

# Iron-Induced Virulence of *Salmonella enterica* Serovar Typhimurium at the Intestinal Epithelial Interface Can Be Suppressed by Carvacrol

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Oral iron therapy can increase the abundance of bacterial pathogens, e.g., *Salmonella* spp., in the large intestine of African children. Carvacrol is a natural compound with antimicrobial activity against various intestinal bacterial pathogens, among which is the highly prevalent *Salmonella enterica* serovar Typhimurium. This study aimed to explore a presumed interaction between carvacrol and bacterial iron handling and to assess the potential of carvacrol in preventing the increase of bacterial pathogenicity during high iron availability. *S. Typhimurium* was cultured with increasing concentrations of iron and carvacrol to study the effects of these combined interventions on growth, adhesion to intestinal epithelial cells, and iron uptake/influx in both bacterial and epithelial cells. In addition, the ability of carvacrol to remove iron from the high-affinity ligand transferrin and an Fe-dye complex was examined. Carvacrol retarded growth of *S. Typhimurium* at all iron conditions. Furthermore, iron-induced epithelial adhesion was effectively reduced by carvacrol at high iron concentrations. The reduction of growth and virulence by carvacrol was not paralleled by a change in iron uptake or influx into *S. Typhimurium*. In contrast, bioavailability of iron for epithelial cells was moderately decreased under these conditions. Further, carvacrol was shown to lack the properties of an iron binding molecule; however, it was able to weaken iron-ligand interactions by which it may possibly interfere with bacterial virulence. In conclusion, our *in vitro* data suggest that carvacrol has the potential to serve as a novel dietary supplement to prevent pathogenic overgrowth and colonization in the large intestine during oral iron therapy.

*Salmonella enterica* serovar Typhimurium is a major cause of gastroenteritis and invasive disease in humans, particularly in susceptible children in tropical Africa (1). Concurrently, there is a high prevalence of iron deficiency among children in developing countries. These children require oral iron therapy to prevent developmental impairment (2). Precariously, growth and colonization of *S. Typhimurium* appear to be stimulated by increased luminal iron availability *in vitro*, as well as *in vivo*, in several but not all published studies (3–6). Thus, there is an unfulfilled need to suppress this common intestinal pathogen.

Carvacrol is a monoterpenoid phenol present in essential oils; it has antimicrobial activity and is one of the main components in oregano and thyme oil (7). It has been shown to be active against various intestinal pathogens, including *S. Typhimurium* (7). Carvacrol appears to target the outer membrane of Gram-negative bacteria and increases membrane permeability, thereby causing depletion of the cellular ATP pool and leakage of other cytoplasmic constituents and outer membrane-associated material (7, 8). However, this process is currently not fully understood, and additional modes of action might exist. It has previously been shown that subinhibitory concentrations of carvacrol can affect virulence traits as it slightly reduced adhesion of *S. Typhimurium* to intestinal epithelial cells and significantly reduced invasion into these cells (9). In contrast, it was shown that increased iron availability promoted *S. Typhimurium* adhesion to, and invasion into, intestinal epithelial cells (3). Further, carvacrol was shown to increase expression of the heat shock protein GroEL and inhibit the synthesis of flagellin in *Escherichia coli* O157:H7 when grown in a standard rich medium (10). On the other hand, moderate to high iron availability reduced GroEL expression and increased flagellin synthesis in *S. Typhimurium* compared to low iron availability

(G. A. M. Kortman, R. W. H. M. Roelofs, and H. Tjalsma, unpublished data). The above-described findings inspired us to speculate that carvacrol may exert (in part) its antimicrobial mode of action through interference with pathways for bacterial iron handling. The present study aimed to investigate this hypothesis and to explore, by an *in vitro* approach, the potential of carvacrol to serve as a food additive to prevent pathogenic overgrowth and colonization in the large intestine during oral iron therapy.

## MATERIALS AND METHODS

**Bacterial strain, media, and growth conditions.** The strain used in this study was *Salmonella* Typhimurium NTB6 (3). This bacterium was cultured at 37°C and 5% CO<sub>2</sub> (standard conditions) in Iscove's modified Dulbecco's medium (IMDM; Invitrogen). This chemically defined medium does not contain iron in its formulation.

**Growth curves and determination of the MIC.** To determine the effects of iron and carvacrol on growth of *S. Typhimurium*, IMDM medium with increasing ferric citrate (Sigma-Aldrich) and carvacrol (Sigma-Aldrich) concentrations was inoculated with a fresh bacterial culture after overnight growth. A stock solution of ferric citrate was made in Milli-Q water (MQ), and a 0.1 mol/liter stock of carvacrol was made in absolute

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ethanol. Final concentrations of ethanol were kept constant at 2.5% (vol/vol) for each condition. Portions of 200  $\mu$ l of culture medium were inoculated in a microplate (in duplicate) and incubated statically at 37°C within a plate reader. To monitor bacterial growth, the optical density was automatically measured at 620 nm ( $OD_{620}$ ) every 20 min.

To determine the lowest concentration of carvacrol that inhibits the visible growth of *S. Typhimurium* NTB6 in IMDM, a serial dilution of carvacrol (0, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, and 2.5 mmol/liter) in IMDM in the presence of 0, 1, 10, or 100  $\mu$ mol/liter ferric citrate was tested. A total of 2 ml of medium in sterile, 75- by 12-mm capped tubes was inoculated with a fresh bacterial culture, and the  $OD_{620}$  was assessed after overnight incubation at 37°C. A negative control containing no bacteria was included. The MIC value, for the specific incubation conditions applied in the present study, was defined as the lowest concentration where no increase in optical density was observed, and concentrations of carvacrol below the MIC (and above 0 mmol/liter) that still supported growth were defined as subinhibitory. It should be noted that we chose to determine the MIC values for the growth medium that was used in this study to sustain consistent growth conditions. If desired in future studies, the MIC should be determined in a more standard reference growth medium (see, e.g., CLSI [<http://www.clsi.org/standards/>] or EUCAST [[http://www.eucast.org/antimicrobial\\_susceptibility\\_testing/mic\\_determination](http://www.eucast.org/antimicrobial_susceptibility_testing/mic_determination)] recommendations).

**Cell line, media, and growth conditions.** The human colorectal adenocarcinoma cell line Caco-2 (obtained from the American Type Culture Collection) was cultured at standard conditions in Dulbecco's modified Eagle's medium (DMEM; Lonza) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 20 mmol/liter HEPES, 100 nmol/liter nonessential amino acids (Invitrogen), and 2 mmol/liter L-glutamine (Lonza). Cells were subcultured every 6 days and used between passage numbers 3 and 6.

**Bacterial adhesion assay.** The influence of iron and carvacrol on adhesion of *S. Typhimurium* to Caco-2 cells was studied by the use of the following adhesion assay.

**(i) Preincubation of bacteria with ferric citrate and carvacrol.** IMDM with increasing ferric citrate concentrations (0 to 50  $\mu$ mol/liter) and carvacrol concentrations (0 to 0.5 mmol/liter) was inoculated with *S. Typhimurium* (overnight culture) and grown to exponential phase. The bacterial cells were pelleted, resuspended, and concentrated in IMDM with 10% glycerol for storage at  $-80^{\circ}\text{C}$  until use. Serial dilutions of thawed stocks were transferred to blood agar plates and incubated overnight to determine the CFU.

**(ii) Culturing of Caco-2 cells.** Caco-2 cells were subcultured in a 24-well plate and maintained until use in adhesion assays. The assays were performed on confluent monolayers between 14 and 16 days after seeding the cells.

**(iii) Bacterial adhesion assay.** The Caco-2 monolayers were washed once with phosphate-buffered saline (PBS). The preincubated stocks of *S. Typhimurium* were pelleted and resuspended in IMDM. Next, bacteria were added to the monolayers at a multiplicity of infection (MOI) of 10:1 in IMDM followed by incubation for 2 h at standard conditions. To determine the number of adherent bacteria, monolayers were washed three times with PBS, and cells were trypsinized and lysed with ice-cold PBS containing 0.025% Triton X-100. Serial dilutions of cell lysates were transferred to blood agar plates for CFU counting.

**Determination of iron uptake by *S. Typhimurium* under the influence of carvacrol.** To determine the effect of carvacrol on iron uptake by *S. Typhimurium*, bacterial cells were grown with iron and carvacrol, after which the total iron content of the bacteria was determined. Cultures (10 ml) of *S. Typhimurium* were incubated with ferric citrate (0 to 50  $\mu$ mol/liter) and carvacrol (0 to 0.5 mmol/liter) under standard conditions. Bacteria were harvested by centrifugation for 5 min at  $3,220 \times g$ , and pellets were resuspended in 5 ml PBS containing 1 mmol/liter deferoxamine (DFO; Sigma-Aldrich) to sequester iron that is loosely bound to the outer membrane of the bacteria. After another centrifugation step, pellets were

resuspended in 1.5 ml PBS and transferred to fresh 2-ml tubes. Tubes were centrifuged for 5 min at  $10,000 \times g$ , and the supernatant was removed. Pellets were snap-frozen in liquid nitrogen ( $3\times$ ), wet pellet weight was noted, and iron content was determined by acid digestion of the bacteria and use of the chromogen bathophenanthroline as described by Torrance and Bothwell (11). To this purpose, bacterial pellets were resuspended in 80  $\mu$ l of acid reagent (3 mol/liter HCl containing 10% trichloroacetic acid) and incubated at 65°C for 20 h with continuous mixing. After incubation, samples were centrifuged to obtain the supernatant, and 20  $\mu$ l was applied to a microplate. Then, 200  $\mu$ l chromogenic reagent containing 127 mg/liter bathophenanthroline sulfonate disodium salt (Fluka), 936 mg/liter thioglycolic acid sodium salt (Sigma-Aldrich), and sodium acetate (diluted 2.2 times from a saturated solution) was added to the samples. After 10 min of incubation at room temperature (RT), color formation was measured at 540 to 560 nm. Iron concentration was determined by comparison to a standard curve made of ferrous sulfate and was corrected for wet pellet weight.

**Determination of iron bioavailability to Caco-2 cells.** To examine the effect of carvacrol on the uptake of iron by enterocytes, Caco-2 cells were exposed to iron and carvacrol. Iron uptake was quantified by measurement of intracellular ferritin levels.

**(i) Culturing of Caco-2 cells.** Culturing was done as described above, with the exception that the assays were performed between 13 and 14 days after seeding the cells.

**(ii) Exposure of Caco-2 cells to iron and carvacrol.** Two days before incubation of the cells with iron and carvacrol, the standard culture medium was replaced by IMDM. Both ferric citrate and ferrous sulfate (20  $\mu$ mol/liter Fe) in combination with carvacrol (0 to 0.3 mmol/liter) or ascorbic acid (200  $\mu$ mol/liter) and tannic acid (2  $\mu$ mol/liter) as the positive and negative control, respectively, were applied to the cells in fresh IMDM. Higher concentrations of carvacrol could not be tested, because of cytotoxic effects to the cells. The cells were incubated for 20 h at standard conditions. At harvesting, cells were washed once with 1 ml PBS containing 1 mmol/liter DFO and washed once with 1 ml PBS. Cells were frozen on dry ice and thawed, after which 100  $\mu$ l of 5 mmol/liter NaOH was added. Cells were then lysed by sonication for 15 min in a sonication bath containing ice water. To neutralize, 50  $\mu$ l of 10 mmol/liter HCl was added, and cells were loosened by scraping. Suspensions were sonicated 5 min and then transferred to fresh tubes. Debris was pelleted by centrifugation, and the supernatant was used for quantification of total protein and ferritin.

**(iii) Quantification of ferritin levels.** Samples were applied to the ferritin enzyme-linked immunosorbent assay (ELISA; Spectro ferritin; Ramco), and the assay was performed according to the manufacturer's instructions. Ferritin levels were normalized for total protein content. Total protein was measured by the use of a Bradford protein assay (Bio-Rad) according to the manufacturer's instructions.

**Iron removal from transferrin assay.** To examine the potential of carvacrol to sequester iron, the ability of carvacrol to remove iron from transferrin was investigated. Incubation buffer containing 100 mmol/liter Tris-HCl (pH 7.5) and 10% glycerol (vol/vol) was depleted of trace iron by pretreatment with Chelex-100 resin (Sigma-Aldrich). Then, 50  $\mu$ g of holo-transferrin (hTf) from human (Sigma-Aldrich) was dissolved in 50  $\mu$ l incubation buffer, and hTf was incubated with carvacrol (0 to 250  $\mu$ mol/liter) and positive controls DFO (500  $\mu$ mol/liter) and tannic acid (Sigma-Aldrich; 100  $\mu$ mol/liter) for 18 h at 37°C. The iron binding status of transferrin (Tf) was investigated by polyacrylamide gel electrophoresis according to the method described by Makey and Seal (12) with a few adaptations. Briefly, a freshly prepared solution containing 6% acrylamide (37.5:1 with bis-acrylamide), 6 mol/liter urea, and TBE buffer (0.1 mol/liter Tris, 0.01 mol/liter boric acid, 0.05 mol/liter EDTA, pH 8.4) was pretreated with Chelex-100 resin and then polymerized in a Bio-Rad Protean II vertical minigel system. The gel was prerun at 70 V for 20 min, and 20  $\mu$ g of the Tf samples was loaded. Partially saturated human transferrin (Sigma-Aldrich) was used as a marker standard (20  $\mu$ g). The gel tray was

placed in ice water, and electrophoresis was performed at 100 V for 7 h. After electrophoresis, the gel was fixed for 15 min in fixative containing 10% (vol/vol) acetic acid, 50% (vol/vol) methanol, and 40% demineralized water (vol/vol). To visualize forms of Tf, it was stained with Coomassie brilliant blue.

**Iron removal from CAS assay.** A universal chemical assay for the detection of iron acquisition siderophores was used to examine the capability of carvacrol to remove iron from a dye-Fe complex. Chrome azurol S (CAS) in complex with ferric iron and hexadecyltrimethylammonium bromide (HDTMA) has a blue color but turns orange when iron is removed from the dye by a stronger ligand. The method used was originally described by Schwyn and Neilands (13). Briefly, a solution of 2 mmol/liter CAS (Tokyo Chemical Industry) was prepared, and 1,875  $\mu$ l was mixed with 375  $\mu$ l ferric iron solution (1 mmol/liter FeCl<sub>3</sub> [Merck] in 10 mmol/liter HCl). Separately, 1.5 ml of 10 mmol/liter HDTMA (Sigma-Aldrich) was placed in an acid-cleaned volumetric flask of 25 ml, and the CAS-iron mixture was slowly added with simultaneous mixing. Then, 1.077 g of anhydrous piperazine (Fluka) was dissolved in 7 ml MQ, and 1,560  $\mu$ l of 12 mol/liter HCl was added. This buffer was rinsed into the volumetric flask, and MQ was added to 25 ml to complete the assay solution. The solution was stored in a plastic tube in the dark. To establish increased speed in iron exchange, a shuttle solution was prepared which involved the addition of 25.42 mg 5-sulfosycilic acid dihydrate (Sigma-Aldrich) to the assay solution before adding the final MQ to a total of 25 ml.

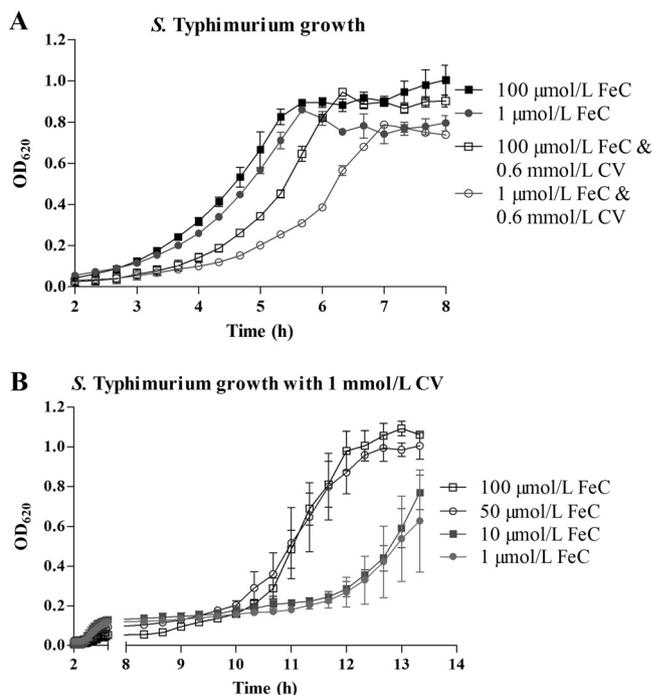
Iron removal capacity and speed of iron removal were tested in a microplate. A total of 100  $\mu$ l carvacrol (0 to 1 mmol/liter in IMDM or MQ), DFO (0 to 20  $\mu$ mol/liter in IMDM or MQ), or tannic acid (0 to 5.9  $\mu$ mol/liter in IMDM or MQ) was mixed with 100  $\mu$ l CAS assay (shuttle) solution. Absorption was periodically monitored at 620 nm.

**Statistical analysis.** To compare means, an unpaired *t* test (2-tailed) was used. In case of unequal variances (as assessed by F-test), an unpaired *t* test with Welch's correction was carried out. To assess the slope of iron-dependent adhesion, linear regression analysis was applied. For comparison of iron uptake by *S. Typhimurium* under influence of carvacrol, two-way analysis of variance (ANOVA) was performed. All analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA. *P* values of <0.05 were considered statistically significant.

## RESULTS

### Growth of *S. Typhimurium* in response to iron and carvacrol.

To evaluate the possibility that the antimicrobial effect of carvacrol can be explained by its interference with bacterial iron handling, the effect of carvacrol on growth of *S. Typhimurium* was tested under moderate iron concentrations and iron-rich conditions. In general, these experiments showed that carvacrol can delay the onset of growth of this bacterium. Growth delay was established under both moderate iron concentrations and high-iron conditions (Fig. 1A; see also Fig. S1 in the supplemental material). In the absence of carvacrol, the high-iron conditions were not influencing growth compared to the moderate iron concentrations. However, in the presence of carvacrol, a shorter lag phase is observed with culture medium containing high iron concentrations than growth with moderate iron concentrations. This suggests that high iron availability partly compensates the postponement effect of carvacrol. Notably, increased carvacrol concentration of 1 mmol/liter strongly retarded growth, but growth was strikingly less retarded in 50 to 100  $\mu$ mol/liter ferric citrate compared to 1 to 10  $\mu$ mol/liter ferric citrate (Fig. 1B). Levels of carvacrol of  $\leq$ 1 mmol/liter were found to be the subinhibitory range under these conditions, as 1 mmol/liter carvacrol was the highest concentration tested that still supported growth under all iron conditions. A slightly higher concentration of 1.25



**FIG 1** Effect of iron and subinhibitory concentrations of carvacrol on growth of *S. Typhimurium*. (A) Effect of 0.6 mmol/liter carvacrol (CV) on *S. Typhimurium* growth (mean  $\pm$  range) for 8 h in medium with 1  $\mu$ mol/liter (open circles) or 100  $\mu$ mol/liter ferric citrate (FeC) (open squares) compared to the same conditions without carvacrol (filled circles or squares, respectively) ( $n = 2$ ; duplicate wells). (B) Effect of 1 mmol/liter carvacrol on growth of *S. Typhimurium* for 13.3 h with 100 (open squares), 50 (open circles), 10 (filled squares), or 1 (filled circles)  $\mu$ mol/liter ferric citrate ( $n = 2$ ). Without addition of any iron, growth is more hampered, as the trace amount of iron in the IMDM limits growth of *S. Typhimurium* (3).

mmol/liter effectively prevented an increase in optical density after overnight growth (MIC).

**Adhesion of *S. Typhimurium* to an epithelial monolayer.** Adhesion to host epithelial cells is an important virulence characteristic for pathogenic bacteria. To test whether iron-induced adhesion, as observed previously (3), can be counteracted by carvacrol, *S. Typhimurium* was first preincubated with increasing concentrations of ferric citrate and carvacrol. To determine the adhesion, preincubated bacteria were added to intestinal epithelial monolayers (Caco-2) under iron-limiting conditions and without carvacrol. Adhesion was expressed as the percentage of the bacterial inoculum and relative to the conditions without iron addition during preincubation. As shown in Fig. 2, iron stimulated the adhesion of *S. Typhimurium*; however, this effect was significantly reduced by carvacrol, especially at the highest iron concentration tested. It should be noted that carvacrol did not significantly affect adhesion under the condition without iron (see Fig. S2 in the supplemental material). Linear regression analysis revealed that the slope of the control conditions without carvacrol ( $2.79 \pm 0.50$ ) was significantly steeper than the slope of the 0.2 mmol/liter carvacrol conditions ( $0.51 \pm 0.20$ ) ( $P = 0.013$ ). Slope of the conditions with 0.5 mmol/liter carvacrol ( $1.47 \pm 0.48$ ) was lower than the controls, although not significantly ( $P = 0.129$ ). Taken together, these experiments show that subinhibitory levels of carvacrol can reduce iron-induced adhesion of *S. Typhimurium* to an epithelial monolayer.

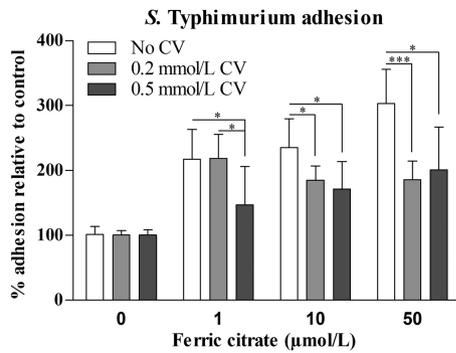


FIG 2 Effect of carvacrol on iron-induced adhesion of *S. Typhimurium* to an epithelial monolayer. Adhesion (mean + SD) of *S. Typhimurium* to a monolayer of Caco-2 cells is given as the percentage of the inoculum and expressed relative to the conditions without iron during preincubation. Data consists of 3 separate experiments in which adherent CFU were determined on different days with duplicate wells ( $n = 6$ ). \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ .

**Iron uptake by *S. Typhimurium* under influence of carvacrol.** One of the mechanisms by which carvacrol could influence bacterial adhesion is the inhibition of iron uptake by *S. Typhimurium*, which consequently would affect adhesion traits. To investigate this, we measured total iron content of *S. Typhimurium* cells which were grown in the presence of increasing concentrations of ferric citrate and carvacrol. The iron content of the bacteria grown without carvacrol was significantly increased in the 10 µmol/liter ferric citrate condition compared to that in the 1 µmol/liter ferric citrate condition ( $P = 0.012$ ) and further increased with 50 µmol/liter ferric citrate ( $P = 0.034$  compared to 1 µmol/liter ferric citrate), indicating increased iron uptake at increasing iron concentration as expected (Fig. 3). Carvacrol appeared not to influence iron uptake or influx significantly at all iron concentrations tested (two-way ANOVA). As carvacrol did not reduce the uptake of iron by *S. Typhimurium*, the inhibiting effect on adhesion can probably not be explained by altered iron uptake.

**Bioavailability of iron to intestinal epithelial cells under influence of carvacrol.** To examine the effect of carvacrol on intestinal iron uptake *in vitro*, iron together with nontoxic concentrations of carvacrol were applied to Caco-2 monolayers. Next to ferric citrate, ferrous sulfate was tested, as this form of iron is

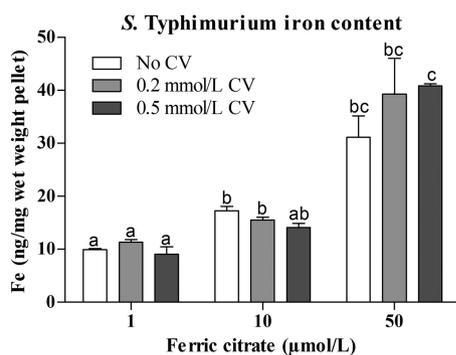


FIG 3 Total iron content of *S. Typhimurium* after growth with iron and carvacrol. *S. Typhimurium* was grown to mid-log phase with increasing concentrations of iron and carvacrol. Iron content (mean + range) is given as ng/mg wet biomass ( $n = 2$ ). Means without a common letter differ significantly ( $P < 0.05$ ).

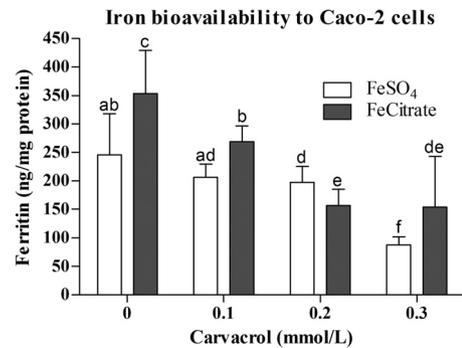
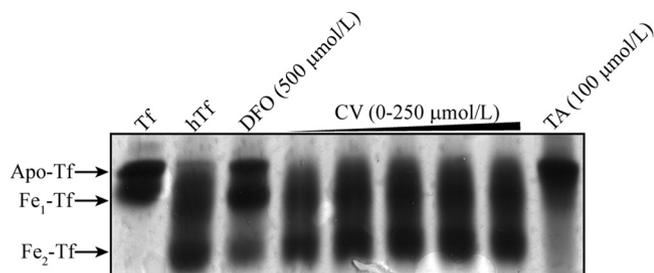


FIG 4 Influence of carvacrol on the bioavailability of iron to an epithelial monolayer. Caco-2 cells were exposed to equimolar concentrations of iron and increasing concentrations of carvacrol. Bioavailability of iron is expressed as total intracellular ferritin content, corrected for total protein (mean + SD). Data consist of 2 separate experiments with measurements performed in triplicate ( $n = 6$ ). Means without a common letter differ significantly ( $P < 0.05$ ).

widely used in iron supplementation studies. Cytoplasmic levels of ferritin were used as a measure for cellular iron uptake. As shown in Fig. 4, in the absence of carvacrol, bioavailability of ferric citrate was higher than ferrous sulfate ( $P = 0.004$ ). Bioavailability of both iron sources was considerably reduced by carvacrol; ferritin formation was about 2.5 times less with 0.3 mmol/liter carvacrol compared to that of the no-carvacrol controls ( $P < 0.0002$  for both iron sources). Notably, carvacrol did not abolish iron uptake, while the addition of the food derived and strong iron binding tannic acid at 2 µmol/liter resulted in a near-complete block of iron uptake (see Fig. S3A in the supplemental material). We note that higher concentrations of carvacrol could not be tested due to toxic effects to the cells, as determined by a lactate dehydrogenase (LDH) release assay (see Fig. S4 in the supplemental material). In contrast, 200 µmol/liter ascorbic acid as a known promoter of iron uptake enhanced iron uptake of both iron sources, especially of ferric citrate ( $P = 0.003$  and  $P < 0.0001$  for ferrous sulfate and ferric citrate, respectively) (see Fig. S3B in the supplemental material).

**Iron binding capacity of carvacrol.** The potential iron binding capacity of carvacrol, or its ability to dissociate iron from iron binding ligands, was investigated in an iron from transferrin removal assay and a universal siderophore CAS assay. The iron from the transferrin removal assay revealed that carvacrol up to 0.25 mmol/liter could not take away iron from transferrin, indicating that carvacrol is not a high-affinity iron binding molecule and cannot dissociate the transferrin-iron complex (Fig. 5). In contrast, use of the strong iron binding molecules DFO and tannic acid, which served as positive controls in this assay, clearly showed their ability to remove 1 or 2 iron atoms from the transferrin molecule.

As shown in Fig. 6A, the siderophore CAS assay revealed that carvacrol can dissociate the Fe-dye complex partly (0.2 mmol/liter) or completely (0.6 mmol/liter); however, this was observed only after a long incubation of 15 h, while dissociation of the Fe-dye complex was already evident after only 3 h for the positive controls DFO (Fig. 6B) and tannic acid (not shown) at low concentrations. CAS-shuttle solution increased the transfer speed of Fe from the dye to DFO, but this was not true for carvacrol (Fig. 6A and B). Together, these results indicate that carvacrol does not



**FIG 5** Iron from transferrin removal assay. hTf was incubated with increasing concentrations of carvacrol and the positive controls DFO and tannic acid (TA) to assess iron removal from hTf. First lane, partially saturated Tf; second lane, hTf control; third lane, 500  $\mu\text{mol/liter}$  DFO; fourth lane, 0  $\mu\text{mol/liter}$  carvacrol (CV) solvent control; fifth lane, 1  $\mu\text{mol/liter}$  CV; sixth lane, 10  $\mu\text{mol/liter}$  CV; seventh lane, 100  $\mu\text{mol/liter}$  CV; eighth lane, 250  $\mu\text{mol/liter}$  CV; ninth lane, 100  $\mu\text{mol/liter}$  TA. Top arrow indicates Tf without iron bound to it, middle arrow indicates Tf with 1 Fe atom bound, and bottom arrow indicates hTf with 2 Fe atoms bound to the protein.

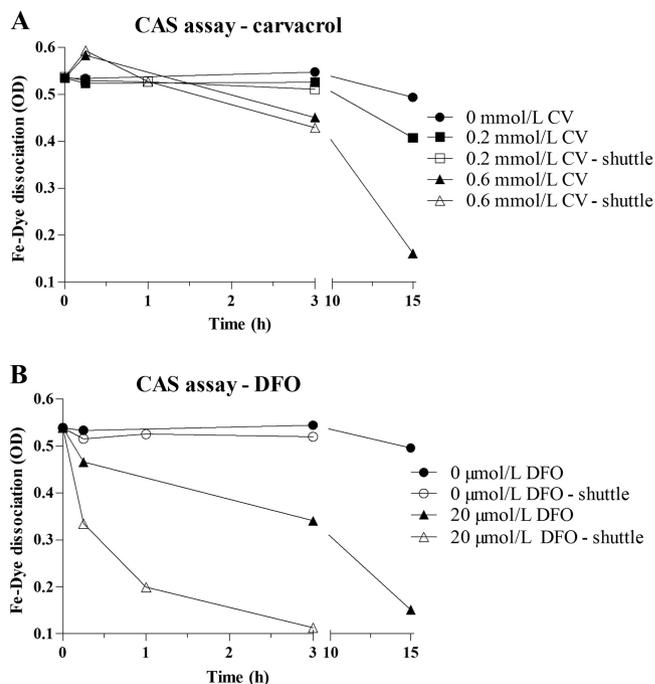
bind iron strongly, but it may be able to disturb iron-ligand complexes under certain conditions.

**S. Typhimurium global lipopolysaccharide (LPS) composition during growth with iron and carvacrol.** As LPS is an important factor in bacterial adhesion (14, 15) and carvacrol is known to induce the release of LPS from *E. coli* O157:H7 (8), carvacrol could affect adhesion characteristics of *S. Typhimurium* by removing LPS from the cell surface. We therefore examined the effect of carvacrol on LPS content and global LPS composition during growth under moderate- to high-iron conditions. As shown in Fig. S5 in the supplemental material, carvacrol up to 0.5 mmol/liter did not detectably influence LPS quantity or global LPS composition. It should be noted that this general analysis cannot visualize LPS in great detail and indicates only that carvacrol did not alter global LPS status of *S. Typhimurium* during growth, which makes it less likely that iron-induced adhesion was reduced by modulating LPS integrity at the bacterial surface.

## DISCUSSION

Oral iron supplementation has potential harmful side effects on gastrointestinal health as a consequence of increased colonization and growth of bacterial pathogens (3, 4). As a result, there is a demand for safer iron supplementation programs. Therefore, we here assessed by an *in vitro* approach whether the simultaneous addition of carvacrol as an antimicrobial agent could counteract the potential adverse effect of iron on the prevalent intestinal pathogen *S. Typhimurium*.

In the present study, we showed that subinhibitory concentrations of carvacrol retarded bacterial growth under all tested conditions. Notwithstanding the presence of a sufficient amount of iron under all conditions, the growth retardation effect of carvacrol was most pronounced under conditions of moderate iron availability. This suggested that its antimicrobial activity is partly related to an iron scavenging effect that is less effectual under high-iron conditions. Nevertheless, we want to emphasize that subinhibitory concentrations of carvacrol delayed growth of *S. Typhimurium* under all conditions. Hence, the previously described antimicrobial activity of carvacrol also applies for conditions with high iron availability (7). Although the ability to replicate is important for bacterial pathogens, the adhesion to host tissues or epithelium is pivotal to establish an infection. Our cur-



**FIG 6** Effect of carvacrol on the stability of the CAS-Fe complex. Removal of iron from the colored complex is displayed as a decrease in optical density. Carvacrol or DFO was incubated with the CAS-Fe complex without and with shuttle solution (filled and open symbols, respectively). (A) Dissociation of the Fe-dye complex in time was monitored without the addition of carvacrol (filled circles) and with the addition of 0.2 mmol/liter (filled and open squares) or 0.6 mmol/liter carvacrol (filled and open triangles). (B) As control conditions, the CAS-Fe complex was incubated without DFO (filled and open circles) and with the addition of 20  $\mu\text{mol/liter}$  DFO (filled and open triangles).

rent data confirm previous findings that iron induces adhesion of *S. Typhimurium* to intestinal epithelial cells *in vitro* (3). Importantly, preincubation of *S. Typhimurium* with subinhibitory concentrations of carvacrol clearly reduced adhesion induced by moderate to high iron concentrations. Together, these observations support the use of carvacrol as a potential additive in oral iron preparations to prevent pathogenic overgrowth and colonization during iron supplementation.

An antimicrobial additive in an iron preparation should not decrease iron uptake from the intestinal lumen or, ideally, should increase it. We therefore investigated the effect of carvacrol on the uptake of iron from different sources by intestinal epithelial Caco-2 cells. Expression of ferritin was used as a readout, which is commonly used as a gold standard to study bioavailability of iron preparations (16). Although carvacrol clearly restricted iron uptake of both ferrous sulfate and ferric citrate, it did not block iron uptake completely like tannic acid, a well-known iron binder, did (17). In general, the bioavailability of ferrous iron is better than that of ferric iron (18–20), but with ferric citrate larger amounts of ferritin were measured than with ferrous sulfate, indicating that in this system the bioavailability of ferric citrate is better. The higher solubility and stability of ferric citrate than those of ferrous sulfate are probably the reasons for this observation. Thus, carvacrol seems not to meet the ideal criteria of an iron additive, as it impairs iron uptake by intestinal cells *in vitro*. Nevertheless, this decreased level of iron bioavailability could still suffice for adequate iron uptake and healthy iron homeostasis *in vivo*, whereas it may be

accompanied by a desirable decrease in virulence of pathogenic gut bacteria.

Iron scavenging by carvacrol could have explained the growth delay and decreased adhesion of *S. Typhimurium* under moderate iron concentrations and the decreased bioavailability of iron to Caco-2 cells in the presence of carvacrol. However, based on the molecular structure of carvacrol, it seems unlikely that it can bind iron ions. Additionally, in the present study, we showed that carvacrol could not remove iron from the high-affinity protein transferrin but was able to dissociate a dye-Fe complex during prolonged incubations. Together, this indicates that carvacrol seems not to bind iron directly but is able to disturb iron-ligand complexes with lower affinity. It can therefore be envisaged that carvacrol may interfere with iron uptake pathways of intestinal epithelial cells rather than making it less bioavailable by scavenging of iron ions. However, it remains to be elucidated by which molecular mechanisms carvacrol could affect cellular iron uptake systems. In contrast to the reduced iron uptake by intestinal epithelial cells, we were not able to show a significant effect of carvacrol on iron uptake or influx into *S. Typhimurium*. Consequently, the observed effects of subinhibitory concentrations of carvacrol on iron-induced growth and adhesion can probably not be explained by interference with bacterial iron uptake or increased influx but instead seem predominantly caused by other (iron-independent) activities of carvacrol.

Although total iron content of *S. Typhimurium* was not altered by carvacrol, the free intracellular iron pool balance could be distorted. A potential elevation of the intracellular “free” iron pool may enhance cell stress via generation of oxygen radicals (Fenton chemistry). This increased iron toxicity may contribute to an overall reduced fitness of the bacteria, which could very well result in the observed adhesion defects under iron-replete conditions in the presence of carvacrol. If true, it remains difficult to explain why *S. Typhimurium* grows slightly better in medium with carvacrol and high iron concentrations but adheres less well under these conditions than bacteria grown under intermediate iron concentrations with carvacrol. Another possible explanation for the carvacrol-induced delay in growth and decreased adhesion of *S. Typhimurium* involves a decrease in outer membrane integrity/stability or an altered surface structure. Carvacrol is known to induce release of LPS from *E. coli* O157:H7 (8). Similarly, iron depletion is associated with reduced LPS content in *Helicobacter pylori* (21). As LPS is a known modulator of bacterial adhesion, both iron concentration and the addition of carvacrol could therefore theoretically affect *S. Typhimurium* adhesion. However, the outcome of our first LPS characterization experiments did not show a clear alteration of LPS content or structure when *S. Typhimurium* was grown in the presence of iron and carvacrol. Nevertheless, carvacrol and high concentrations of iron could still destabilize the bacterial outer membrane through interference with divalent cations such as  $Mg^{2+}$  and  $Ca^{2+}$  that bind to the anionic LPS and thereby stabilize the outer membrane (22). Carvacrol and iron could somehow distort this delicate balance and thereby indirectly influence LPS-mediated adhesion. To assess this hypothesis, the potential interaction of iron and carvacrol with LPS needs to be investigated further in greater detail. This could involve the detection and characterization of LPS shed into the medium by using additional LPS characterization techniques.

In summary, the natural antimicrobial carvacrol can potentially be used as an additive during oral iron therapy to prevent

pathogenic overgrowth and colonization in the large intestine. Future *in vivo* research should reveal whether carvacrol can indeed improve the safety of oral iron therapy. The success of this approach will depend on defining an optimal dose of carvacrol which exerts its antimicrobial effect in the host's intestines but does not inhibit improvement of host iron status. One challenge in dosing is the absorption of carvacrol by the host upper GI tract, which likely results in low concentrations in the colon and reduced effectiveness in this target organ. This is probably the reason that oral carvacrol administration was previously shown to have little effect on colonic microbiota composition in piglets (23). Nevertheless, gastrointestinal health in these piglets was improved, which could, for instance, relate to a reduction in virulence of low-abundance gut pathogens, whereas other modes of carvacrol delivery, e.g., in encapsulated form, still need to be examined (23). Importantly, the latter approach could be used for the targeted delivery of antimicrobial activity in the human colon during oral iron administration with minimal effect on iron uptake in the ileum of the host. Subinhibitory concentrations of carvacrol are nevertheless likely to occur in the colon. Nonetheless, subinhibitory concentrations are effective in reducing bacterial virulence, as we show in the present study and which is supported by previous findings (9, 24). Under the experimental culture conditions of our study, a concentration of carvacrol of  $\geq 0.2$  mmol/liter was already effective in reducing iron-induced adhesion, while concentrations of  $\leq 0.3$  mmol/liter were well tolerated by the differentiated intestinal epithelial cell line Caco-2. The latter is supported by previous findings (25), and previous *in vivo* studies have shown that carvacrol as a feed additive is well tolerated and does not show signs of great cytotoxicity (23, 26). Another challenge is to simultaneously find the optimal iron preparation and dose for effective improvement of host iron status, with minimal effect on the gut microbiota. Although we did not test the effect of iron-carrying substances other than ferric citrate on bacterial virulence, we believe that most soluble iron sources will enhance the virulence of intestinal pathogens, as we previously showed for ferric citrate (3). Taken together, carvacrol is a known natural dietary additive, with the potential to prevent gastrointestinal side effects during oral iron therapy in regions where infection is endemic. Nevertheless, it goes without saying that future *in vivo* studies are required to further evaluate the real therapeutic utility of carvacrol as an anti-infective agent in combination with dietary iron supplements.

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## REFERENCES

1. Graham SM. 2010. Nontyphoidal salmonellosis in Africa. *Curr. Opin. Infect. Dis.* 23:409–414. <http://dx.doi.org/10.1097/QCO.0b013e3283dd25d>.
2. WHO, UNICEF. 2004. Focusing on anaemia: towards an integrated approach for effective anaemia control. WHO, Geneva, Switzerland.
3. Kortman GA, Boleij A, Swinkels DW, Tjalsma H. 2012. Iron availability increases the pathogenic potential of *Salmonella Typhimurium* and other enteric pathogens at the intestinal epithelial interface. *PLoS One* 7:e29968. <http://dx.doi.org/10.1371/journal.pone.0029968>.

4. Zimmermann MB, Chassard C, Rohner F, N'Goran EK, Nindjin C, Dostal A, Utzinger J, Ghattas H, Lacroix C, Hurrell RF. 2010. The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire. *Am. J. Clin. Nutr.* 92:1406–1415. <http://dx.doi.org/10.3945/ajcn.110.004564>.
5. Dostal A, Chassard C, Hilty FM, Zimmermann MB, Jaeggi T, Rossi S, Lacroix C. 2012. Iron depletion and repletion with ferrous sulfate or electrolytic iron modifies the composition and metabolic activity of the gut microbiota in rats. *J. Nutr.* 142:271–277. <http://dx.doi.org/10.3945/jn.111.148643>.
6. Dostal A, Fehlbaum S, Chassard C, Zimmermann MB, Lacroix C. 2012. Low iron availability in continuous in vitro colonic fermentations induces strong dysbiosis of the child gut microbial consortium and a decrease in main metabolites. *FEMS Microbiol. Ecol.* 83:161–175. <http://dx.doi.org/10.1111/j.1574-6941.2012.01461.x>.
7. Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. *Int. J. Food Microbiol.* 94:223–253. <http://dx.doi.org/10.1016/j.ijfoodmicro.2004.03.022>.
8. Helander IM, Alakomi H-L, Latva-Kala K, Mattila-Sandholm T, Pol I, Smid EJ, Gorris LGM, von Wright A. 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. *J. Agric. Food Chem.* 46:3590–3595. <http://dx.doi.org/10.1021/jf980154m>.
9. Inamuco J, Veenendaal AK, Burt SA, Post JA, Tjeerdma-van Bokhoven JL, Haagsman HP, Veldhuizen EJ. 2012. Sub-lethal levels of carvacrol reduce *Salmonella* Typhimurium motility and invasion of porcine epithelial cells. *Vet. Microbiol.* 157:200–207. <http://dx.doi.org/10.1016/j.vetmic.2011.12.021>.
10. Burt SA, van der Zee R, Koets AP, de Graaff AM, van Knapen F, Gaastra W, Haagsman HP, Veldhuizen EJA. 2007. Carvacrol induces heat shock protein 60 and inhibits synthesis of flagellin in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 73:4484–4490. <http://dx.doi.org/10.1128/AEM.00340-07>.
11. Torrance JD, Bothwell TH. 1968. A simple technique for measuring storage iron concentrations in formalinised liver samples. *S. Afr. J. Med. Sci.* 33:9–11.
12. Makey DG, Seal US. 1976. The detection of four molecular forms of human transferrin during the iron binding process. *Biochim. Biophys. Acta* 453:250–256. [http://dx.doi.org/10.1016/0005-2795\(76\)90270-1](http://dx.doi.org/10.1016/0005-2795(76)90270-1).
13. Schwyn B, Neilands JB. 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160:47–56. [http://dx.doi.org/10.1016/0003-2697\(87\)90612-9](http://dx.doi.org/10.1016/0003-2697(87)90612-9).
14. Bravo D, Hoare A, Silipo A, Valenzuela C, Salinas C, Alvarez SA, Molinaro A, Valvano MA, Contreras I. 2011. Different sugar residues of the lipopolysaccharide outer core are required for early interactions of *Salmonella enterica* serovars Typhi and Typhimurium with epithelial cells. *Microb. Pathog.* 50:70–80. <http://dx.doi.org/10.1016/j.micpath.2010.11.001>.
15. Kong Q, Yang J, Liu Q, Alamuri P, Roland KL, Curtiss R, III. 2011. Effect of deletion of genes involved in lipopolysaccharide core and O-antigen synthesis on virulence and immunogenicity of *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* 79:4227–4239. <http://dx.doi.org/10.1128/IAI.05398-11>.
16. Glahn RP, Lee OA, Yeung A, Goldman MI, Miller DD. 1998. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an *in vitro* digestion/Caco-2 cell culture model. *J. Nutr.* 128:1555–1561.
17. South PK, Miller DD. 1998. Iron binding by tannic acid: effects of selected ligands. *Food Chem.* 63:167–172. [http://dx.doi.org/10.1016/S0308-8146\(98\)00040-5](http://dx.doi.org/10.1016/S0308-8146(98)00040-5).
18. Hernandez M, Sousa V, Moreno A, Villapando S, Lopez-Alarcon M. 2003. Iron bioavailability and utilization in rats are lower from lime-treated corn flour than from wheat flour when they are fortified with different sources of iron. *J. Nutr.* 133:154–159.
19. Santiago P. 2012. Ferrous versus ferric oral iron formulations for the treatment of iron deficiency: a clinical overview. *ScientificWorldJournal* 2012:846824. <http://dx.doi.org/10.1100/2012/846824>.
20. Yokoi K, Konomi A, Otagi M. 2009. Iron bioavailability of cocoa powder as determined by the Hb regeneration efficiency method. *Brit. J. Nutr.* 102:215–220. <http://dx.doi.org/10.1017/S0007114508149182>.
21. Keenan JI, Davis KA, Beaugie CR, McGovern JJ, Moran AP. 2008. Alterations in *Helicobacter pylori* outer membrane and outer membrane vesicle-associated lipopolysaccharides under iron-limiting growth conditions. *Innate Immun.* 14:279–290. <http://dx.doi.org/10.1177/1753425908096857>.
22. Nikaido H. 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* 67:593–656. <http://dx.doi.org/10.1128/MMBR.67.4.593-656.2003>.
23. Michiels J, Missotten J, Van Hoorick A, Obyn A, Fremaut D, De Smet S, Dierick N. 2010. Effects of dose and formulation of carvacrol and thymol on bacteria and some functional traits of the gut in piglets after weaning. *Arch. Animal Nutr.* 64:136–154. <http://dx.doi.org/10.1080/17450390903499915>.
24. van Alphen LB, Burt SA, Veenendaal AK, Bleumink-Pluym NM, van Putten JP. 2012. The natural antimicrobial carvacrol inhibits *Campylobacter jejuni* motility and infection of epithelial cells. *PLoS One* 7:e45343. <http://dx.doi.org/10.1371/journal.pone.0045343>.
25. Fabian D, Sabol M, Domaracka K, Bujnakova D. 2006. Essential oils—their antimicrobial activity against *Escherichia coli* and effect on intestinal cell viability. *Toxicol. In Vitro* 20:1435–1445. <http://dx.doi.org/10.1016/j.tiv.2006.06.012>.
26. Windisch W, Schedle K, Plitzner C, Kroismayr A. 2008. Use of phyto-genic products as feed additives for swine and poultry. *J. Anim. Sci.* 86: E140–E148.