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.

Keywords

Shiga toxin producing *E. coli*, Carvacrol, Cell permeability, ATP, Adhesion, Intestinal cells

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are zoonotic food-borne 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 et al., 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, 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 1×10^6 CFU/mL in MHB with 0.15% agar. Afterwards, each tube was inoculated with approx. 5×10^5 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.).

2.7. Quantitative adhesion assay

The human ileocecal carcinoma cell line HCT-8 was used to investigate the STEC adhesion levels after treatment with carvacrol. HCT-8 cells were grown in RPMI supplemented with 10% fetal bovine serum (FBS, Gibco, U.K.) without antibiotics at 37 °C in 5% CO_2. Approximately 10^5viable cells were seeded for each bacterial infection in 6 well plates and the adherence assay took place after a 70–80% confluence was reached. MHB broths, supplemented with carvacrol (0.0156%) and thoroughly vortexed, were inoculated with approximately 10 CFU/ml and incubated for 24 h at 37 °C. Afterwards, bacteria were washed in PBS (5000 ×*g* for 20 min) and harvested by centrifugation (5000 ×*g* for 20 min) and re-suspended in RPMI. The HCT-8 cells were washed three times with PBS (pH 7.4) and then infected with 10^7 bacteria for 3 h at 37 °C at a multiplicity of infection of 100. The number of bacteria for each inoculum was confirmed by spread plating on MHA plates. Subsequently, the cells were gently scraped off with the use of 0.1% triton X-100 (Sigma, U.K.) in PBS. The lysate was diluted and spread plated on MHA plates and the CFU were enumerated after 24 h at 37 °C. The ratio between

the total number of adhered bacteria and the initial inoculum was used to calculate the percentage adherence (Brown et al., 2010). The experiments were performed in triplicate.

2.8. Statistical analysis

The experiment was performed three times on different occasions in order to obtain three independent replicates. The data are presented as the mean ± SD. Differences between means were determined by *t*-test and ANOVA. The Tukey's test was used to compare differences amongst means. Differences were defined as significant at $p \leq 0.05$.

3. Results and discussion

3.1. Antimicrobial activity of carvacrol against *E. coli* O157, O145, O104, O103, O45, O121, O26 and O111

In the present study, DIZ, MIC and MBC parameters were used to evaluate the antimicrobial efficiency of carvacrol against the 8 STECs. Results in Table 1 show that carvacrol had a potent antibacterial effect against all STECs, with the antibacterial effect being dose dependent. Strain O157 was the most susceptible and strain O104 being the least susceptible to carvacrol with diameters of inhibition zones of 17.7 and 14 mm, respectively. Also, the MIC values for carvacrol were found to be 0.031% for all strains. MBC values were 0.0625% for all strains except O104 which had an MBC of 0.125% (Table 2). Similar MIC and MBC values have been found in other studies in different growth media (Du et al., 2015, Pei et al., 2009, Rivas et al., 2010). Variations in the MICs and MBCs between studies are to be expected to a certain extent due to differences in experimental methods and bacterial strains used (Rivas et al., 2010). Most of the studies have focused primarily on the effect against *E. coli* O157. The results presented here extend the observations of the effects of carvacrol to several non-O157 serogroups as well. STEC growth curves were also determined during 24 h incubation in MHB in the presence of carvacrol (results not shown). At a 0.031% carvacrol concentration, bacterial growth was inhibited, as expected, since it was found to be the MIC for all strains. At a 0.0156% carvacrol concentration, there was no growth inhibition for all strains, therefore, this concentration was determined as the sub-inhibitory concentration (concentration that has no effect on growth) and used in further experiments.

3.2. Effect of carvacrol on cell permeability of *E. coli* O157, O145, O104, O103, O45, O121, O26 and O111

The cell permeability of the 8 STECs was studied by measuring the changes in the relative electric conductivity. The results presented in Fig. 1 revealed that carvacrol concentrations at the MIC level significantly increased the electric conductivity ($p < 0.05$) of all strains. When carvacrol was applied at a higher concentration (MBC) electric conductivity values increased even further, at least two-fold, ($p < 0.05$) compared to the MIC treatment. This dose dependent increase in the electric conductivity revealed that there was leakage of ions (e.g. Na^+, H^+, K^+) even at the a concentration that was not bacteriocidal (MIC). The low electric conductivity values observed for the control treatments can be attributed to normal cell lysis. Zhang et al. (2016) also observed an increase in electric conductivity for *E. coli* and *S. aureus* after treatment with cinnamon essential oil. A gradual increase in the electric conductivity of *E. coli* and *S.* Typhimurium suspensions was also observed by Sadiq et al. (2017) after treatment with acacia extracts throughout an 8 h period. Ion homeostasis is of great importance for cells as it regulates processes such as solute transport, metabolism, turgor pressure and motility (Trumpower & Gennis, 1994). Therefore, changes in cell permeability that lead to release of electrolytes can negatively affect cell functions and result in cell death. These results clearly show an

increase in cell permeability due to carvacrol for all strains. Carvacrol possesses a hydroxyl group which plays an important role in its antimicrobial activity as it can penetrate the cytoplasmic membrane alter its physical and chemical properties and affect stability of the lipid bilayer (Ben Arfa et al., 2006, Xu et al., 2008). In general, carvacrol was found to interfere with ion homeostasis of all 8 STECs tested at both MIC and MBC levels.

3.3. Effect of carvacrol on protein and DNA release of *E. coli* O157, O145, O104, O103, O45, O121, O26 and O111

Fig. 2, Fig. 3 present the effect of different concentrations of carvacrol on the protein and 260 nm absorbing material release, respectively, on the 8 strains. Determination of leakage of cell components can provide evidence on the integrity of the cell membrane and further elucidate the antimicrobial mechanism of carvacrol against STECs. Proteins are present throughout the membrane and cytoplasm of the bacterial cells and have major structural and functional roles. In the present study carvacrol applied at the MIC and MBC levels led to significantly increased protein release ($p < 0.05$) in the cell suspension of all 8 STEC strains. When carvacrol was applied at the MBC level protein concentrations increased even further ($p < 0.05$) compared to the MIC treated cells showing the deleterious effect of carvacrol on all strains. Fennel seed essential oil has also been shown to cause rapid losses of proteins from treated *Shigella dysenteriae* cells, indicating damage to the cell membrane (Diao, Hu, Zhang, & Xu, 2014). A significant release of 260-nm-absorbing material has been reported to suggest release of nucleic acids through a damaged membrane (Carson, Mee, & Riley, 2002). In the present study when carvacrol was applied at the MBC level the absorbance values (OD at 260 nm) exhibited a multi-fold increase ($p < 0.05$) revealing that nucleic acids was released in the cell suspension. In this case as well, when carvacrol was applied in the MIC level, it was still able to cause a significant leakage of nucleic acids ($p < 0.05$) suggesting the occurrence of membrane structural damage even at a sub-lethal carvacrol concentration. These results are in agreement with the study of Bajpai et al. (2013) which showed that exposure of *B. cereus*and *E. coli* O157:H7 to *Cudrania tricuspidata* essential oil caused rapid loss of 260-nm absorbing materials from the cells. Cinnamon essential oil has also been found to increase the release of 260-nm absorbing materials (nucleic acids) from *E. coli* and *S. aureus* cells (Zhang et al., 2016). Overall, carvacrol was able to compromise the integrity of the cell membrane in all the STECs studied leading to growth inhibition and cell death.

3.4. Effect of carvacrol on intracellular ATP levels of *E. coli* O157, O145, O104, O103, O45, O121, O26 and O111

The effect of carvacrol on the intracellular ATP levels of the 8 strains is presented in Fig. 4. Intracellular ATP is required for storing and providing metabolic energy, for enzymatic reactions and signalling processes (Mempin et al., 2013) and therefore it constitutes a parameter to be studied in order to investigate the antimicrobial mechanism against the 8 strains. In this experiment carvacrol was applied at a sub-lethal concentration (MIC) and at a ½ MIC level (i.e. 0.0156%) in an effort to better understand the effects of carvacrol at a sub-inhibitory concentration. Carvacrol significantly reduced the intracellular ATP of all STECs (Fig. 4) ($p < 0.05$) at both concentrations used compared to the untreated samples. Results also showed that ATP levels for strains treated with carvacrol at the MIC level were significantly lower ($p < 0.05$) compared to the 1/2MIC treatment. The reduction of ATP of STECs observed here can be explained by ATP leakage through the cell membrane rendered permeable by carvacrol or by an increased rate of ATP hydrolysis within the cells. ATP hydrolysis could be attributed to a change in the ATP hydrolysis reaction equilibrium due to inorganic phosphate loss through the

compromised membrane or depletion of the intracellular ATP pool and dissipation of proton motive force (Sánchez et al., 2010, Ultee et al., 1999). The loss of intracellular ATP has been observed in other studies as well. For *Bacillus cereus*, the bactericidal effect of carvacrol has been attributed to a depletion of intracellular ATP and a dissipation of ion gradients due to increased permeability of the cytoplasmic membrane (Ultee, Bennik, & Moezelaar, 2002). Carvacrol at bactericidal concentrations has also been found to reduce the ATP levels of *Listeria monocytogenes*, *E. coli* and *Lactobacillus* sakei (Gill & Holley, 2006) but did not have any effect on the ATP levels of *Campylobacter* jejuni when applied at a sub-inhibitory concentration (Van Alphen, Burt, Veenendaal, Bleumink-Pluym, & Van Putten, 2012). In the present study, results showed that both sub-lethal and sub-inhibitory concentrations of carvacrol decrease intracellular ATP levels in all STECs studied.

3.5. Effect of carvacrol on *E. coli* O157, O145, O104, O103, O45, O121, O26 and O111 adherence to HCT-8 cells

In order to further investigate the spectrum of biological activities offered by carvacrol, a sub-inhibitory concentration (0.0156%; concentration that does not inhibit growth) of the antimicrobial was used to assess its effect on adherence to HCT-8 cells. Results for the quantitative adhesion of the 8 STECs are presented in Fig. 5. Different STEC strains have been found to possess different adherence capabilities (Pradel et al., 2015). In the present study, all of the strains tested harboured *stx1* and/or *stx2* genes (Table 3) and were able to adhere to HCT-8 cells, showing their virulent nature. Strains O157, O145, O104, O103, O45, O121 and O111 grown for 24 h in the presence of 0.0156% carvacrol and subsequently incubated with the HCT-8 cells for 3 h showed a significant reduction in the level of adherence compared to control treatments ($p < 0.05$). The reduction in adherence was 21.60, 15.64, 7.41, 10.50, 12.64, 10.17 and 9.17%, respectively. Strain O26 although exhibited only a slightly reduced adherence on HCT-8 cells (2.34%), it did not differ significantly from the control ($p > 0.05$). Many pathogenic bacteria including STECs need to colonise host epithelial cells in order to initiate the development of disease (Boerlin et al., 1999). The ability of STECs to adhere to cells also allows efficient toxin release on the surface or entry inside the intestinal cells (Cossart and Sansonetti, 2004, Pizarro-Cerdá and Cossart, 2006). Therefore, a possible way to reduce the risk from STECs in food is by reducing their adherence capacity to intestinal cells. Carvacrol at sub-inhibitory level was shown to interfere with the colonisation process and effectively reduce adherence of 7 out 8 STECs used in this study. In an effort to link the results from the adherence assay to the presence of absence of intimin, all 8 STECS were screened for the presence of the *eae* gene (Table 3). All strains were *eae* positive (including O26) except *E. coli* O104. Thus, the fact that O26 adherence was not significantly reduced suggests i) that the presence or not of the *eae* gene, was not responsible for the difference in adherence observed and ii) the importance of different adherence mechanisms in different strains of STECS (Cordeiro et al., 2013, Pradel et al., 2015). At least 25 different proteinaceous colonisation factors have been identified in STECs (Clements, Young, Constantinou, & Frankel, 2012), therefore the mechanism by which carvacrol affects adherence is likely to be complicated and further studies would be required to fully understand the process. Mith, Clinquart, Zhiri, Daube, and Delcenserie (2015)investigated the effect of oregano essential oil and carvacrol on the virulence gene transcription of *E. coli* O157:H7 and found that both were able to significantly down-regulate genes involved in toxin production, quorum sensing, attaching/effacing lesions and motility. Upadhyay et al. (2017) also found that sub-inhibitory concentrations of *trans*-cinnamaldehyde, carvacrol and eugenol reduce the attachment and invasion of some *C. jejuni* strains in Caco-2 cells by interfering with the expression of motility and attachment genes although the effect was strain dependent. Therefore, one possible explanation for the reduced adherence observed in this study is the production of aflagellate cells or cells with impaired flagella function.

4. Conclusions

In conclusion, carvacrol has a potent antimicrobial activity against the clinically relevant *E. coli* O157, O145, O104, O103, O45, O121, O26 and O111 and it exerts its effect by inducing changes in cell membrane permeability, release of proteins and nucleic acid and reduction in ATP levels. It was also shown that carvacrol can reduce the adherence to intestinal epithelial cell of 7 out 8 of STECs used and thus offer protection against shiga toxin *E. coli* cellular infection. The findings presented here indicate the potential application of carvacrol even at sub-inhibitory levels as a means to control Shiga toxin *E. coli* by applying it as a food additive.

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Tables

Table 1. Antibacterial activity of carvacrol against STECs. Values represent the diameter[a] of inhibition zone in mm ± standard deviation.

	Carvacrol Concentrations			
	8%	**4%**	**2%**	**1%**
O145	15 ± 1.7	13 ± 0	12.3 ± 0.6	12 ± 0
O104	14 ± 0	12.3 ± 0.6	11.7 ± 1.2	10 ± 0.6
O103	16 ± 1.7	14.7 ± 0.3	14.3 ± 0.6	13.7 ± 0.6
O45	15 ± 0.7	13.7 ± 0.3	12.7 ± 0.6	12.3 ± 0.6
O121	15.7 ± 0.6	14.3 ± 0.6	13.7 ± 0.6	13 ± 0
O26	15.7 ± 0.6	15 ± 0.9	14.3 ± 0.6	13.7 ± 0.6
O157	17.7 ± 0.6	15.7 ± 0.6	15 ± 0	14.3 ± 0.6
O111	16.3 ± 0.3	14.7 ± 0.6	13.7 ± 0.6	12.3 ± 0.6

[a] Diameter of the disc is included (6 mm).

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of carvacrol against STECs.

Serotype	**MIC (%)**	**MBC (%)**
O145	0.031	0.0625
O104	0.031	0.125
O103	0.031	0.0625
O45	0.031	0.0625
O121	0.031	0.0625
O26	0.031	0.0625
O157	0.031	0.0625
O111	0.031	0.0625

435 Table 3. Virulence characteristics of STECs used in this study.

Serotype		Virulence genes		
		Stx1	***Stx2***	***eae***
O145	In house collection	+	+	+
O121	In house collection	–	+	+
O111	In house collection	+	–	+
O45	In house collection	+	–	+
O157	EDL 933[a]	+	+	+
O104	ATCC BAA-2326	–	+	–
O103	In house collection	+	+	+
O26	In house collection	+	–	+

436 a
437 Information on presence/absence of genes was already available therefore analysis was not
438 performed for this strain.

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Figures

Figure 1

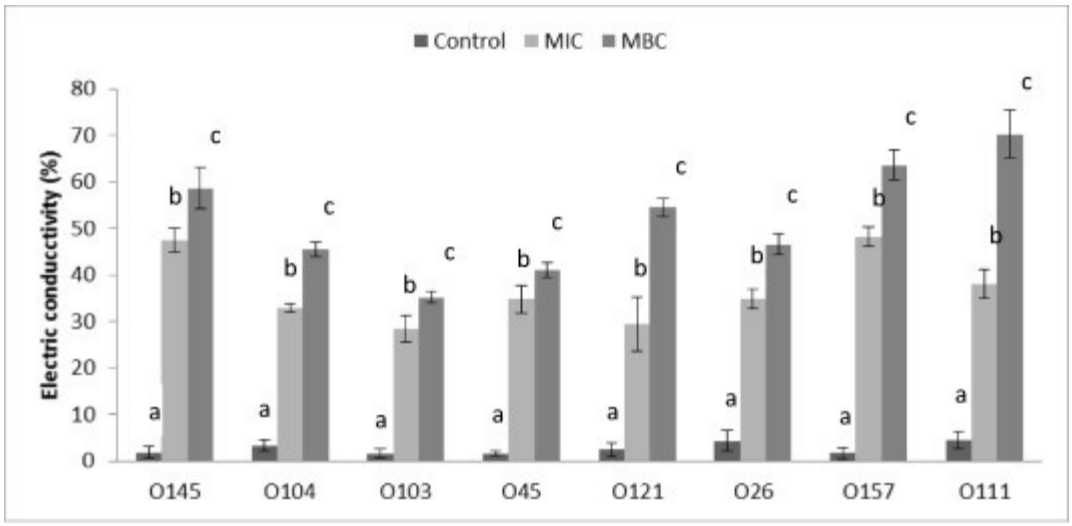

Figure 2

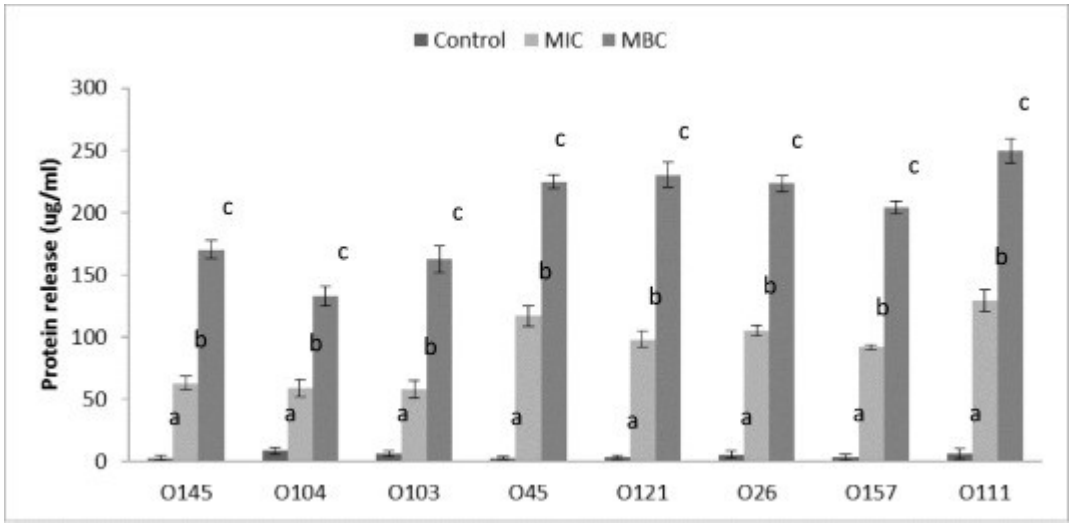

Figure 3

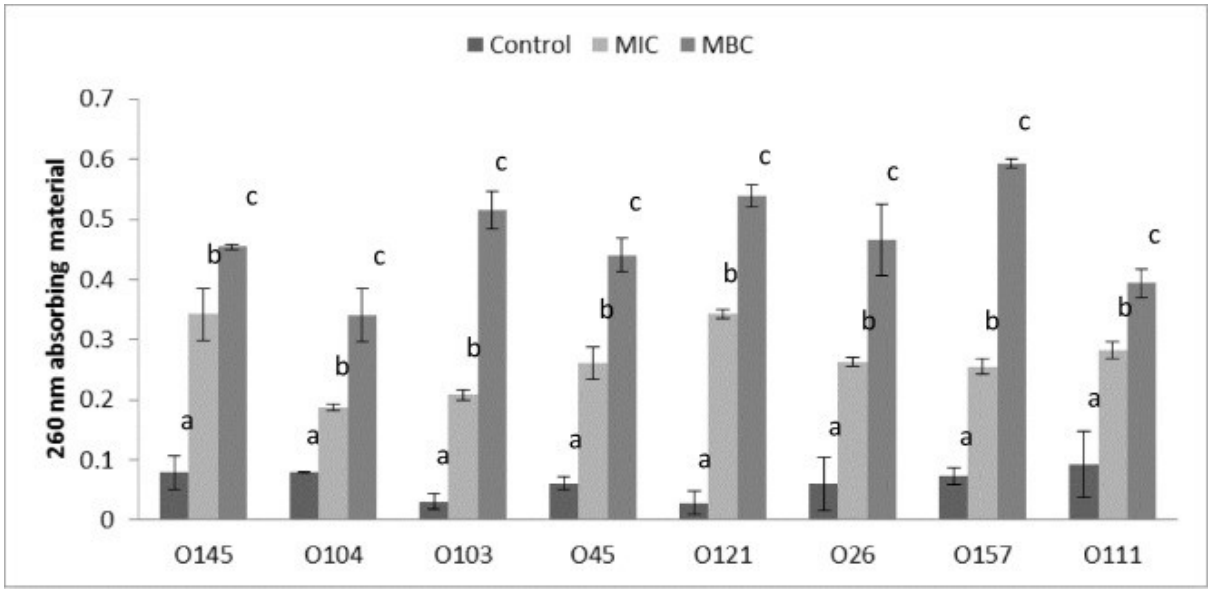

Figure 4

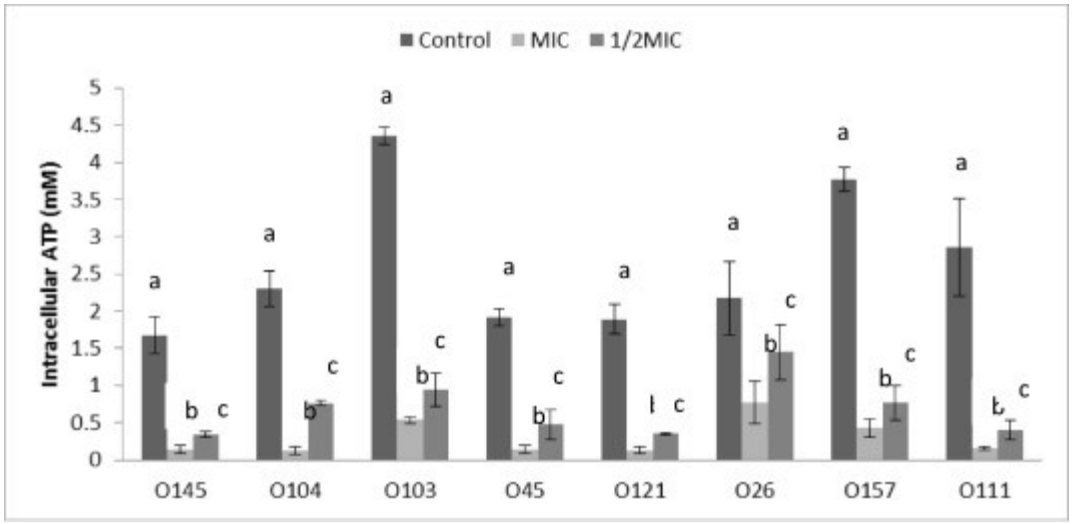

Figure 5

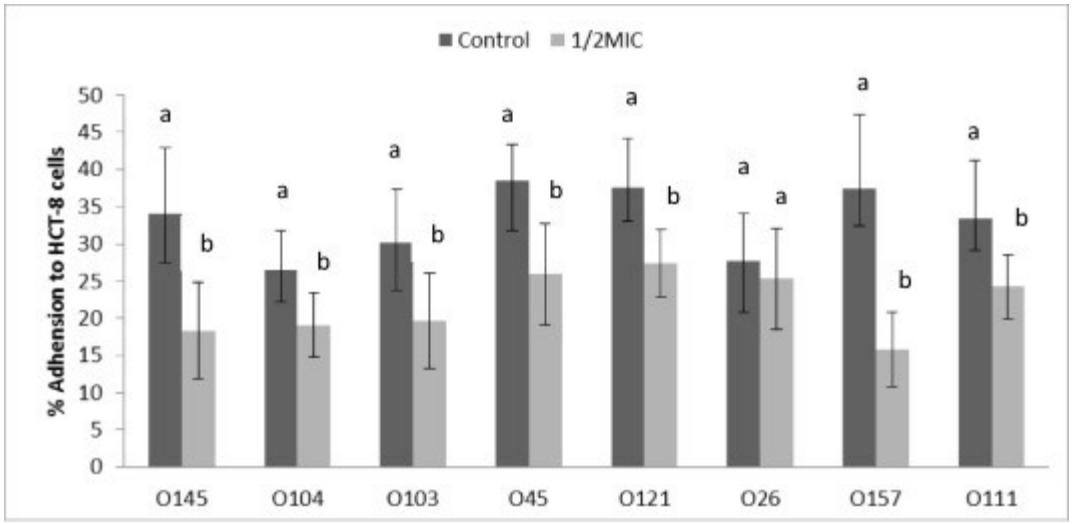

Figure legends

Fig. 1. Effect of different concentrations of carvacrol (0, MIC, MBC) on cell membrane permeability of STECs in terms of electric conductivity. Each point represents the mean ± standard deviation. Different letters for each strain denote statistically significant differences ($p < 0.05$).

Fig. 2. Leakage of proteins from STECs treated with different concentrations of carvacrol (0, MIC, MBC). Each point represents the mean ± standard deviation. Different letters for each strain denote statistically significant differences ($p < 0.05$).

Fig. 3. Leakage of 260 nm absorbing materials from STECs treated with different concentrations of carvacrol (0, MIC, MBC). Each point represents the mean ± standard deviation. Different letters for each strain denote statistically significant differences ($p < 0.05$).

Fig. 4. Effect of different concentrations of carvacrol (0, MIC, 1/2MIC) on the intracellular ATP levels of STECs. Each point represents the mean ± standard deviation. Different letters for each strain denote statistically significant differences ($p < 0.05$).

Fig. 5. Adhesion of STECs on cultured human intestinal epithelial cells (HCT-8). STECs were grown in MHB in the presence of carvacrol. Each point represents the mean ± standard deviation. Different letters for each strain denote statistically significant differences ($p < 0.05$).