The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/138357

Please be advised that this information was generated on 2019-02-17 and may be subject to change.
Iron-Induced Virulence of *Salmonella enterica* Serovar Typhimurium at the Intestinal Epithelial Interface Can Be Suppressed by Carvacrol

Guus A. M. Kortman,a Rian W. H. M. Roelofs,a Dorine W. Swinkels,a Marien I. de Jonge,a,b Sara A. Burt,c Harold Tjalsmaa

Department of Laboratory Medicine, Nijmegen Institute for Infection, Inflammation, and Immunity (N4I), and Institute for Genetic and Metabolic Disease (IGMD) of the Radboud University Medical Center, Nijmegen, The Netherlands; Department of Pediatrics, Laboratory of Pediatric Infectious Diseases of the Radboud University Medical Center, Nijmegen, The Netherlands; Institute for Risk Assessment Sciences, Veterinary Public Health Division, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

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 deprivation of the heat shock protein GroEL and increased flagellin expression 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% CO2 (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 alcohol.

Received 20 September 2013 Returned for modification 8 October 2013 Accepted 20 December 2013

Address correspondence to Harold Tjalsma, harold.tjalsma@radboudumc.nl.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.02060-13.

Copyright © 2014, American Society for Microbiology. All Rights Reserved. doi:10.1128/AAC.02060-13
the presence of 0, 1, 10, or 100 carvacrol (0, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, and 2.5 mmol/liter) in IMDM in visible growth of...
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. Chromo azur 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 Neillands (13). Briefly, a solution of 2 mmol/liter CAS (Tokyo Chemical Industry) was prepared, and 1,875 μl was mixed with 375 μl ferric iron solution (1 mmol/liter FeCl₃, 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 μl 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 μl carvacrol (0 to 1 mmol/liter in IMDM or MQ), DFO (0 to 20 μmol/liter in IMDM or MQ), or tannic acid (0 to 5.9 mmol/liter in IMDM or MQ) was mixed with 100 μl 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 μmol/liter ferric citrate compared to 1 to 10 μmol/liter ferric citrate (Fig. 1B).

Levels of carvacrol of ≤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 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 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 ± 0.50) was significantly steeper than the slope of the 0.2 mmol/liter carvacrol conditions (0.51 ± 0.20) (P = 0.013). Slope of the conditions with 0.5 mmol/liter carvacrol (1.47 ± 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.
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 mmol/liter ferric citrate condition (P < 0.01) and further increased with 50 mmol/liter ferric citrate (P = 0.034 compared to 1 mmol/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 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
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 current 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 ≥0.2 mmol/liter was already effective in reducing iron-induced adhesion, while concentrations of ≤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.

ACKNOWLEDGMENTS

This work was in part supported by the Dutch Digestive Diseases Foundation (project WO 10-53). We thank Annemarie Boleij, Ger Bongaerts, Coby Laarakkers, Betty Jongerius-Gortemaker, and Primrose Freestone for useful discussions and/or technical assistance and the three anonymous reviewers for their excellent suggestions and comments that were very useful in shaping the final article.

REFERENCES


