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# The *in vitro* effect of carvacrol, a food additive, on the pathogenicity of O157 and non-O157 Shiga-toxin producing *Escherichia coli*

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ABSTRACT

Shiga toxin producing *Escherichia coli* (STEC) are important pathogens implicated in foodborne outbreaks and severe human infections. *E. coli* O157:H7 is the most common strain amongst STECs however non-O157 STECs have been connected with numerous outbreaks worldwide. The use of natural plant extracts to reduce the risk from foodborne pathogens is gaining increasing importance. Therefore, the aim of the study was to investigate the effect of carvacrol against O157, O26, O45, O103, O111, O121, O145 and O104 at different concentrations. Changes in membrane permeability, membrane integrity and intracellular ATP levels were determined to further elucidate the possible antimicrobial mechanism. The effect of carvacrol on the phenotypic expression of virulence in terms of adhesion to human intestinal cells was also studied. Carvacrol had potent antibacterial effect against all strains. Treatment with carvacrol at different concentrations significantly affected the cell membrane permeability and reduced intracellular ATP levels for all STECs. It was also shown that exposure of STECs to carvacrol at sub-inhibitory concentrations reduces adherence to intestinal cells. The data presented here offer further insight into the antimicrobial activity of carvacrol and show that it has the potential to be used as a natural food antimicrobial against clinically relevant STECs even at sub-inhibitory concentrations.



## 1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are zoonotic foodborne pathogens of important public health concern. They are one of the leading causes of bacterial enteric infections worldwide, capable of causing life threatening complications such as hemolytic-uremic syndrome (Schulz et al., 2015; Wang, Jiang, & Ge, 2012). Although *E. coli* O157:H7 is the most common serogroup linked to disease in humans, the clinical significance of non-O157 STEC is also increasing at an international level. O26, O45, O103, O111, O121, and O145 are considered the top 6 non-O157 serogroups associated with sporadic and epidemic infections in the United States (Schulz et al., 2015). The STEC route of transmission is mainly via consumption of contaminated food (beef, milk, cheese, juice, produce), water, contact with animal carriers and from person to person (Gyles, 2007). Given the importance of STECs in human illness, it is important that effective strategies to control the risk from O157 and non-O157 STECs are available. The increasing resistance of bacteria to conventional chemicals and drugs as well as consumer demands for natural food preservatives have encouraged research for the identification of novel natural antimicrobials (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013). Essential oils have been gaining importance as natural food preservatives as many studies have found that they possess significant antimicrobial properties against a broad range of foodborne pathogens such as *Listeria monocytogenes*, *Bacillus cereus*, *Salmonella enteritidis*, *E. coli* and *Staphylococcus aureus* (Zhang, Liu,

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Wang, Jiang, & Quek, 2016). Carvacrol is the main component of oregano essential oil and although its exact mode of action remains to be established, it has been found to have antimicrobial as well and antifungal activity against a broad range of microorganisms (Burt, 2004; Van Alphen et al., 2012). During application of carvacrol as a food or feed additive, it is possible that its concentration in the food can be reduced due to dilution or due to binding to proteins and lipids (Perricone, Arace, Corbo, Sinigaglia, & Bevilacqua, 2015). Therefore, it is important to investigate the effects of carvacrol also at sub-lethal and sub-inhibitory concentrations. Exploring how exposure of STECs to carvacrol can affect adherence to intestinal cells can help to elucidate more clearly any additional beneficial mechanisms of action.

Most studies on the antimicrobial effects of plant components have focused on *E. coli* O157 however studies on non-O157 STECs, including serogroup O104, are very limited. Data on the effect of plant components on the phenotypic expression of virulence are also lacking. To fill this gap, the aim of this study was to provide a systematic analysis of the antimicrobial activity of different concentrations of carvacrol against different clinically relevant STECs (i.e. O157, O145, O104, O103, O45, O121, O26 and O111). Also, we aimed to explore the antimicrobial mechanism of carvacrol against these strains by measuring membrane permeability, membrane integrity and intracellular ATP levels and to investigate, for the first time, the effect of carvacrol on the adhesion of O157 and non-O157 STECS on human epithelial cells.

## 2. Materials and methods

### 2.1. Bacterial strains and carvacrol

STECs (O157, O145, O104, O103, O45, O121, O26 and O111) were maintained in Tryptone Soya Agar plus 0.6% yeast extract (TSAYE, Oxoid, UK) slopes at 4 °C. When necessary they were cultured in Mueller-Hinton broth (MHB) at 37 °C for 24 h. Food grade carvacrol was purchased from Sigma-Aldrich (U.K.).

### 2.2. Characterisation of STECs by PCR detection of stx1, stx2 and the adherence-factor intimin (eae) genes

The foodproof® STEC screening Lykit (Biotecon, UK) was used to detect the shiga toxin genes (*stx1*, *stx2*) and intimin gene (*eae*) in a multiplex real-time PCR reaction for all the strains that were used in this study, according to the manufacturer's instructions. The real-time PCR was performed in a LightCycler 96 (Roche, U.K.). PCR cycling conditions were: Pre-incubation step – 1 cycle (37 °C for 4 min, 95 °C for 5 min; Amplification step – 50 cycles (95 °C for 5 s, 60 °C for 60 s); cooling - 1 cycle (37 °C for 60 s).

### 2.3. Disc diffusion assay, minimum inhibitory concentration and minimum bactericidal concentration

Antimicrobial activity was evaluated by the disc diffusion method according to Bajpai, Sharma, and Baek (2013) by measuring the diameter of the inhibition zone (1, 2, 4 and 8% v/v) around (DIZ) the disc (6 mm) for each of the different carvacrol concentrations used. The two-fold tube dilution method was used to determine the lowest concentration of carvacrol that can inhibit growth of bacteria (MIC) and the lowest concentration that results in bacterial death (MBC) according to Zhu, Du, Fox, and Zhu (2016). Carvacrol was diluted (8% down to 0.015625% v/v) in MHB containing 0.15% (w/v) agar and thoroughly vortexed. Individual overnight bacterial cultures were harvested by centrifugation, washed with PBS and diluted to approximately $1 \times 10^6$ CFU/mL in MHB with 0.15% agar. Afterwards, each tube was inoculated with approx. $5 \times 10^5$ CFU/mL of respective bacterial culture. Non-inoculated tubes containing the same growth medium were used as negative controls and tubes inoculated with individual bacterial cultures in MHB + 0.15% agar without carvacrol were used as positive controls. Subsequently, the tubes were incubated at 37 °C for 24 h. Tubes without visible growth were considered as the MIC. One hundred millilitres were taken from the tubes that showed no growth and inoculated onto MHA plates, the highest dilution with no microbial growth was considered as the MBC. Each assay was repeated thrice for each strain.

### 2.4. Cell membrane permeability

The cell membrane permeability was assessed according to Zhang et al. (2016) by determining the changes in electric conductivity of cell cultures treated with different concentrations of carvacrol (0, MIC, MBC) for 6 h. An electric conductivity meter (Jenway 4200, U.K.) was used to determine changes in electric conductivity. After incubation at 37 °C for 24 h each STEC culture was centrifuged and bacteria separated at 5000 g for 10 min. Subsequently, the bacteria were washed with 5% of glucose until their electric conductivity was near to that of 5% glucose (isotonic bacteria). Carvacrol was added to 5% glucose (0, MIC, MBC) and the electric conductivities of the mixtures were marked as $L_1$. The same concentrations of carvacrol were also added into the isotonic bacteria and the conductivities of the individual cultures were measured after 6 h incubation at 37 °C ($L_2$). As a control, the conductivity of bacteria cells in 5% glucose treated in boiling water for 5 min was used ($L_0$). The permeability of cell membrane was calculated using the equation: Relative electric conductivity (%) = 100 ($L_2 - L_1$)/$L_0$.

### 2.5. Membrane integrity

Membrane integrity was determined my measuring the leakage of proteins and 260 nm absorbing materials into the cell suspension according to Sadiq, Tarning, Cho, and Anal (2017), after treatment with different concentrations of carvacrol (0, MIC and MBC) for 6 h. The protein concentration in the supernatants was assessed using the Pierce BCA protein kit (ThermoScientific, U.K.). Nucleic acid leakage in terms of optical density of 260 nm absorbing materials was performed using a UV-vis spectrophotometer. Untreated samples were used as controls.

### 2.6. Intracellular ATP levels

The method described by Shi et al. (2016) was used, with minor modification to determine the effect of carvacrol on the intracellular ATP levels. Individual overnight STEC cultures were centrifuged for 5 min at 5000 × g and the supernatant was removed, the cell pellets were washed three times with PBS and the cells were collected by centrifugation. One millilitre of the individual cell suspension (approx. $10^9$ CFU/ml) was placed into Eppendorf tubes containing PBS supplemented with carvacrol at different concentrations (0, MIC, 1/2MIC). Then the samples were maintained at 37 °C for 6 h. To extract the intracellular ATP, cells were centrifuged and then treated with a lysis buffer (Roche, U.K.) for 5 min at room temperature and centrifuged at 5000 × g for 5 min. Intracellular ATP was measured with an ATP assay kit (ATP bioluminescence assay kit HS II, Roche, U.K.), after adding 100 μL of ATP luciferace reagent to 100 μL of supernatant in white 96-well plates. The ATP concentrations were measured with a microplate reader (FLUOstar Omega, BMG Labtech, U.K.).