

The antimicrobial effect of a commercial mixture of natural antimicrobials against *Escherichia coli* O157:H7

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Abstract

Ruminants are important reservoirs of *E. coli* O157:H7 and are considered as the major source of the most foodborne outbreaks (e.g. 2017 outbreak in Germany, 2014 and 2016 outbreaks in U.S.A., all linked to beef products). A promising strategy to reduce *E. coli* O157 is using antimicrobials to reduce the pathogen levels and/or virulence within the animal gastrointestinal tract and thus foodborne disease. The aim of the study was to determine the efficacy of a commercial mixture of natural antimicrobials against *E. coli* O157. The minimum inhibitory concentration and minimum bactericidal concentration of the antimicrobial were quantitatively determined and found to be 0.5 and 0.75% (v/v) of the natural antimicrobial, respectively. Microbial growth kinetics were also employed to determine the effect of the antimicrobial on the pathogen. The natural antimicrobial affected the cell membrane of *E. coli* O157, as demonstrated by the increase in relative electric conductivity and increase in protein and nucleic acid release. The antimicrobial was also able to significantly reduce the concentration of *E. coli* O157 in a model rumen system. Biofilm assays showed that sub-inhibitory concentrations of the antimicrobial significantly reduced the *E. coli* O157 biofilm forming capacity without influencing pathogen growth. Also, the natural antimicrobial was able to reduce motility and exopolysaccharide production. Sub-inhibitory concentrations of the antimicrobial had no effect on AI-2 production. These findings suggest that the natural antimicrobial exerts an antimicrobial effect against *E. coli* O157 *in vitro* and in a model rumen system and could be potentially used to control this pathogen in the animal gut. The results also indicate that sub-inhibitory concentrations of the antimicrobial effectively reduce biofilm formation, motility and exopolysaccharide production.

Introduction

Escherichia coli O157:H7 is an important foodborne pathogen that has been linked to many outbreaks worldwide. Shiga toxins are major virulence factors of this pathogen and their production in the human gut along with other virulence factors can cause clinical manifestations that range in severity, even leading to life threatening hemolytic uremic syndrome (Luck et al. 2005; Smith et al. 2014). Ruminants, in particular cattle, are considered an important source of *E. coli* O157:H7 most frequently colonising the lower gastrointestinal tract of cattle (Xu et al. 2014). Quantitative risk assessments have brought to light the association between levels of *E. coli* O157:H7 in faeces and hides of cattle and the contamination of beef carcasses (Duffy et al. 2006). Treating patients with *E. coli* O157 infection is problematic since many antibiotics are shown to enhance shiga toxin production *in vitro* and *in vivo* and thus increase the risk of hemolytic uremic syndrome development (Zhang et al. 2000). Owing to the potentially severe public health concerns, both researchers and industry are continuously investigating different strategies to control this pathogen. Different studies have sought to identify effective ways of reducing foodborne pathogens at pre-harvest and post-harvest stages including plant derived compounds, bacteriophages, vaccines and antimicrobial peptides (McDonnell et al. 2012; Shi et al. 2017; Zhang et al. 2000). However, the frequent foodborne disease outbreaks linked to meat and meat products demands further research. *E. coli* O157 cells are able to form complex aggregations which are enclosed in a matrix of exopolysaccharides (EPS) called biofilms. EPS plays a key role in the biofilm architecture and provides increased resistance to different types of stresses (e.g. host immune responses, osmotic and oxidative stresses antimicrobial agents) (Hentzer et al. 2003; Rivas et al. 2010; Silva et al. 2014). Quorum sensing (QS) constitutes a global regulatory mechanism for different physiological functions of *E. coli* as well as for virulence factors including biofilm formation (Sperandio et al. 2001). These virulence characteristics pose a significant challenge in clinical, veterinary and industry settings and identifying ways of controlling them are of great importance. It has been found that different natural plant extracts and compounds (e.g. carvacrol, brazilin) although they can be bactericidal, in sub-lethal concentrations can potentially increase the phenotypic expression of virulence

and virulence gene expression of pathogenic *E. coli* (García-Heredia et al. 2016). Therefore, in addition to determining the bactericidal effect of different antimicrobial agents, it is also important to determine the effect of varying concentrations of these agents on virulence expression since these pathogens can be transferred, during the slaughter process, from the animal gut to meat processing surfaces and meat, ultimately reaching the consumer.

There are no reliable commercially available strategies to control the carriage of *E. coli* O157 in ruminants (Rivas et al. 2010).

The objectives of this pilot study were to determine the antimicrobial effect of a commercial mixture of natural antimicrobials against different strains of *E. coli* O157:H7. Also, to investigate its antimicrobial mechanism and also to determine its effect on motility, biofilm formation, exopolysaccharide production and quorum sensing at sub-inhibitory concentrations.

Materials and Methods

Bacterial strains and antimicrobial

E. coli O157:H7 EDL 933 (ATCC 43895), *E. coli* O157 VSD 400 and *E. coli* O157 PHL 09 were maintained in Tryptone Soya Agar plus 0.6% yeast extract (TSAYE, Oxoid, UK) slopes at 4°C. When necessary the pathogens were cultured in Mueller-Hinton broth (MHB) at 37 °C for 24 h. The antimicrobial product Auranta 3009 was obtained from Envirotech Innovative Products Ltd and consisted of lactic acid, E330 citric acid and citrus extract. The manufacturer of this product is Bio Science Nutrition Ireland Ltd, Ireland, DAFM No.IELK241361.

Growth curves

The three individual *E. coli* O157 culture (6.0 log CFU/ml in MHB) were transferred into 96 well microtiter plates with final concentrations of the natural antimicrobial of 0, 0.0625, 0.125, 0.25, 0.5 and 0.75% (v/v). The inoculum level was determined after plating out onto MHA plates and incubation at 37 °C for 24 h. Cultures were incubated at 37°C, and optical density was monitored at 600 nm at intervals of 1 hour, over 24 h, using an automatic plate reader (FLUOstar Omega, BMG Labtech, U.K.). The concentrations of the antimicrobial that did not inhibit bacterial growth were chosen as the sub-inhibitory concentrations and used for the subsequent phenotypic virulence assays.

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentrations (MBC)

In order to determine MIC and MBC we have used a methodology as previously described by Zhu et al. (2016). Briefly, the antimicrobial was diluted from 8% to 0.015% (v/v) in MHB. An overnight bacterial culture was diluted to approximately 6 Log CFU/mL in MHB. A tube containing each dilution of the natural antimicrobial in MHB was inoculated with 5.49 ± 0.16 log CFU/ml of bacterial culture. Non-inoculated tubes were used as positive controls. Subsequently, the tubes were incubated at 37°C for 24 h. The highest dilution of the natural antimicrobial in the tube without visible bacterial growth was considered as the MIC. To determine the MBC, 100 µl of the

cultures that showed no growth were spread plated onto Mueller-Hinton Agar (MHA) plates and incubated at 37 °C for 24 h.

Cell membrane permeability

E. coli O157:H7 cell membrane permeability was determined by assessing the electric conductivity of cultures treated with different concentrations of the natural antimicrobial for 2, 6 and 24 h, as described by Stratakos et al. (2018). A conductivity meter (Jenway 4200, U.K.) was used to quantify changes in electric conductivity. Bacteria were washed with 5% glucose until their electric conductivity was near to that of 5% glucose (isotonic bacteria). The antimicrobial was added to 5% glucose (0, MIC, MBC) and the electric conductivities were measured (L_1). The same concentrations of the antimicrobial were also prepared with the isotonic bacteria and the conductivities were measured after 2, 6 and 24 h incubation at 37 °C (L_2). The conductivity of bacteria cells in 5% glucose treated in boiling water for 5 min was used (L_0) as control. The permeability of cell membrane was calculated using the equation: Relative electric conductivity (%) = $100 (L_2 - L_1)/L_0$.

Membrane integrity

Membrane integrity for the three *E. coli* strains was evaluated in terms of protein leakage and 260 nm absorbing materials into the cell suspension as previously described (Stratakos et al. 2018), after a 6 h treatment with different concentrations of natural antimicrobial (0, MIC and MBC). The protein content 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 (FLUOstar Omega, BMG Labtech, U.K.). Untreated samples were used as controls.

***In vitro* rumen assay**

The antimicrobial efficiency of the natural antimicrobial (0, MIC, MBC and 2xMBC) against *E. coli* O157 EDL 933 was tested in the assay. Three dairy cows (Swedish-Red × Jersey/Holstein-Friesian) fitted with rumen cannula were used as rumen fluid donors. The rumen fluid was collected 3 h after the morning feeding. At the laboratory, the rumen contents (solid and liquid phases) were homogenised, filtered through four

layers of cheesecloth and maintained at 39°C under anaerobic conditions. The filtered rumen fluid was dispensed into bottles and diluted with four parts of a buffer solution. The buffer solution was prepared by combining two solutions: buffer A and buffer B (5:1). The following quantities were used to prepare one litre of buffer A: 10 g KH₂PO₄; 0.5 g MgSO₄·7H₂O; 0.5 g NaCl; 0.1 g CaCl₂·2H₂O and 0.5 g Urea. One litre of buffer B was prepared using the following: 15 g Na₂CO₃ and 1 g Na₂S·9H₂O. After mixing at 39°C, the combined buffer solution was adjusted to a final pH of 6.8. An overnight culture of *E. coli* O157 was prepared and serially diluted in 9 ml of PBS and inoculated into the rumen at approximately 6 Log CFU/ml. All bottles were flushed with CO₂ for approximately 1 min prior to incubation at 39°C, with agitation. Samples were taken from each bottle at 0, 4, 18 and 24 h. After sampling at each time point, all bottles were flushed with CO₂ for approximately 1 min before reincubation at 39°C. For spread plating, 1 ml aliquots taken from each bottle were serially diluted in 9 ml volumes of MRD and 0.1 ml aliquots of each dilution were spread onto plates of TSA which were incubated at 37°C for 2 h. Subsequently, they were overpoured with CT-SMAC and incubated for another 48 h to allow for the recovery of injured cells. The suspected *E. coli* O157 positive colonies were confirmed by latex agglutination (Prolex™ *E. coli* O157 Latex test reagent kit, Prolab Diagnostics, U.K.). Treatments and control samples (without antimicrobial addition) were incubated in triplicate. The assay was performed in duplicate, using fresh rumen fluid on two separate days.

Biofilm formation and motility assay

A biofilm formation assay for *E. coli* EDL 933 was performed in 96-well polystyrene plates according to the method described by Lee et al. (2014). Briefly, overnight cultures were inoculated into Luria-Bertani (LB) broth (Oxoid, U.K.) with known concentrations (0, 0.0625 and 0.125%) of the antimicrobial and cultured for 24 h without shaking at 37°C. To quantify biofilm formation, wells were washed three times with PBS to remove all non-adherent cells. Cells were fixed with 200 µl of methanol for 20 min. Biofilms were stained with crystal violet (0.4% w.v) for 15 min and washed three times with PBS. The formed biofilm was then dissolved with 200 µl 96% ethanol for 30 min and absorbance was measured (570 nm) in a microtiter plate reader. Cultures with no added antimicrobial served as positive controls. Motility was determined as described by Kulshreshtha et al. (2016). Briefly five microliters of *E. coli* O157 culture (~7.0 log CFU) were stabbed into plates with semisolid medium

(MHB supplemented with 0.5% agar) with and without 0.0625 and 0.125% of the natural antimicrobial. Plates with no added antimicrobial served as positive controls.

Exopolysaccharide (EPS) content determination

An *E. coli* O157 EDL 933 culture grown in the presence and absence of sub-MIC of the antimicrobial was centrifuged and the supernatant was filtered through a 0.22 µm filter. Three volumes of chilled 100% ethanol were added to the filtered supernatant and incubated overnight at 2°C to precipitate the EPS. The ethanol wash and centrifugation steps were repeated three times (Al-Shabib et al. 2016; Huston et al. 2004). EPS was quantified using the colorimetric phenol-sulphuric acid method as previously described (DuBois et al. 1956)

Autoinducer-2 (AI-2) assay

To determine whether AI-2 production in the *E. coli* O157 EDL 933 is affected by sub-inhibitory concentrations of the novel antimicrobial, the autoinducer-2 (AI-2) assay was performed as previously described (Castillo et al. 2015; Duan and March 2010). Briefly, approximately 7 Log CFU/mL of *E. coli* O157 were inoculated and cultured in LB broth with 0.5% (w/v) glucose containing 0, 0.0625 and 0.125% of the antimicrobial at 37°C for 6 h. *Vibrio harveyi* BB170 was grown overnight at 30°C with agitation in Autoinducer Bioassay medium (AB) and diluted 1:3,000. Cultures to be tested for CAI-1 activity were centrifuged (12,000 × g for 5 min). The supernatant was filtered and 20 µL of cell-free supernatant was added to 80 µL of diluted *V. harveyi* BB 170 reporter culture in a sterile 96-well plate and incubated at 30°C with shaking at 500 rpm. The luminescence values of the samples were measured in an automatic plate reader (FLUOstar Omega, BMG Labtech, U.K.) every 0.5 h. The analysis was repeated three times. In addition the *E. coli* cultures were enumerated by plating on brain-heart infusion agar. The aforementioned concentrations of the antimicrobial were also tested against growth of *V. harveyi* BB170 to ensure that decrease in bioluminescence was not caused by growth inhibition.

Statistical analysis

All experiments were performed in triplicate. To measure the statistical significance we used two tailed Student's *t* test. A group difference was assumed to be statistically significant when $P < 0.05$. All results were expressed as means \pm S.D.

Results

Antimicrobial activity and mechanism

The antimicrobial exerted a potent effect against all *E. coli* O157 strains tested, with MIC and MBC values of 0.5 and 0.75% (v/v) of the natural antimicrobial. The antimicrobial activity can be attributed to the lactic acid, citric acid and citrus extract present in the antimicrobial solution. The results revealed (Fig 1) that the electric conductivity of all treated samples increased significantly ($P < 0.05$) with exposure time (2 to 24 h) and with increasing concentrations of the antimicrobial ($P < 0.05$). These results indicate that the gradual increase in cell membrane permeability leads to cellular leakage. Membrane integrity was also studied by monitoring protein (Fig. 2A) and nucleic acids release (Fig 2B) in terms of 260 nm absorbing materials. When the antimicrobial was applied at MIC and MBC levels, protein release was found to progressively increase with time ($P < 0.05$). Also, when the antimicrobial was applied at the MBC level, protein release was significantly higher compared to the MIC treated *E. coli* O157. A very similar trend was observed for nucleic acid release (OD_{260nm}). Application of the natural antimicrobial at MIC and MBC concentrations resulted in 5-12 fold increase in 260 nm materials release ($P < 0.05$) after 24 h. There was also a gradual increase in the 260 nm materials release during the 24h incubation, with the release of MBC treated cells being always higher compared to the MIC treatment ($P < 0.05$). Protein and nucleic acid release for control samples remained stable in all cases. The above results suggest that membrane structural damage is taking place at both MIC and MBC concentrations for all three *E. coli* O157 strains tested. Since the three strains had the same MIC and MBC values and exhibited similar behaviour in terms of cell membrane integrity and permeability only *E. coli* EDL 933 was used in subsequent experiments as it is widely used in other studies thus allowing better comparisons.

Growth curves

Growth curves revealed complete inhibition of growth at MBC and MIC, as expected. The 1/2 MIC (0.25% v/v) concentrations caused a prolongation of the lag phase of all three *E. coli* O157 strains tested for 1 hour whereas 1/4 MIC (0.125% v/v) and 1/8 MIC (0.625% v/v) showed a very similar growth curve to the control showing that these concentrations had no effect on growth (Fig 3). Therefore these two concentrations were selected to study the effect on motility, biofilm formation, exopolysaccharide production and quorum sensing.

***In vitro* rumen system**

Application of the antimicrobial at the MIC level did not influence the growth of the pathogen, which was very similar to the control ($P > 0.05$). Application at the MBC level resulted in a gradual reduction in the *E. coli* O157 counts, with the levels of the pathogen being significantly lower (5.15 ± 0.38 log CFU/ml) compared to the control and MIC treatment after 18 h of incubation (Fig 4). After 24h, the pathogen levels were reduced by 1.63 log CFU/ml for the MBC treatment. The 2xMBC reduced the levels of *E. coli* O157 by approx. 1 log CFU/ml within 4h, compared to the control and MIC treatments ($P < 0.05$). The pathogen levels were reduced by 3.15 log CFU/ml after 24h, which was significantly lower compared to the pathogen levels at 24h for the MBC treatment ($P < 0.05$).

Effect on biofilm and motility

Addition of the natural antimicrobial at sub-inhibitory levels significantly decreased ($P < 0.05$) biofilm formation by *E. coli* O157, in a dose dependent manner by 43.20 and 52.13 % compared to the control (Fig 5). The effect of the novel antimicrobial on the pathogen motility is shown in figure 6. The natural antimicrobial at both sub-inhibitory concentrations resulted in a clear reduction in the motility of the pathogen (34.11 ± 3.95 mm) compared to 68.77 ± 5.50 mm for the control ($P < 0.05$).

Effect on EPS production

Spectrophotometric determination of the extracted EPS revealed that the concentration of EPS decreased when the antimicrobial was applied (Figure 7). A statistically significant reduction in the EPS production was recorded at both sub-inhibitory concentrations tested. The natural antimicrobial treated samples showed

a 48.17 ± 5.67 and $52.07 \pm 11.50\%$ reduction in EPS production. However, no difference was found between the two treatments ($P < 0.05$).

Effect on Quorum sensing

The effect of the natural antimicrobial on AI-2 production by *E. coli* O157:H7 was assayed using the *V. harveyi* BB170 reporter strain. The reporter strain is bioluminescent in response to the presence of AI-2. Results showed that a six-hour treatment of *E. coli* O157:H7 with sub-inhibitory concentrations of the antimicrobial did not result in any significant changes ($P < 0.05$) in AI-2 production compared to the control (Fig. 8A). The antimicrobial at the concentrations used (0.0625 and 0.125%) did not have any effect on the growth of *E. coli* O157 (Fig. 8B). Also, the antimicrobial showed no antimicrobial activity against *V. harveyi* at the concentrations used in this study (results not shown).

Discussion

Enterohemorrhagic *E. coli* O157:H7 infection is a worldwide problem linked to many foodborne outbreaks (Vygen-Bonnet et al. 2017; CDC 2018). Since ruminants are an important source of this pathogen (EFSA 2010), reducing its ability to reside in the animal gastrointestinal tract would be desirable. In the present study we demonstrated that the natural antimicrobial is effective against *E. coli* O157:H7 with MIC and MBC values of 0.5 and 0.75% (v/v), respectively.

When bacteria are exposed to antimicrobial agents, different physical changes occur to their cell membranes (Bajpai et al. 2013). We show that the bactericidal effect observed is linked to the ability of the antimicrobial to disrupt *E. coli* O157:H7 membranes as described in figure 1. This indicates a potential leakage of ions even at the MIC level because the bacterial membrane acts as a barrier to the movement of electrolytes that take part in cell functions and metabolism (Diao et al. 2014). Different studies have investigated the effect of plant extracts on *E. coli* O157. Exposing *E. coli* O157 to eugenol has also been observed to cause membrane damage and subsequent leakage of intracellular components (Di Pasqua et al. 2007). Commercial citrus fruit extracts have also been found to cause physical damage to *Salmonella enterica* and *E. coli* membranes and leakage of cellular materials (Alvarez-Ordóñez et al. 2013).

Stratakos et al. (2018) also found that carvacrol was able to increase cell membrane permeability in different STEC strains leading to cell death. Release of proteins and nucleic acids (260 nm absorbing materials) is used as an indirect way to assess membrane damage after application of an antimicrobial treatment (Bajpai et al. 2013; Stratakos et al. 2018). The natural antimicrobial caused a rapid release of proteins and 260 nm absorbing material, which increased over time, suggesting damage to the cytoplasmic membrane of the pathogen, which is consistent with the increase in relative electric conductivity. Zhang et al. (2017) also observed a dose dependent increase (0, MIC, 2xMIC) in the protein and nucleic acid release of non-pathogenic *E. coli* cells after treatment with black pepper essential oil. Carvacrol and cinnamon essential oil has also been found to cause protein and nucleic acid release in *E. coli* and *Staphylococcus aureus* (Zhang et al. 2016). In general, the novel antimicrobial was found to interfere with *E. coli* O157 ion homeostasis and membrane integrity. The *in vitro* rumen model showed that it is possible to reduce *E. coli* O157 EDL 933 shedding in ruminants with the natural antimicrobial. The 2xMBC was used in the experimental design as it has been found in other studies that antimicrobials exhibit significantly reduced activity when applied in complex systems since these systems can contain different inhibitors (Burt 2004; Rydlo et al. 2006). McDonnell et al. (2012) found that although caseicin A was able to reduce *E. coli* O157 in a broth system the same concentrations (≥ 2 mg/ml) had no effect when applied in an *in vitro* model system. The study of Rivas et al. (2010) showed that carvacrol applied at a concentration of 1000 μ g/ml reduced *E. coli* O157 by > 5 log CFU/ml in an *in vitro* rumen system after 24 h incubation. However, more work is required to elucidate further the effects of the antimicrobial on rumen microbiota and thus rumen fermentation.

It is possible that during ingestion of an antimicrobial present in the feed or water, its concentrations can be reduced due to partial inactivation when coming in contact with other compounds in the animal digestive system or simply by dilution. These lower concentrations might not have an effect on growth of pathogens residing in the gut but can potentially have an effect on different virulence factors (García-Heredia et al. 2016). Although a potential increase in the virulence of the pathogen due to exposure to sub-inhibitory concentrations of the antimicrobial will not cause any clinical symptoms to the animal, it could potentially result in cells with increased virulence being transferred to the meat or food producing surfaces during the slaughter process,

increasing the risk and/or severity of human infection. Therefore, the present study also investigated the effect of sub-inhibitory concentrations of the antimicrobial on different virulence factors of *E. coli* O157:H7.

E. coli O157 is also able to form biofilms on various biotic and abiotic surfaces. These biofilms are resistant to host immune responses, antimicrobial agents and other stresses (Kim et al. 2016). Therefore, sub-inhibitory concentrations of the natural antimicrobial were used to investigate the effect on biofilm formation. Both concentrations used (0.0625 and 0.152%) reduced *E. coli* O157 biofilm formation in a dose dependent manner without affecting the growth of planktonic cells (Fig. 5). Previously, eugenol was found to inhibit biofilm formation by *Listeria monocytogenes* and *E. coli* O157 (Perez-Conesa et al. 2006; Upadhyay et al. 2013). Similarly, sub-inhibitory concentrations of citral have been found to inhibit *Cronobacter sakazakii* biofilm formation at 12°C and 25°C (Shi et al. 2017). Carvacrol has also been found to inhibit biofilm formation in *Campylobacter jejuni* (Burt et al. 2014).

Motility plays an important role in *E. coli* biofilm formation (Ren et al. 2001) and also contributes to colonisation of host tissues and enables pathogens to move towards favourable environments and away from repellents (Zhu et al. 2015). Therefore, the motility of *E. coli* O157:H7 during exposure to sub-inhibitory concentrations of the antimicrobial was also investigated. Both sub-inhibitory concentrations of the antimicrobial significantly reduced the motility of the pathogen (Fig. 6). The presence of functioning flagella is required for motility and biofilm formation in Gram-negative bacteria (Chelvam et al. 2014). Burt et al. (2016) showed that organic acids, specifically cinnamic (0.2%), lactic (0.6%) and propionic acid (0.4%) can reduce motility in *Salmonella typhimurium*. Mith et al. (2015) also found that oregano essential oil and carvacrol are able to significantly down-regulate genes involved in motility of *E. coli* O157:H7. Carvacrol has also been found, after overnight incubation, to prevent the development of flagella in *E. coli* O157:H7 leading to aflagellate and non-motile cells. Coumarins were also found to suppress curli genes and motility genes by causing reductions in fimbriae production and swarming motility in *E. coli* O157:H7 (Lee et al. 2014). Thus, a possible explanation for the reduced motility observed is inhibition of the flagellar synthesis by the sub-inhibitory concentrations of the antimicrobial.

To further elucidate the effect on *E. coli* O157:H7 virulence, EPS production by treated and non-treated bacteria was determined. *E. coli* O157:H7 produces EPS which acts

as a physical barrier to protect cells against different stresses. EPS is also involved in cell adhesion and the formation of three-dimensional biofilm structures (Ryu and Beuchat 2005). Therefore, any changes in the EPS production by the pathogen can have an effect on biofilm formation and architecture and also reduce resistance to drugs. In this study our results revealed that EPS was reduced significantly and to the same extent by the two sub-inhibitory concentrations of the antimicrobial (Fig. 7). The study of Al-Shabib et al. (2016) also showed that EPS was reduced significantly when *Listeria monocytogenes* and *E. coli* were treated with sub-inhibitory concentrations of zinc oxide nanostructures from *Nigella sativa*. Cellulose is one of the EPS components that enable biofilm formation in Enterobacteriaceae (Priester et al. 2006). Shi et al. (2017) found that treating *Cronobacter sakazakii* cells with citral down-regulated the expression of *bcsA* and *bcsG* that encode the cellulose biosynthesis operon. In the present study, we hypothesise that the novel antimicrobial could potentially affect the synthesis of components (e.g. cellulose) which EPS consists of and thus lead to inhibition of *E. coli* O157:H7 biofilm formation.

Accordingly our results showed that sub-inhibitory concentrations of the novel antimicrobial can reduce the pathogen's biofilm forming capability, motility and exopolysaccharide production. The data also suggest that the antimicrobial does not interfere with the AI-2 synthetic pathway and thus the anti-biofilm effect of the antimicrobial is not closely linked to AI-2 production.

Conclusion

Our study showed that the natural antimicrobial has a potent antimicrobial effect against all tested *E. coli* O157:H7 strains and exerts its antimicrobial effect by cell membrane disruption. Our data also shows that the antimicrobial can reduce the levels of the pathogen in an *in vitro* rumen model and revealed that sub-inhibitory concentrations of the antimicrobial attenuate *E. coli* O157:H7 reduced biofilm formation, motility and exopolysaccharide production, potentially reducing the risk and severity of disease. However, expression of virulence could be influenced by other factors during the slaughter process which should be also investigated in the future. Future studies should also elucidate the effect of the antimicrobial on Shiga toxin production as it is one of the main *E. coli* O157:H7 virulence factors. These findings indicate that the natural antimicrobial can potentially be used as an antimicrobial to

reduce the presence of *E. coli* O157:H7 in the gastrointestinal tract of ruminants and help control human infection.

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Figure legends

Figure 1. Effect of different concentrations of the antimicrobial on the three *E. coli* O157:H7 strains cell permeability as determined by measuring changes in relative electric conductivity. Each point represents the mean \pm standard deviation. MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

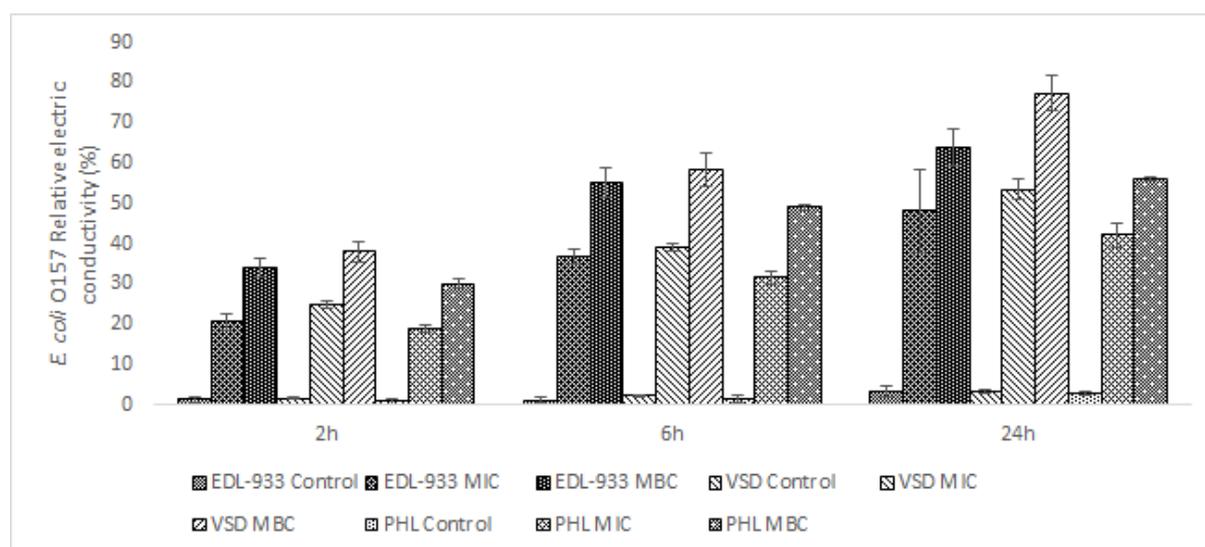


Figure 2. Effect of different concentrations of the antimicrobial on the three *E. coli* O157:H7 strains protein (A) and nucleic acid release (B). Each point represents the mean \pm standard deviation. MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

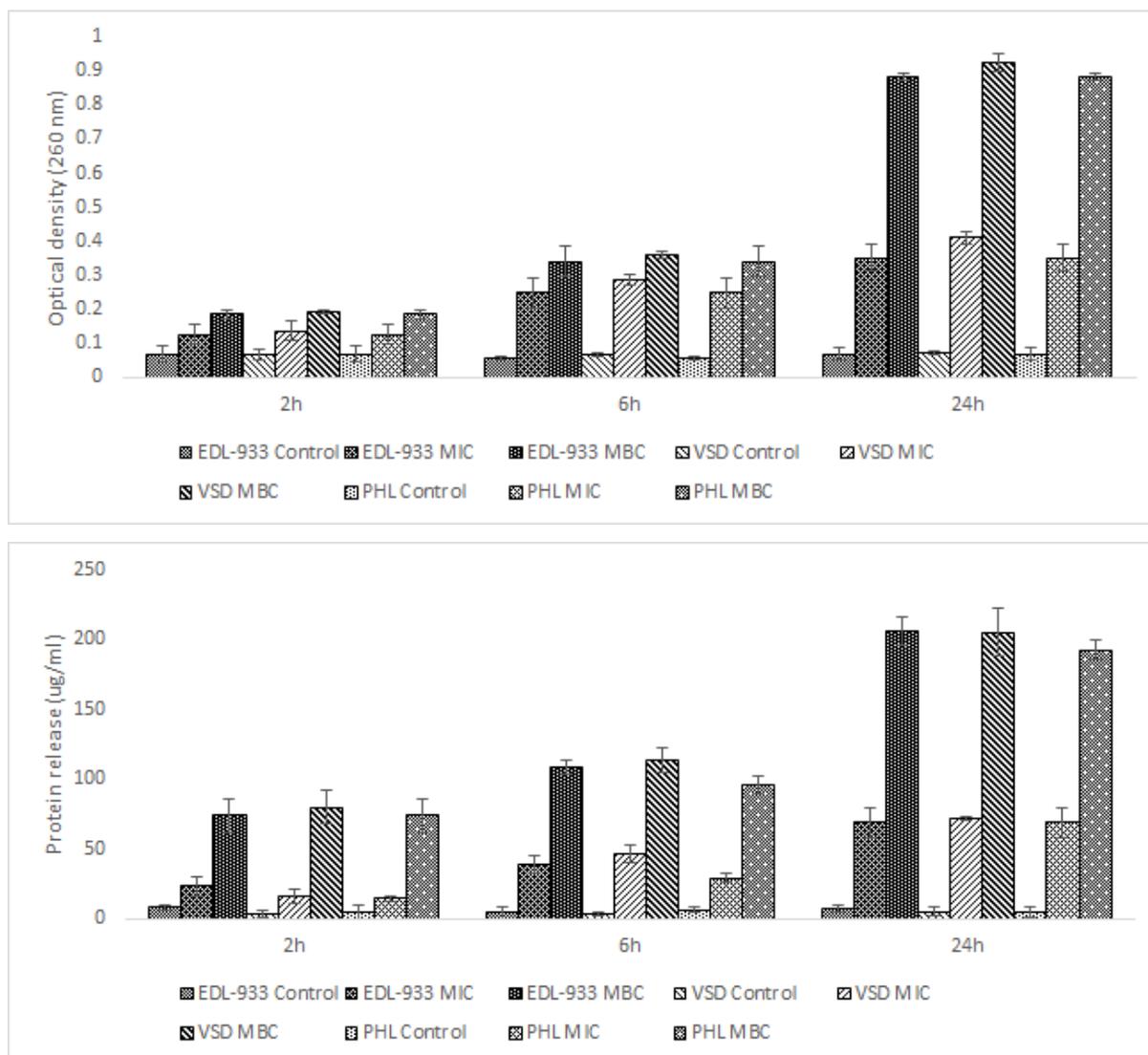


Figure 3. Growth of *E. coli* O157:H7 EDL 933 (A), VSD 400 (B) and PHL 09 (C) in MHA with various concentrations of the antimicrobial. Each value represents the mean of three different measurements.

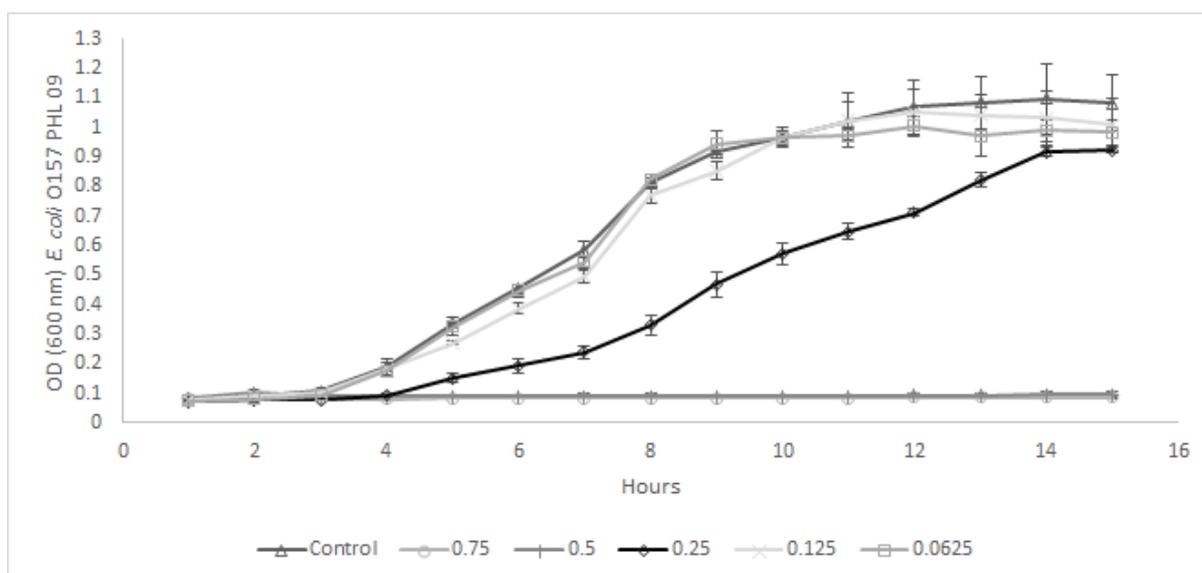
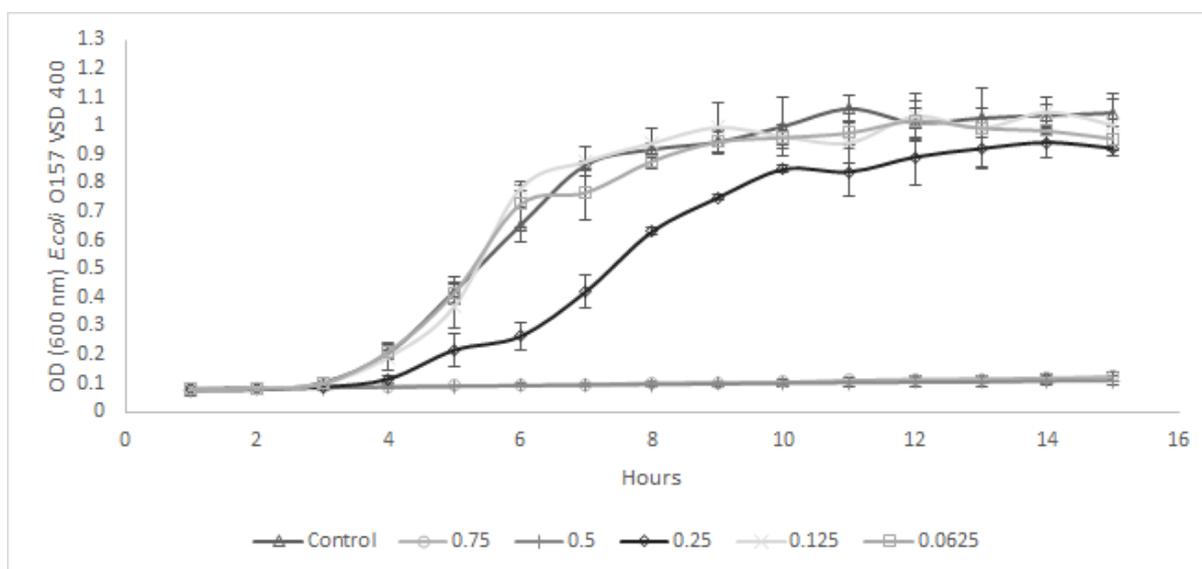
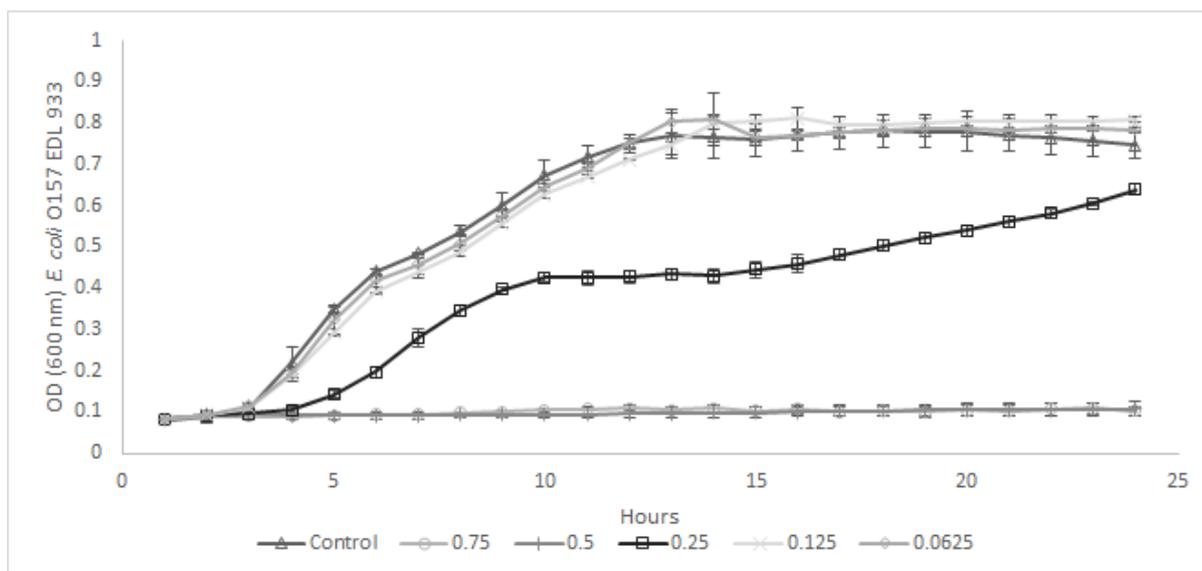


Figure 4. Levels of *E. coli* O157:H7 EDL 933 following treatment with different concentrations of the antimicrobial in an *in vitro* rumen system. Each point represents the mean \pm standard deviation.

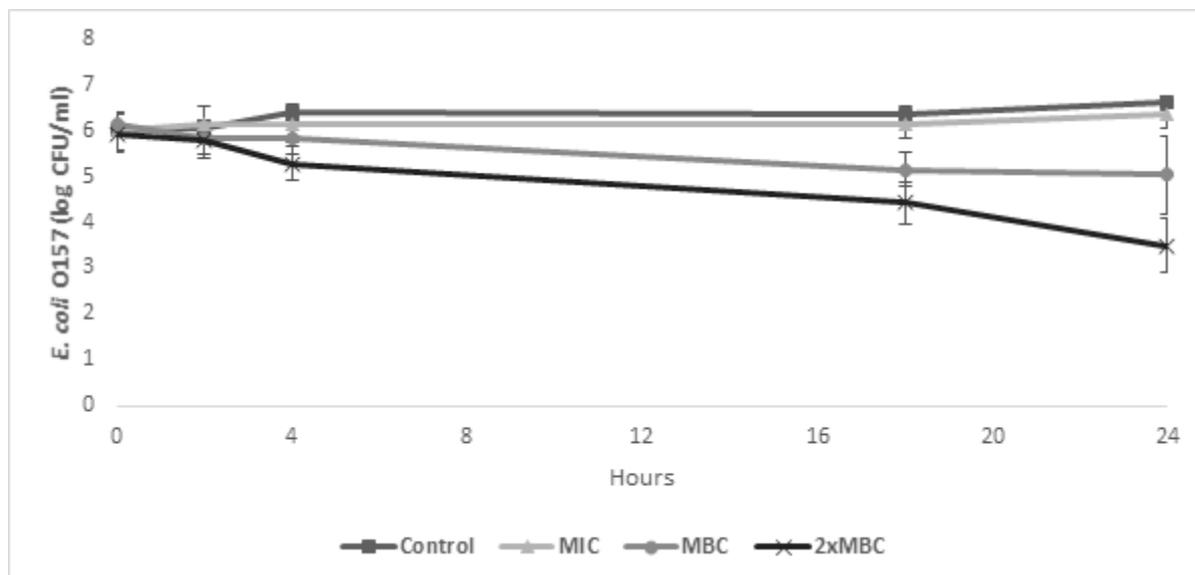


Figure 5. Effect of different sub-inhibitory concentrations of the antimicrobial on biofilm formation of *E. coli* O157:H7 EDL 933. Each point represents the mean \pm standard deviation.

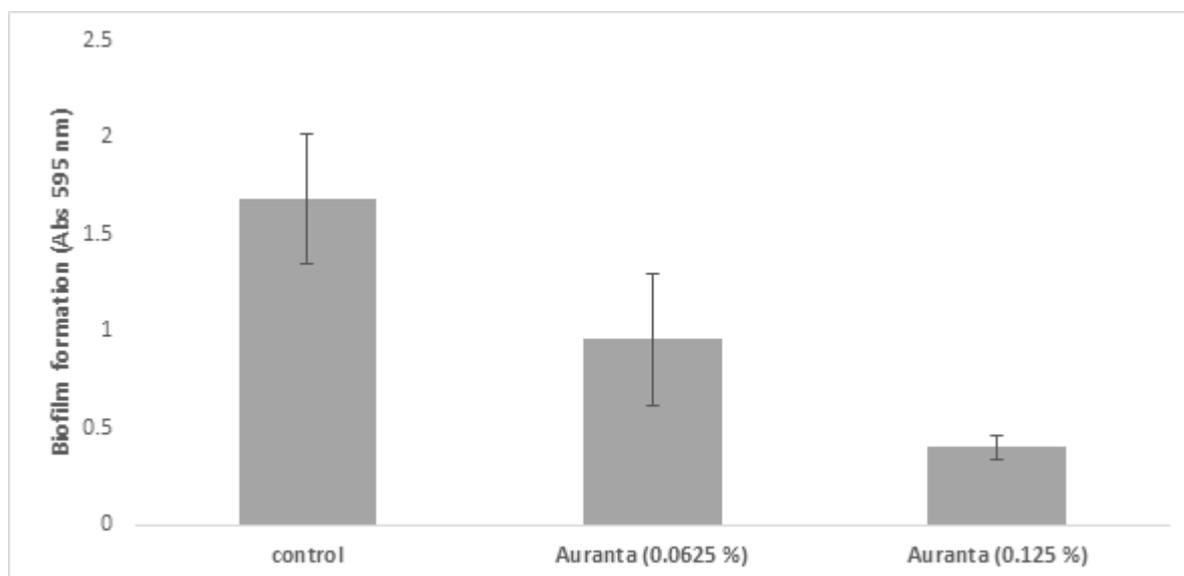


Figure 6. Effect of different sub-inhibitory concentrations of the antimicrobial on *E. coli* O157:H7 EDL 933 motility. Each point represents the mean \pm standard deviation.

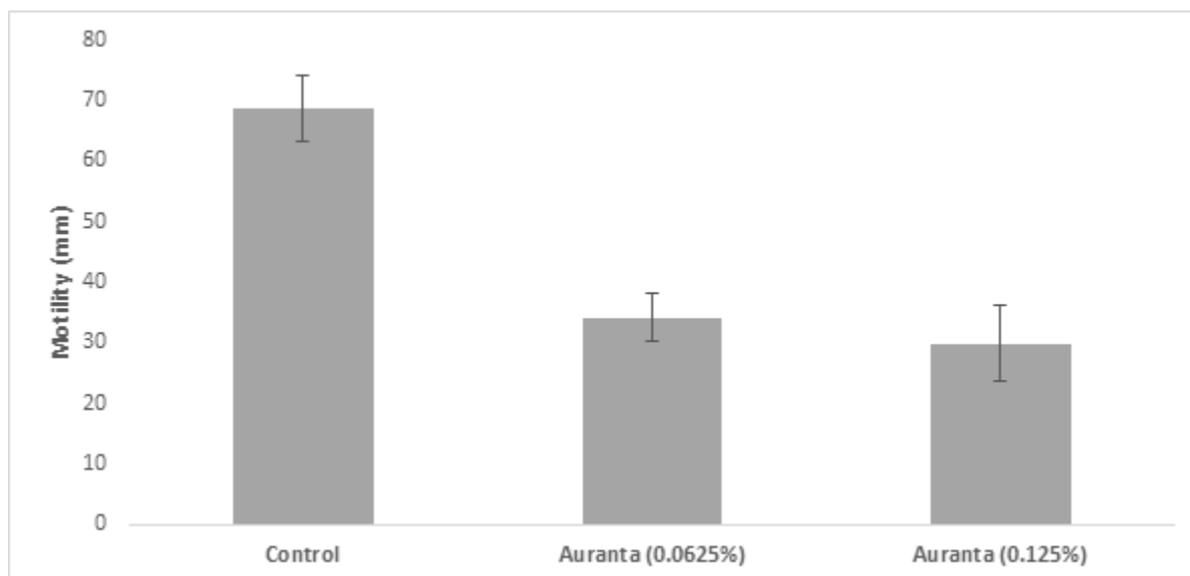


Figure 7. Quantitative determination of EPS inhibition *E. coli* O157:H7 EDL 933 after exposure to different sub-inhibitory concentrations of the antimicrobial. Each point represents the mean \pm standard deviation. Results are expressed in percentages of the control treatment.

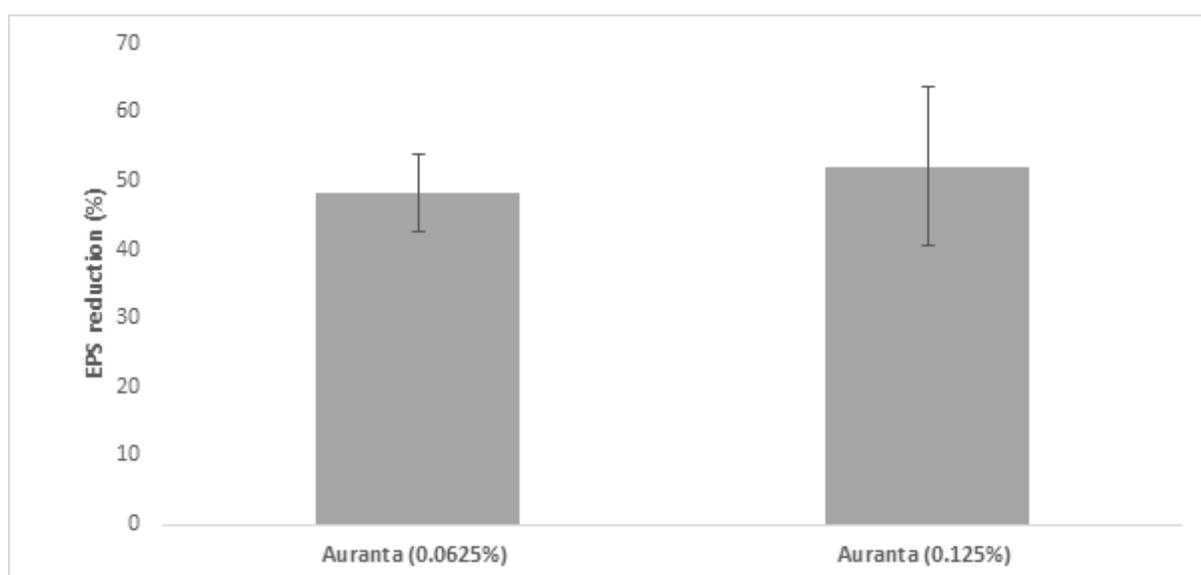
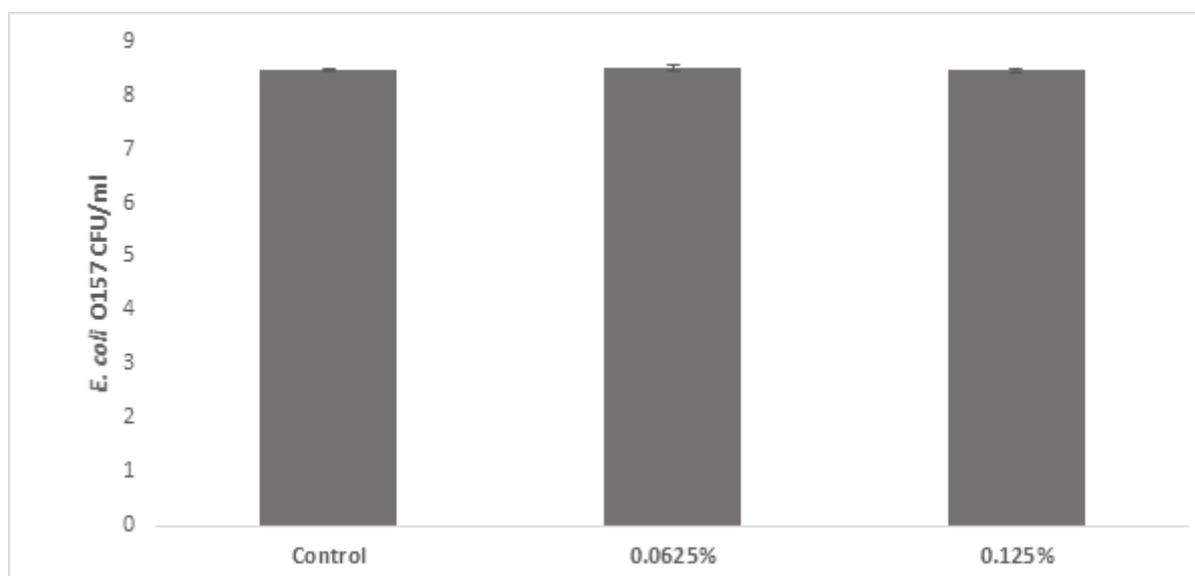
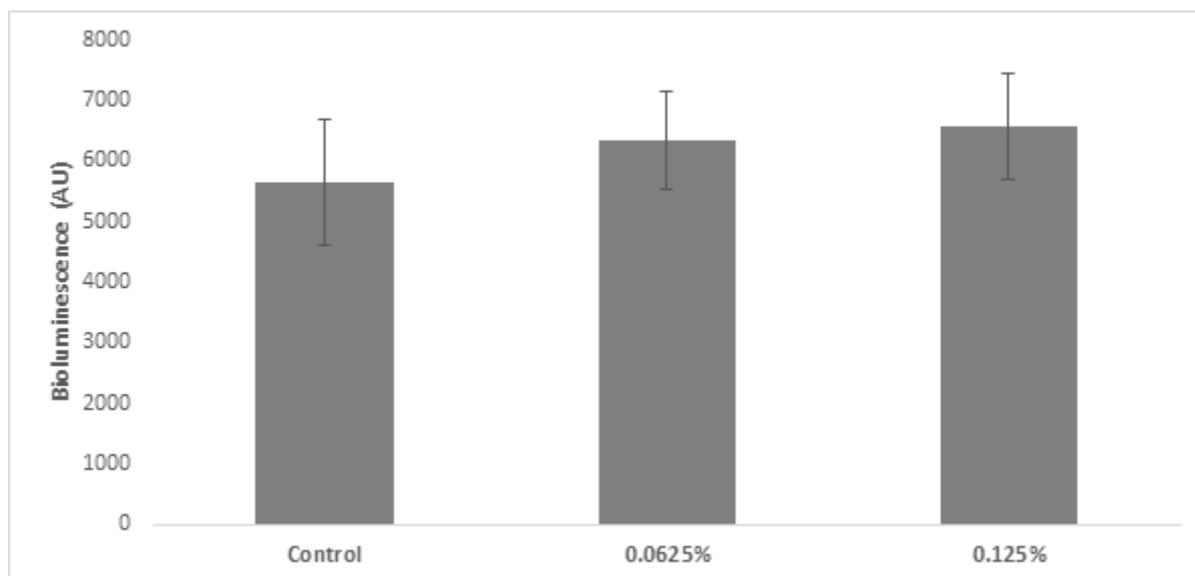


Figure 8. Production of the autoinducer (AI-2) by *E. coli* O157:H7 EDL 933 after exposure to different sub-inhibitory concentrations of the antimicrobial (A). Levels of *E. coli* O157:H7 EDL 933 after exposure to different sub-inhibitory concentrations of the antimicrobial (B). Each point represents the mean \pm standard deviation.



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