epithelial cells.

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1. Introduction

The European ban on the use of antibiotic growth promotors has increased the search for new alternatives to prevent pig intestinal microbial diseases and to stimulate growth. The addition of essential oils or components thereof, such as carvacrol, to pig feed is a promising alternative. Carvacrol is one of the main components of oregano oil, and has antibacterial but also antifungal and insecticidal activity against a wide range of microorganisms (Burt, 2004; Chami et al., 2005; Panella et al., 2005; Tampieri et al., 2005). The exact mode of action of carvacrol

is not known yet, but most studies point towards membrane disruption as the main mechanism.

If carvacrol is to be applied as an additive in animal feed, minimal inhibitory concentration (MIC) or minimal bactericidal concentration (MBC) levels are not likely to be reached in the gastrointestinal tract where it should exert its function. Not only will carvacrol be diluted while traveling through the intestinal tract, *in vitro* studies have also shown that it can bind to protein and lipid components of feed, lowering the effective concentration of carvacrol available for activity against microorganisms (Si et al., 2006; Veldhuizen et al., 2007). In this report we describe the effect of sub-lethal concentrations of carvacrol on *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). At concentrations where growth of *S.* Typhimurium was not inhibited the effect of carvacrol on motility and on adhesion and invasion characteristics of this bacterium were determined. In addition, the cytotoxic

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effect of carvacrol on intestinal epithelial cells and indirect effects on the host response towards *Salmonella* invasion were investigated.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Four *S. Typhimurium* strains and one *S. enterica* serovar Enteritidis (*S. Enteritidis*) strain were used: *S. Typhimurium* phage type 510, *S. Typhimurium* phage type 656 (both clinical isolates from pig intestine), *S. Typhimurium* DT104 (Hendriksen et al., 2004), *S. Typhimurium* ATCC 14028, and *S. enterica* serovar Enteritidis 90-13-706 (Van Asten et al., 2000). Overnight (o/n) cultures of *Salmonella* strains were prepared freshly for every experiment by cultivation from frozen stock at 37 °C for 16 h in Tryptone Soy Broth (TSB, Oxoid Basingstoke, United Kingdom). Before use in the antimicrobial assays described below, the optical density of the suspension was measured using a Pharmacia Ultraspec III spectrophotometer (Pharmacia Biotech, Roosendaal, the Netherlands) at 620 nm, and the suspensions were diluted in TSB to the appropriate bacterial density needed in the experiments.

2.2. Cell culture

The IPEC-J2 cell line (Schierack et al., 2006) was maintained in 50% Dulbecco's Modified Eagle Medium and 50% Nutrient Mixture F12 (Ham) (1:1 DMEM/F12) (GIBCO, Invitrogen, Carlsbad, CA), supplemented with 10 U/ml penicillin, 10 mg/ml streptomycin, 2 mM L-glutamine and 5% (v/v) fetal bovine serum (all provided by GIBCO, Invitrogen, Carlsbad, CA). Cells were grown in monolayers at 37 °C in 5% CO₂ atmosphere. Medium was changed every other day and cells were passaged once a week. For infection experiments, cells were cultured in 12-well cell culture plates (Costar, Corning Incorporated, Corning, NY) and grown to confluent monolayers in 7 days. In all experiments IPEC-J2 cells were used between passages 80 and 100. Human-derived enterocyte-like Caco-2 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 0.03% L-glutamine (Invitrogen life technologies, Carlsbad, CA), 1% non-essential amino acids, 10 mM sodium bicarbonate, 25 mM HEPES, 10 U/ml penicillin, 10 mg/ml streptomycin, and 20% fetal bovine serum in monolayers at 37 °C in a 5% CO₂ atmosphere.

2.3. Growth curves

Growth curves of bacteria in TSB containing carvacrol were determined using a Bioscreen C optical density (OD) reader (Oy Growth Curves AB Ltd., Helsinki, Finland) using software version 2.28. A stock preparation of 1 M carvacrol was prepared in 96% ethanol and diluted in TSB to the appropriate concentration. Final ethanol concentrations never exceeded 2% (v/v), to avoid an antibacterial effect of ethanol itself. From the 1 M stock in ethanol a 5 mM working solution was prepared in TSB, which was shaken vigorously for 2 h at 37 °C to completely dissolve carvacrol in the growth medium. In a typical experiment, aliquots of

100 µl of *Salmonella* suspension containing 2×10^6 colony forming units (CFU)/ml were added to 100 µl carvacrol in TSB. All wells were incubated for 18 h at 37 °C with continuous shaking. Optical density (OD) measurements (broad filter 420–580 nm) were obtained automatically every 30 min. Each experiment was carried out three times with at least three replicates each time. Ten samples containing carvacrol concentrations between 0 and 2.5 mM and a negative control containing no bacteria were tested in each experiment. The MIC value was defined as the lowest concentration where no increase in optical density was observed after 18 h.

2.4. Motility assay

To assess the effect of carvacrol on bacterial migration, a colony grown o/n on TSA was stabbed into semi-solid medium (1% Bacto tryptone (Difco laboratories, Detroit), 0.5% (w/v) NaCl, 0.35% Agar (Difco laboratories, Detroit)) with or without carvacrol at final concentrations ranging from 0.0 to 1.0 mM. Bacterial swarming was assessed after incubation at 37 °C for 24 h. Motility of *Salmonella* was also qualitatively determined by the hanging-drop technique (Burt et al., 2007). For this, a droplet of the culture was suspended from a glass coverslip over a microscope slide with a central concavity and observed under a light microscope. The bacterial cells were observed at a magnification of 1000.

2.5. Detection of flagellin in carvacrol treated *S. Typhimurium* DT104

S. Typhimurium DT104 cultures were grown to log phase in the presence of 0–0.8 mM carvacrol. After growth, cultures were normalized for optical cell density. One hundred microliter of cells were centrifuged (10 min, 16,000 × g), washed twice with PBS and separated on a 12% SDS PAGE gel and blotted onto nitrocellulose. Flagellin was detected on the western blot using anti-flagellin antibodies as described before (Burt et al., 2007).

2.6. Electron microscopy

S. Typhimurium DT104 cultures were grown to log phase in the presence of 0, 0.5 or 0.8 mM carvacrol. Carbon coated copper grids with a Formvar support film were glow discharged just before the bacterial samples were placed on the grids. Bacteria attached to the grids during a 3 min incubation, which was followed by a rinsing step of 10 s upside down on a 30 µl droplet of water. Subsequently, negative staining was achieved by an incubation of 3 min on 2% uranyl acetate in water and removal of the excess with filter paper. Samples were examined in a FEI Tecnai 12 microscope at an accelerating voltage of 100 kV.

2.7. ATP leakage assay

To determine whether sub-MIC concentrations of carvacrol induce release of ATP, both intra and extra-cellular ATP levels were measured. Carvacrol (0.5 or 0.8 mM) or the equivalent amount of ethanol (0 mM

carvacrol control) was added at the start of *Salmonella* growth in TSB medium. Cultures were grown at 37 °C with agitation for 3 h or 18 h. After growth, cultures were normalized for optical cell density and 1 ml centrifuged (10,000 × g, 10 min, 4 °C). Culture supernatants were centrifuged (15,000 × g, 15 min, 4 °C) to remove remaining cells and cell debris and then kept on ice. Cell pellets were resuspended in lysis buffer (100 mM Tris, 4 mM EDTA, pH 7.75) to an OD_{600 nm} = 2. To 100 µl of suspension, 900 µl of boiling lysis buffer was added. Samples were incubated for 2 min at 100 °C, placed on ice and centrifuged (1000 × g, 1 min, 4 °C). One hundred microliters of cell lysates and culture supernatants were mixed with an equal amount of luciferase reagent from the ATP Bioluminescence Assay CLS II kit (Roche Diagnostics, Mannheim, Germany) and luciferase activity was measured using a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA, USA). The ATP concentration was calculated from an ATP standard curve.

2.8. Cytotoxicity of carvacrol

Susceptibility of IPEC-J2 cells to cytotoxic effects of carvacrol was evaluated using the trypan blue exclusion assay. IPEC-J2 cells were grown to confluency in microtiter plates and subjected to 0–1.0 mM carvacrol in plain DMEM/F12 (without serum, L-glutamine or antibiotics) for 2 h. Subsequently, cells were washed 3 times with PBS and detached from the well bottom using Trypsin/EDTA (Invitrogen life technologies, Carlsbad, CA). After inhibition of trypsin activity by addition of full medium, IPEC-J2 cells were centrifuged and resuspended in plain DMEM/F12 medium, mixed 1:1 with 0.4% trypan blue and the percentage of non-viable (blue) cells were counted after 1 min but within 5 min. Experiments ($n=5$) were performed in triplicate on separate days and on average 300 cells were counted for each condition.

2.9. Adhesion and invasion of porcine epithelial cells

S. Typhimurium DT104 was grown to log phase in the presence of 0, 0.5 or 0.8 mM carvacrol. Bacteria were centrifuged and resuspended in plain DMEM/F12 medium to a density of 1×10^8 CFU/ml. IPEC-J2 cells were grown to confluency in 12-well tissue-culture plates, washed three times with plain medium, and incubated with 1 ml of the bacterial solution for 1 h at 37 °C. The monolayers were washed three times with plain medium and lysed in 1% Triton X-100 in PBS at room temperature for 5 min in order to release the bacteria. The suspensions were serially diluted and 100 µl of each dilution was plated on Trypticase Soy Agar (TSA, Oxoid Limited). The plates were incubated for 24 h at 37 °C. Numbers of cell associated bacteria were calculated as total (adhering and intracellular) bacterial CFU. In the invasion assay, the IPECJ2 monolayers in the wells were washed once with 1 ml warm plain medium after 1 h incubation with bacteria and then incubated for 2 h with 1 ml of 300 µg/ml colistin in warm plain DMEM/F12 to kill extracellular bacteria. Cells were washed three times with plain DMEM/F12, and finally lysed in 1% Triton X-100. The number of intracellular bacteria was determined by colony-plating as

described above. Similar experiments were performed with Caco-2 cells.

2.10. Effect of carvacrol treatment of *Salmonella* on porcine β defensin-2 gene expression of IPEC J2 cells

S. Typhimurium DT104 was grown to log phase in the presence of 0, 0.5 and 0.8 mM carvacrol. Cells were centrifuged and resuspended in plain DMEM/F12 medium to a density of 1×10^8 CFU/ml and added to a confluent monolayer of IPEC-J2 cells in a 12-wells plate. After 3 h of incubation, cells were washed twice and incubated with full medium containing 300 µg/ml colistin. After 6 and 24 h, IPEC-J2 cells were dissolved in 1 ml TRIzol (Invitrogen Life Technologies, Carlsbad, CA). Total cellular RNA was isolated according to the supplier's recommendation. Purity and quality of the RNA extracts was checked on 1% agarose gels and using UV absorption at 260/280 nm. Approximately 500 ng RNA was used to produce cDNA, using iScript (Bio-rad Laboratories BV, Veenendaal, The Netherlands) according to the manufacturer's recommendations. Quantitative determination of gene expression of porcine β -defensin 2 (pBD-2) was performed as described before (Veldhuizen et al., 2009).

2.11. Detection of cytokine expression

The effect of carvacrol treatment on the ability of *S. Typhimurium* to modulate cytokine production in IPEC-J2 cells was measured by means of enzyme-linked immunosorbent assay (ELISA). The expression levels of porcine TNF- α , IL-1 β , IL-6, and IL-8 were measured using the commercially available DuoSet ELISA kits (R&D Systems). IPEC-J2 cells were grown and infected as described in Section 2.10, subsequently after 3 h of incubation cells were washed twice and incubated with full medium containing 300 µg/ml colistin. After 4, 6 and 24 h, culture supernatants were collected and used for cytokine detection following the protocols provided by the manufacturer. All samples were centrifuged briefly at 500 for 3 min at room temperature to remove cell debris prior to use. The microtiter plates were read at an absorbance of 450 nm.

2.12. Statistical analyses

Statistical analysis of variance was performed using SPSS Version 16.0 for Windows. All data were analyzed by the post hoc Bonferroni test. Significant differences between means were defined as $p < 0.05$.

3. Results

3.1. Growth curves

The effect of carvacrol on the growth of *S. Typhimurium* DT104 is shown in Fig. 1. Up to 1 mM, carvacrol did not affect the growth rate of *S. Typhimurium* DT104 while at concentrations higher than 1 mM, a significant reduction in growth rate was observed. The minimal inhibitory concentration was 2 mM while the MBC (as determined by plating out those wells without observable growth) was

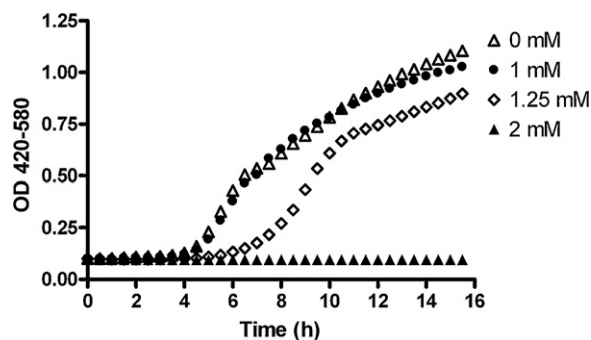


Fig. 1. Representative growth curves of *S. Typhimurium* DT104 in TSB in the presence of different concentrations of carvacrol. For clarity, only a selection of the tested carvacrol samples is depicted. Shown is the mean of quadruplicate samples in one experiment.

similar to the MIC. Similar results were obtained for the other four *Salmonella* strains, with only *S. Enteritidis* having a slightly lower MIC and MBC (1.75 mM, data not shown) indicating that the effect of carvacrol on growth is not *Salmonella*-strain specific.

3.2. Motility

Using soft agar plates, motility of all five *Salmonella* strains was determined in the presence of carvacrol. As depicted in Fig. 2 for *S. Typhimurium* DT104, motility was slightly reduced at 0.4 mM and completely abolished at 1 mM carvacrol. The four other strains showed similar reduced motility at these carvacrol concentrations (data not shown). These results show that motility is affected at lower concentrations than growth. Non-quantitative determination of motility using a light microscope showed complete loss of motility in solution at concentrations >0.6 mM, for all *Salmonella* strains confirming the soft agar results (data not shown).

3.3. Flagella presence and flagellin production

The effect of carvacrol on the production of flagellin in *S. Typhimurium* DT104 was determined using electron

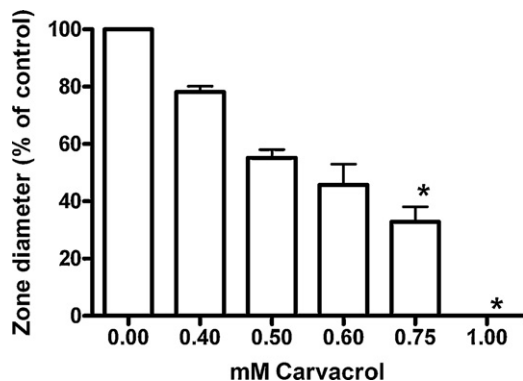


Fig. 2. Swarming of *S. Typhimurium* DT104 in soft agar plates. The relative zone diameter compared to the control (0 mM carvacrol, set at 100%) is presented as mean + SEM of three independent experiments. * indicates statistical significance.

microscopy and western blot. Bacteria incubated with 0.5 mM (not shown) or 0.8 mM carvacrol possessed flagella as shown by negative staining of the *Salmonella* (Fig. 3). Using western blot, all samples showed an immunoreactive band at the expected size (± 50 kDa) for flagellin with no observable difference in intensity (data not shown). This indicates that the reduced motility is not due to the loss of the flagellum of *S. Typhimurium*.

3.4. ATP leakage

In order to determine whether sub-lethal concentrations of carvacrol could result in energy depletion of the bacterium, ATP levels were measured after 3 and 18 h of growth in carvacrol containing medium. For log phase *Salmonella* (3 h growth) extracellular ATP levels were significantly higher for the 0.8 mM carvacrol treated *Salmonella* indicating that ATP had indeed leaked out of the bacterium (Fig. 4A). Intracellular levels were not lowered showing that lost ATP was regenerated. For bacteria grown o/n in the presence of carvacrol, intracellular levels were actually higher than the no-carvacrol control, while extracellular levels were slightly reduced

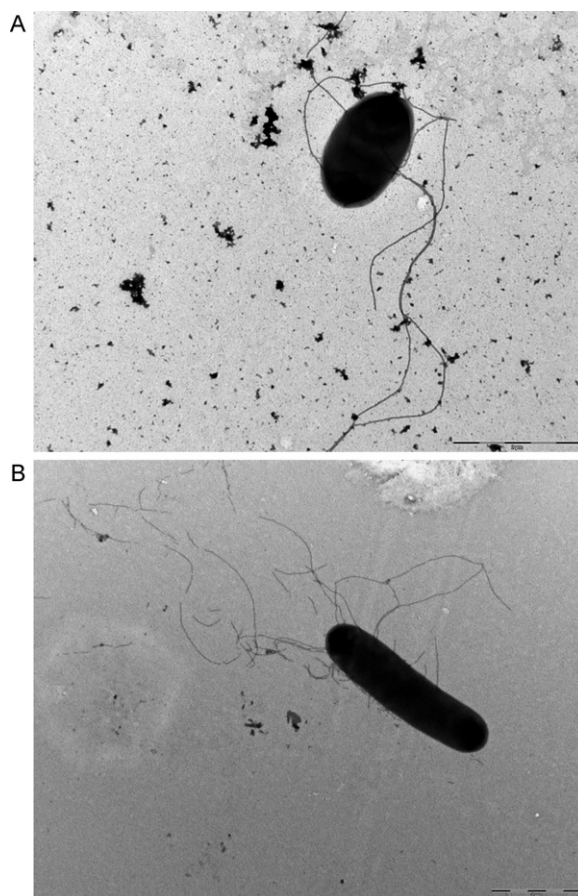


Fig. 3. Flagella are present on carvacrol-incubated *S. Typhimurium*. *S. Typhimurium* was grown for 3 h in the absence (A) or presence (B) of 0.8 mM carvacrol. Bacteria were visualized using electron microscopy by negative staining. Scale bar represents 2 μ m.

(Fig. 4B). However, these differences were not statistically significant.

3.5. Cytotoxicity of carvacrol

The cytotoxic effect of carvacrol on IPEC-J2 intestinal cells is shown in Table 1. Although standard deviations were relatively high for this set of experiments, a clear increase in the number of dead cells with higher carvacrol concentrations was observed. At 1 mM carvacrol, the highest concentration tested, 48% of IPEC cells were dead indicating that under these culture conditions carvacrol is toxic to this porcine epithelial cell line.

3.6. Adhesion and invasion of intestinal cells

Since motility of *Salmonella* is correlated to invasion, the adhesion and invasion characteristics of carvacrol treated bacteria were determined. The growth to log phase of *S. Typhimurium* DT104 in the presence of 0.5 mM carvacrol resulted in a significant reduction in invasion (34% of control) of IPEC-J2 cells, but had no significant effect on adhesion (Fig. 5A). At 0.8 mM adhesion was also reduced twofold compared to the control while invasion was further lowered to 14%. In order to check that this was

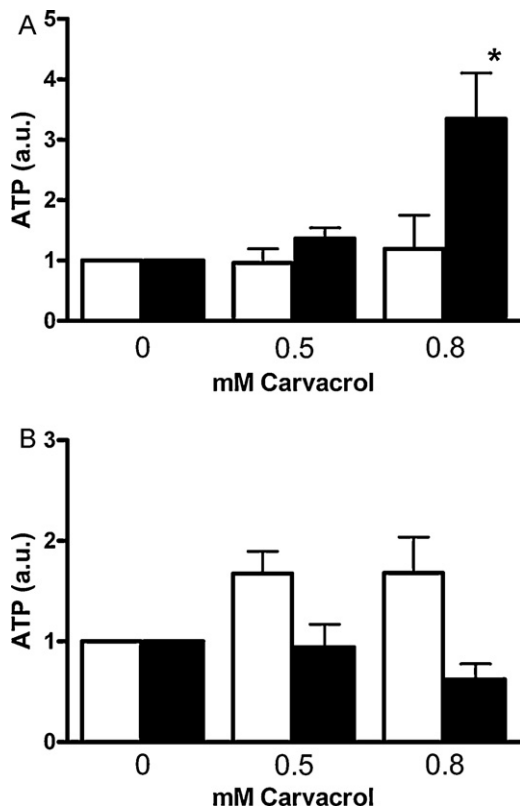


Fig. 4. Intracellular (white bars) and extracellular (black bars) ATP levels of *Salmonella* Typhimurium grown in the presence of 0, 0.5 and 0.8 mM carvacrol. Shown is the mean and SEM of three independent experiments of quadruplicate samples. (A) *S. Typhimurium* DT104 grown to log phase (3 h) in the presence of carvacrol, (B) *S. Typhimurium* DT104 grown o/n in the presence of carvacrol. * indicates statistical significance.

Table 1

Cytotoxic effects of carvacrol on epithelial IPEC-J2 cells.

| Carvacrol (mM) | % Dead cells (mean \pm SEM) |
|----------------|-------------------------------|
| 0 | 2.1 \pm 0.5 |
| 0.3 | 5.2 \pm 1.1 |
| 0.7 | 16.7 \pm 4.0 |
| 1.0 | 48.5 \pm 15.9 |

Depicted are the mean and SEM of 5 independent experiments.

not a cell-specific phenomenon, similar experiments were performed with human Caco-2 cells. In these experiments a similar trend was observed (Fig. 5B) with a reduced invasion of cells upon carvacrol treatment of *S. Typhimurium* but no difference in adhesion.

3.7. pBD-2 gene expression

Upon infection with *Salmonella* Typhimurium, IPEC-J2 cells increase the gene expression of host defense peptides such as pBD-2 (Veldhuizen et al., 2009). After 6 h of infection, pBD-2 gene expression levels of *S. Typhimurium* DT104 infected IPEC-J2 cells (0 mM carvacrol) were approximately 9 times higher than the non *Salmonella*-infected control cells, while after 24 h pBD-2 levels were >250 times higher (Fig. 6A and B). When *S. Typhimurium* DT104 was grown in the presence of 0.5 mM or 0.8 mM carvacrol before infection of IPEC-J2 cells, this pBD-2 upregulation was partially inhibited. At both time points a

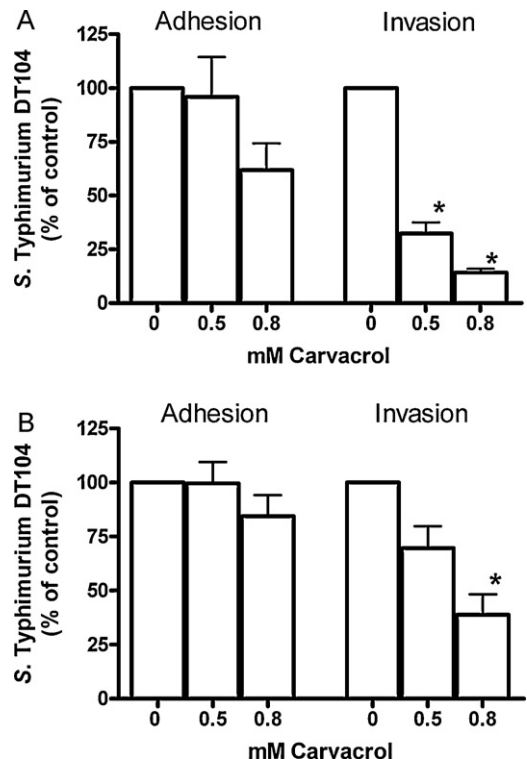


Fig. 5. Adhesion and invasion of IPEC-J2 (A) and Caco-2 (B) cells by *S. Typhimurium* DT104. Adhesion and invasion are shown as percentages relative to the control (0 mM carvacrol, set at 100%). Shown are the mean and SEM of five independent experiments. * indicates statistical significance.

twofold decrease in pBD-2 gene expression compared to the 0 mM carvacrol sample was observed, although only the 24 h levels reached statistical significance.

3.8. Detection of cytokines

Infection of IPEC-J2 cells with *S. Typhimurium* DT104 resulted in a clear upregulation after 4, 6 and 24 h of IL-8 (Fig. 7A). Control samples of non-infected cells showed increased IL-8 levels as well, indicating that IPEC-J2 cells have a constitutive expression of this chemokine under our culture conditions. *S. Typhimurium* DT104 grown to log phase in the presence of carvacrol caused a slightly lower level of IL-8 but this did not reach statistical significance. Similarly, the TNF α , IL-6 or IL-1B expression was measured upon *Salmonella* infection with and without carvacrol treatment (Fig. 7B–D). Infection only had a minor effect on the expression of these cytokines with observed increases in the pg/ml range compared to ng/ml for IL-8. In addition no significant effect of carvacrol could be determined.

4. Discussion

The European ban on prophylactic use of antibiotics in order to reduce antimicrobial resistance development has

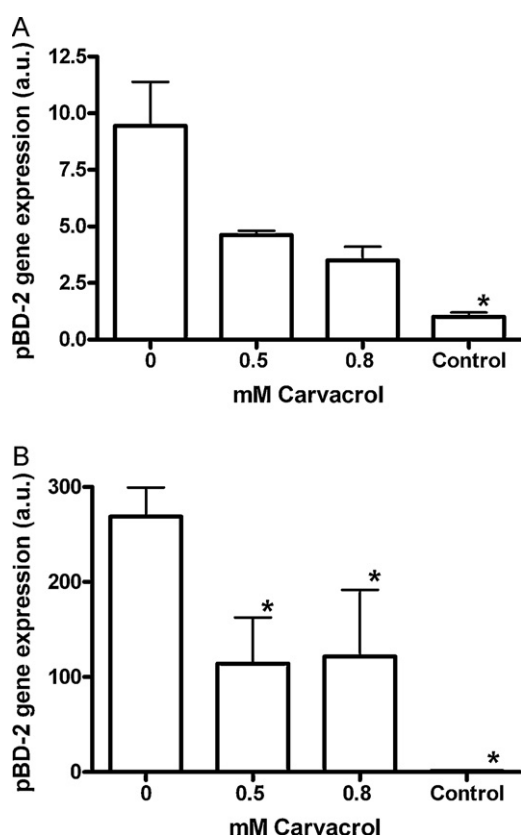


Fig. 6. Gene expression of pBD-2 in IPEC-J2 cells upon *S. Typhimurium* DT104 infection. Shown is the mean and SEM of three independent experiments in duplicate. (A) Gene expression 6 h post infection, (B) gene expression 24 h post infection. * indicates statistically significant difference compared to the 0 mM carvacrol sample.

raised the interest in the use of phytochemicals, such as carvacrol, as feed additives to increase the intestinal health of swine and prevent infections. In order to determine the exact mechanism of action of carvacrol or other antibacterial chemicals, studies are usually performed at concentrations close to, or higher than, the minimal inhibitory concentration (MIC) of this compound. However sub-lethal concentrations of chemicals can already have important effects on bacteria. Ultee and Smid showed that at low concentrations of carvacrol toxin production was inhibited in *Bacillus cereus*, while viability of this bacterium was unchanged (Ultee and Smid, 2001). Similarly, low sub-lethal concentrations of small and medium chain fatty acids affected gene expression of several virulence genes in *Salmonella* (Boyen et al., 2008).

In this study we determined the effect of sub-lethal concentrations of carvacrol on the virulence of *S. Typhimurium* DT104. Growth of *S. Typhimurium* was reduced at >1 mM carvacrol while it was completely inhibited at 2 mM. These MIC values are similar to most other studies describing direct antimicrobial effects of carvacrol *in vitro* (Burt, 2004). Interestingly, motility of *Salmonella* was greatly reduced at lower concentrations than growth inhibition, between 0.4 and 1 mM carvacrol (Fig. 2), as has been described for *E. coli* (Burt et al., 2007). In these latter studies, the lack of motility of *E. coli* after incubation with 1 mM carvacrol was attributed to the absence of the flagellum. For *S. Typhimurium* the effect of carvacrol seems to be different since no reduction in the number of flagella and the presence of flagellin was observed upon incubation with carvacrol (Fig. 3). This indicates that the loss of motility cannot be attributed to the loss of flagella but possibly to the loss of functionality of the flagellum.

Sub-lethal levels of carvacrol did not lead to decreased intracellular ATP levels in log phase or o/n cultures (Fig. 4). In log phase bacteria extracellular ATP levels were higher than non carvacrol-treated controls indicating that carvacrol caused some ATP leakage but that lost intracellular ATP was regenerated. Similar ATP leakage due to carvacrol has been described before but only at bactericidal concentrations of carvacrol (Ultee et al., 1999; Gill and Holley, 2006) and not at sub-lethal conditions. From the ATP results it is clear that loss of motility of *S. Typhimurium* is not related to intracellular ATP levels.

S. Typhimurium grown to log phase in the presence of 0.5 and 0.8 mM carvacrol showed reduced invasion of intestinal cells, while adhesion was relatively unaffected (Fig. 5). The correlation between motility and invasion has been shown before, although other factors such as the type III secretion system encoded by genes on the *Salmonella* pathogenicity island I are equally important (Shah et al., 2011). In addition, we tested whether the host response to *S. Typhimurium* invasion was affected. The lowered invasion characteristic of carvacrol treated *S. Typhimurium* indeed led to a twofold lower pBD-2 gene expression (Fig. 6). In earlier studies, we hypothesized that, based on our experiments using heat or colistin-killed *S. Typhimurium*, invasion of live bacteria was required for the induction of pBD-2 gene expression (Veldhuizen et al., 2009). The results described in this report confirm that invasion and not adhesion is important for the level of

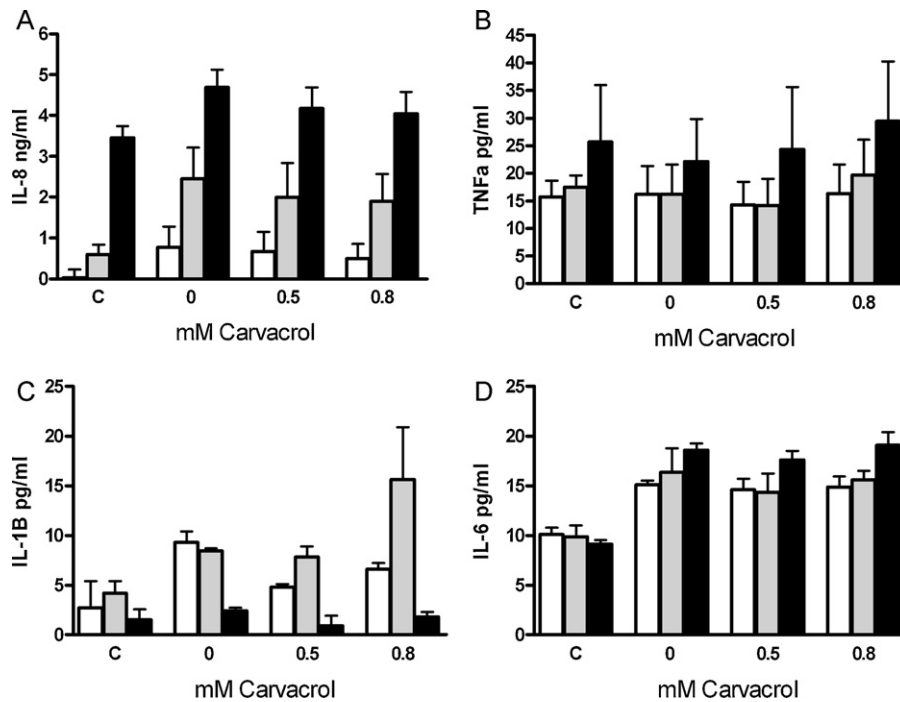


Fig. 7. Cytokine production of IPEC-J2 cells upon *S. Typhimurium* infection. IPEC-J2 were infected with carvacrol treated *S. Typhimurium*, IL-8 (A), TNF α (B) IL-1B (C) and IL6 (D) production was measured after 4 (white) 6 (grey) and 24 h (black bars). Shown is the mean and SEM of three independent experiments.

pBD-2 gene expression of the porcine epithelial cells. These results indicate that cytosolic immunosensors, such as NOD or NOD-like receptors (Delbridge and O'Riordan, 2007) could be involved in the host response towards *S. Typhimurium*. Reduced invasion of *Salmonella* did not lead to a changed cytokine expression profile of IPEC-J2 cells (Fig. 7), indicating that other signal transduction pathways lead to cytokine production compared to pBD-2 production.

A major concern for the potential of carvacrol, or in general any other feed additive, is the potential toxicity towards mammalian cells. Our experiments showed that a sub-lethal concentration of carvacrol has toxic effects on the porcine intestinal epithelial cell line IPEC-J2. A similar cytotoxic effect of carvacrol, or reduced proliferation of cells under the influence of carvacrol has been shown for several other cells lines (Fabian et al., 2006; Karkabounas et al., 2006; Lampronti et al., 2006). However, one report describes that up to 5 mM carvacrol did not affect the transepithelial electric resistance of porcine small intestinal epithelial cells (IPEC-1 cells) *in vitro*, implying that the toxicity is likely cell-type dependent (Roselli et al., 2007). In addition, several *in vivo* studies have been performed using carvacrol containing feed additives and these have not shown signs of great cytotoxicity (Windisch et al., 2008). This indicates that the use of cell lines is not always a good model to predict toxic effects *in vivo*. Another explanation is that the effectively available amount of carvacrol to interact with host cells is lower than calculated due to dilution effects along the gastrointestinal tract, and/or carvacrol binding to feed components. In

addition, some studies have shown that small lipophilic compounds such as carvacrol are absorbed by the upper part of the intestine, after which they are metabolized (Kohlert et al., 2002; Stoni et al., 2006).

A lower amount of freely available carvacrol could also explain why *in vivo* the beneficial effects of carvacrol and other phytochemicals are less pronounced. Although several studies show effects of (carvacrol containing) feed additives on food conversion and growth, (reviewed in Burt, 2004; Baser, 2008; Windisch et al., 2008), the effects often seem marginal compared to the expectations based on *in vitro* data. This implicates that much optimization in the experimental set-up is required. This needs to include dose and composition of the phytochemicals given, but also timing of administration (for example pre or post weaning) could turn out to be crucial. Moreover, it would be beneficial to protect the active components until they can be released at the part of the intestine where they are required. Finally, determination of clear, reliable and reproducible read-out parameters is needed in order to fully compare and understand the described activities of phytochemical activity.

In conclusion, we have shown that a sub-inhibitory concentration of carvacrol affects virulence of *Salmonella* leading to reduced motility and invasion of epithelial cells. This is an important finding since sub-inhibitory concentrations are likely to occur *in vivo* when carvacrol is used as a feed additive. However, it is of the highest importance to better map the interplay of carvacrol with other feed and/or intestinal components to understand its effect *in vivo* in the pig intestine.

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