The Role of Transport Channels in the Mechanism of Action of a Novel Antimicrobial Formulation

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Abstract

Globally, antimicrobial resistance (AMR) is emerging as an increasing health threat without available effective antimicrobials. This creates a critical need for novel and effective antimicrobials with new mechanisms of action to circumvent AMR. *Enterococcus faecium* is one of the main causes of nosocomial infections exhibiting resistance to almost all available antibiotics. A potential formulation to combat resistance in both vancomycin-sensitive and vancomycin-resistant *E. faecium* (VSE, VRE), is the formula of 1.98 mM carvacrol and 4.20 mM cuminaldehyde with 0.031 mg/l vancomycin. The mechanism of action of this formulation is yet to be determined. Transcriptomic analysis showed that many genes were differentially regulated when exposed to the formulation; including genes related to transportation in the bacterial cell. Five of these genes were chosen for further investigations; *bcr*, *ecfA1*, *ecsA-1*, *yloB* and *nhaC_2.* The current study aims to investigate the involvement of transport channels in the antimicrobial mechanism of action of a novel antimicrobial formulation.

The involvement of channels similar to TRPV1 mammalian channels in the mechanism of action of the formulation was investigated by conducting growth assays and viable counts with all treatment conditions (carvacrol, cuminaldehyde, carvacrol and cuminaldehyde together, vancomycin and the formulation) and with either 1.6 mM capsaicin or 30 nM AMG517. The effects of the formulation/components on the expression levels of *bcr*, *ecfA1*, *ecsA-1*, *yloB* and *nhaC_2* were established by conducting qPCR under the effect of all treatment conditions and in the presence of either 1 mM calcium or 0.1 mM EDTA over the time points 10, 30, 60, 120 or 360 minutes. The development of strains of *E. faecium* containing gene knockout of either *bcr* or *yloB* were attempted.

The data obtained showed that AMG517 did not reverse the action of either capsaicin, carvacrol or cuminaldehyde on VSE elucidating that the channels investigated in this study are not similar to TRPV1 mammalian channels. Of the five genes analysed by qPCR, the main changes happened at 10 and 30 minutes with significant alterations in the expression level of *yloB* by -5.67 and 3.68-fold, and *bcr* by -13.5 and 2.16-fold, respectively, and in the expression levels of *ecfA1* by 2.85-fold at 120 minutes, *ecsA-1* by 25.87-fold at 360 minutes, and *nhaC_2* by 4.7-fold at 120 minutes. Ca^{2+} has induced a significant increase in the expression of *yloB* by 11.18-fold with capsaicin at 10 minutes; and by -50, -4.4, 6.6 and 3.08-fold with the formulation at 10, 30, 60 and 120 minutes, respectively, all in comparison to those in the absence of Ca^{2+}. The addition of EDTA to the formulation induced non-significant downregulation by 1.06-fold and significant downregulation by 10-fold of *bcr* at later time points of 120 and 360 minutes, respectively, in comparison to being upregulated by 15.03 and 15.71-fold when with the formulation alone at these similar time points. Construction of plasmids containing Δ*yloB* and Δ*bcr* was successfully established in *Escherichia coli* TG1 (*repA*$^{+}$); however, isolation of chloramphenicol/ampicillin-resistant Δ*yloB* and Δ*bcr* VRE strains was not successful following electroporation of pGhost9-*cat*/*bla* constructs.

Overall, the findings of the current study suggest the involvement of transportation channels in the mechanism of action of the formulation against *E. faecium* VRE; however, it is yet to be established if the novel antimicrobial formulation will be effective in combating VRE in both hospital and community environments.

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Declaration

The composition and experiments involved in the thesis are the work of the author unless otherwise stated. No portion of the work referred to in this thesis has been submitted in support of an application of another degree.

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List of Abbreviations

Abbreviation	Meaning
ABC	ATP-Binding Cassette
AMR	Antimicrobial Resistance
APases	Alkaline Phosphatases
ATP	Adenosine Triphosphate
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
BSI	British Standards Institute
Ca^{2+}	Calcium Ion
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CM	Cytoplasmic Membrane
CRE	Carbapenem-Resistant Enterobacteriaceae
Cq	Threshold Cycle
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
cDNA	Complementary DNA
EDTA	Ethylenediamine Tetraacetic Acid
EO	Essential Oil
ESBL	Extended Spectrum β-Lactamase

EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
g	Gram
gDNA	Genomic DNA
H^+	Hydrogen Ion
IGR	Intergenic Region
K^+	Potassium Ion
KO	Knockout
MBC	Minimum Bactericidal Concentration
Mb	Mega base pair
MDR	Multi-Drug Resistance
MFS	Major Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
MID	Minimum Inhibitory Dose
Mg^{2+}	Magnesium Ion
mg	Milligram
mL	Millilitre
mM	Milli molar
mRNA	Messenger RNA
MRSA	Methicillin-Resistant *Staphylococcus aureus*

MRSE	Methicillin-Resistant *Staphylococcus epidermidis*
NA	Nutrient Agar
Na^+	Sodium Ion
NCTC	National Collection of Type Cultures
nM	Nano Molar
NNIC	National Nosocomial Infectious Surveillance
NRTC	No reverse transcriptase control
NTC	No template control
OD	Optical Density
OM	Outer Membrane
OMP	Outer Membrane Protein
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHE	Public Health England
psi	Pounds Per Square Inch of Pressure
qPCR	Quantitative Real Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
RPM	Round Per Minute
RR	Ruthenium Red
SCO	Single Crossover
SPSS	Statistical Package for Social Sciences Software

TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TF	Transcription Factor
Tm	Melting Temperature
TRP	Transient Receptor Potential
TRPV1	Transient Receptor Potential Vanilloid1
µg	Microgram
µl	Micro Litre
µM	Micro Molar
UTI	Urinary Tract Infection
UV	Ultra Violet
VRE	Vancomycin-Resistant *Enterococcus faecium*
VSE	Vancomycin-Sensitive *Enterococcus faecium*
WHO	World Health Organisation
WT	Wild Type
σ	Sigma Factor
Δ	Mutant
$[Ca^{2+}]$	Intracellular Concentration of Calcium Ion

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Chapter 1

1 Introduction

1.1 Overview

In 1945, Sir Alexander Fleming raised the first concerns over the overuse of antibiotics, as reviewed by Ventola, 2015. Antibiotic resistance has been named by the World Health Organisation (WHO) as one of the three most important public health threats of the 21st century (WHO, 2014). On 21st of September 2016, antimicrobial resistance (AMR) was elevated to the level of crisis by the United Nations, and the heads of the member states signed up to a programme to eliminate the causes of AMR (Buckland, 2017).

Microorganisms that are multidrug resistance (MDR) such as methicillin-resistant *S. aureus* (MRSA), carbapenem-resistant *Enterobacteriaceae* (CRE), vancomycin-resistant *Enterococci* (VRE) usually cause nosocomial infections in hospitals and in particular intensive care units (Leong *et al.*, 2018; Rohraff and Morgan, 2014). One of the leading causes of nosocomial infections has been recognised as *Enterococci*, with a predominance of *E. faecalis* and *E. faecium* (90-95 % and 5-10 %, respectively), due not only to the virulence of the *Enterococci,* but the hospitals themselves being a hub, in which *Enterococci* can survive for several days (Peng *et al.*, 2017; Furtula *et al.*, 2013).

In 2018, in England, Wales and Northern Ireland, the overall rate of bacteraemia cases caused by *Enterococcus spp*. was 13.3 per 100,000 population. With 39 % caused by *E. faecium* and 45 % caused by *E. faecalis* (Public Health England, 2019).

The first report of vancomycin-resistant *Enterococci* (VRE) was in the 1980s, and since then it has been reported in healthcare settings worldwide. With *E. faecium* (VRE)

infections in particular are steadily increasing (Lam *et al.*, 2012), in the United States, Europe and South America in the last decade (Qin *et al.*, 2012).

Due to difficulties combatting antibiotic resistance, novel antimicrobial strategies are being sought, and particular interest paid to new antimicrobials effective against AMR microorganisms. Essential Oils are plant extracts (wide range of organic compounds) without direct effect on the plant growth and with known antimicrobial activities and multiple targets inside the bacterial cell (Mangalagiri *et al*., 2021; Simpkin *et al*., 2017). With their antimicrobial actions are mainly due to their chemical composition and concentration (Rao *et al*., 2019). They have less side effects and are of low cost compared with commercial antibiotics, and they represent one such potential area for research (Ju *et al*., 2019; Simpkin *et al.*, 2017; Wikaningtyas and Sukandar, 2016).

For over 2000 years EOs have been used for medicinal purposes, having their roots in ancient Egypt, Persia and India (Burt, 2004). Their use for pharmaceutical purposes has been documented in Europe as long ago as the 13th century (Fei *et al.*, 2011), although it was not until the 16th century that they became more widely used, particularly in London (Burt, 2004). In the 17th century the French physician, Du Chesne, reported that EOs were commonly used in medical practice in Europe, and at that time between 15 and 20 different EOs were stocked in pharmacies. By the 19th and 20th centuries, EOs had been introduced into foods to act as fragrances and flavourings (Fei *et al.*, 2011).

In addition to that EOs are antimicrobials per se, combining EOs with commercial antibiotics has been assessed for reversing antibiotics resistance, reducing the minimum inhibitory concentration (MIC) of antibiotics, and minimising the side effects of

commercial antimicrobials being used alone (Chouhan *et al.*, 2017; Yang *et al.*, 2017; Yap *et al.*, 2013).

Previously, EOs of cumin (*Cuminum cyminum*), oregano (*Origanum compactum*) and rosewood (*Aniba roseadora*) were established to inhibit an array of pathogenic bacteria including methicillin-sensitive *Staphylococcus aureus* and MRSA (Laird *et al*., unpublished data), elucidating the possibility of using these EOs as antimicrobials against a range of infectious bacteria. The EOs of cumin, oregano and rosewood and their individual components cuminaldehyde, carvacrol and linalool, respectively, were found to inhibit VSE and VRE, and were investigated for potential synergistic EO-vancomycin combinations to restore the efficacy of vancomycin against VSE and VRE (Owen *et al*., 2019). Screening the synergistic interactions between carvacrol, cuminaldehyde and linalool has shown that carvacrol and cuminaldehyde were the most potent EO components, and they therefore were considered for further investigations of antimicrobial activity and interactions with antibiotics (Owen and Laird, 2019). Synergistically, carvacrol and cuminaldehyde enhanced the antimicrobial activity of vancomycin against VSE and VRE, including clinical isolates (Owen *et al*., 2020). Nevertheless, the mechanism of action of this combination of EO components and vancomycin needs to be fully established and understood.

1.2 *Enterococcus*

1.2.1 The enterococcal genus

It was until 1984 when the enterococcal species were separated from *Streptococcus* genetically as their own genus (Schleifer and Kilpper-Balz, 1984). The *Enterococcus*

genus was previously known as the faecal or Lancefield group D *Streptococci*. In 1984 the *Streptococcus* genus was divided on the basis of 16S rRNA cataloguing three genera: *Enterococcus*, *Lactococcus* and *Streptococcus*. The revised genus *Enterococcus* was created, and a number of bacteria were reclassified. The *Streptococcus* species; *S. faecalis, S. faecium*, *S. avium* and *S. gallinarum,* were re-designated as *Enterococcus species* (Giraffa, 2002). Based on the 16S rRNA nucleotide sequence the enterococcus species are classified into species groups. The *faecium* group comprises of *E. faecium*, *E. hirae*, *E. durans*, and *E. mundtii* (Oana *et al.*, 2002).

1.2.2 Epidemiology

The genus of *Enterococcus* consists of more than fifty species and can be found in different environments (Mundt, 1963; Euzéby, 1997). *Enterococcus spp*. are abundant in human and animal faeces as they are an important commensal in the gastrointestinal tract of human and animal (Byappanahalli *et al.*, 2012). Wastewater and animal waste act as reservoirs for antibiotic-resistant pathogens that threaten the human health (Furtula *et al.*, 2013). The environmental distribution of *Enterococcus spp*. is wide, and includes many different habitats such as soil, sediments, aquatic environments (rivers, streams and creek waters) (Byappanahalli *et al.*, 2012).

1.2.3 Growth

The Gram-positive *Enterococci* are facultative anaerobes, found in chains or pairs, catalase negative, and do not form spores (Facklam, 1973; Sherman, 1937).

E. faecium can grow in the basic media used in microbiological laboratories, such as brain heart infusion (BHI) agar and nutrient agar (NA), at a range of temperature (10-

45˚C) with optimal growth occurs at 37˚C and pH (4.5-10.5) (Čermák *et al.*, 2009), and hydrolyse esculin in the presence of bile salts at 40 % (Facklam, 1973). Bacterial growth is enhanced by 10 % CO_2 during incubation; nevertheless, *Enterococcus spp*. are able to replicate in aerobic conditions (Carlos *et al.*, 2010; Čermák *et al.*, 2009).

1.2.4 The enterococcal genome

The size of enterococcal genome ranges between 2.7 and 3.6 Mb, and the number of open reading frames varies from 2587 to 3118 (van Schaik and Willems, 2010).

The genome of *Enterococcus faecalis* V583 was the first enterococcal genome to be published in 2002, and is a vancomycin-resistant clinical strain with mobile or foreign DNA constructing more than a quarter of the genome (Paulsen *et al.*, 2003).

In March 2012, the genome of *E. faecium* Aus0004 strain was the first to be sequenced, which is also a vancomycin-resistant strain, and was discovered in the blood of a patient in Melbourne, Australia, in 1998 (Lam *et al.*, 2012; Palmer *et al.*, 2014). The strain investigated in the current study, vancomycin-resistant *E. faecium* NCTC12202, was the first to be isolated in the UK (Uttley *et al*., 1989), and the whole genome sequence of *E. faecium* strain NCTC12202 was submitted in 2018 as reported on NCBI with accession number UFYT01000003.1.

1.2.5 Pathogenicity and antibiotic resistance in *E. faecium*

Previously the organisms have been considered to be non-pathogenic to humans (Čermák *et al.*, 2009; Fisher and Phillips, 2009). However, infections by *Enterococcus spp.* were accounted for 60 % of all healthcare-associated infections with *E. faecium* responsible for 35 %, including 15-20 % of Urinary tract infections, bloodstream

infections with *E. faecium* causing 40 %, endocarditis with *E. faecium* was checked in 5-15 %, intraabdominal collections and peritonitis, meningitis, and less common wound and soft tissue infections, diabetic and decubitus ulcers, endophthalmitis, prosthetic joint infections and dental and root canal failure infections (Said *et al*., 2021).

In 2016 Public Health England reported that *E. faecalis* was responsible for 42 % of bacteraemia cases caused by *Enterococcus spp*. in England, Wales and Northern Ireland. *E. faecium* accounted for a further 33 % of the cases during this time period. In 2017 Public Health England reported that *E. faecium* was 90.2 % resistant to ampicillin/amoxicillin, 22.2 % to vancomycin, 23.4 % to teicoplanin and 1.1 % to linezolid. However, in the case of *E. faecalis*, resistance to antibiotics was rare (Publich Health England, 2017).

Enterococci spp. have been reported to be resistant to a wide range of antimicrobials due to intrinsic or acquired mechanisms or due to the plasticity of their genomes to acquire horizontally transferred genes (Tian *et al.*, 2019), and as reviewed by Selleck *et al*. (2019) that *Enterococci* are intrinsically resistant to aminoglycosides, lincosamides, streptogramins and cephalosporins.

The *Enterococcus* genus, especially *E. faecium,* has a wide range of natural and acquired antibiotic resistance (Klare *et al.*, 2003). Vancomycin, a glycopeptide antibiotic first used in 1972; however, it was only 15 years later that the first vancomycin-resistant *Enterococcus* was observed. Vancomycin resistance increased by 7.6 % between 1989 and 1993, as reported by the National Nosocomial Infectious Surveillance (NNIS) (Metan *et al.*, 2005).

Late in the 1980s, *Enterococci* have emerged as serious nosocomial pathogens due to glycopeptide-resistance. The glycopeptide antibiotics, vancomycin and teicoplanin, act by forming hydrogen bounds to the D-Ala-D-Ala terminus of pentapeptidic precursors of the peptidoglycan growing layer at the site of polymerisation, and thereby inhibit the cross-linking by trans-peptidase and prevent the cell wall formation. Consequently, cytolysis and death of the bacterial cell happen due to inability of the weakened cell wall to stand the positive osmotic pressure within the cell (Cetinkaya *et al*., 2000; Wang *et al*., 2018). The glycopeptide-resistance is mediated by seven types of *van* gene clusters (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG* and *vanL*) were described in *Enterococci spp*. based on DNA sequence, organisation and according to the ligase gene for the synthesis of peptidoglycan precursors (D-Ala:D-Lac and D-Ala:D-Ser) with low affinity for glycopeptides (Xu *et al.*, 2010). The result of forming D-Ala-D-Lac leads to a decrease of about 1000-fold in the affinity of vancomycin for its binding sites in *Enterococcus spp.* (Miller *et al.*, 2014).

1.3 Antibiotic resistance

The misuse and overuse of antibiotics are the major factors behind the development of widespread antibiotic resistance and resulted in the reduced effectiveness of available antimicrobials. AMR microbes have become an increasing dilemma for hospitals and general practice surgeries in the UK and worldwide (Buckland, 2017; Djeussi *et al.*, 2013), and this has created an urgent need to assess new strategies and develop novel antimicrobials (Rohraff and Morgan, 2014).

1.3.1 Strategies

Recommendations in The Standing Medical Advisory Committee report `Path of Least Resistance` (1998) led to the first UK AMR Strategy, published in 2000. The strategy resulted in improvements in antibiotic use. An increase in funding for drug discovery programmes and the field of research of AMR in the UK was given extra support, via considering AMR a priority issue, needs better understanding of the AMR origin, spread, evolution and development, and an urgent global cross-sectoral response and research are demanding (Simpkin et al., 2017).

Recently, an updated national five-year action plan to tackle antimicrobial resistance in the UK from 2019-2024 has been published by Public Health England in 2019. The plan described actions to contain and control antimicrobial resistance to ensure progress towards 20-year vision on AMR. The actions included; 1) reducing the unintentional exposure to and the need for antimicrobials, through decreasing the problem of human and animal infections, facilitating global access to clean water and facilities, increasing the food safety and reducing the AMR spread in the environment. 2) optimal use of antimicrobials in humans, animals and agriculture. 3) investing in innovation, stream and admission to tackle AMR, such as basic research, evolve new remedies, novel diagnostic methods, health products and vaccines to be developed and accessed (Courtenay *et al.*, 2019).

1.3.2 Costs

The antibiotic resistance increased the cost of antibiotic treatment and the mortality rate worldwide (Laxminarayan *et al.*, 2016). It has been estimated that by 2050 there

will be 10 million deaths due to the multidrug-resistant microbes every year and the suspected cost around $ 100 trillion (O'Neill *et al.*, 2017).

The antibiotics market has become financially unattractive to developers due to many factors. Firstly, since the national conservation programs limit sales, antibiotics have become less profitable than other drug categories, and available antimicrobials are no longer effective due to AMR being increased. Secondly, in the United States and European Union uncertainty surrounding the regulatory requirements for market approval is creating additional development risk (Renwick *et al.*, 2016). Lastly, is the sheer investment required to develop a new targeted antibiotic, with an estimated global cost of $ 1681 million (Towse *et al.*, 2017).

Moreover, due to that antibiotics being used to cure diseases that require short-courses of treatment, big companies moved to run investments focusing on drugs of every day dose which are taken for the rest of the patient's life, such as treating hypertension, cholesterol, diabetes mellitus, acid reflux, arthritis, dementia and HIV, and when comparing with drugs that affect the morbidity (rate of disease in population) and mortality (death on a large scale) like cancer therapies, antibiotics prices are not competitive (Spellberg, 2014).

1.3.3 Lack of new antibiotics in the pipeline

WHO has confirmed a serious lack of new antibiotics to tackle resistant pathogens (WHO, 2017). In March 2017 it was estimated by the Pew Trust that there are 48 new antimicrobials in the development pipeline phases, but research has indicated that this insufficient to match demand (Simpkin *et al.*, 2017).

To re-enhance the antibiotics pipeline, it is essential to establish new methods in research and development of new antibiotics. One of the strategies being followed is looking at natural sources as a backbone for novel antibiotic formulations (Spellberg, 2014).

1.4 Essential oils

1.4.1 Essential oils

The European Pharmacopoeia (7th edition) defines EOs as: "Odorant products with a complex composition, can be obtained from plant raw material, either by driving by steam of water, by dry distillation or by a suitable mechanical method without heating" (El Asbahani *et al.*, 2015). And due to that they represent the main essence and the very important part of the plant, are named essential (Yap *et al.*, 2014).

EOs are extracted from plants as secondary metabolites, with at least 2000 plant species producing around 3000 known oils; around 300 of these are commercially available in the flavour and fragrances market (Stefanakis *et al.*, 2013; Raut and Karuppayil, 2014). Different parts of plants are used as sources to extract EOs, including flowers, buds, fruits and roots (Nazzaro *et al.*, 2013), where EOs are stored in secretory cells, cavities, canals, glandular trichomes or epidermic cells (Stefanakis *et al.*, 2013).

Due to their hydrophobic nature, EOs do not dissolve in water; however, they can be dissolved in alcohol or in non-polar or weakly polar solvents, such as waxes and oils (Bouyahya *et al.*, 2016).

Worldwide, EOs have been used in food preservation, and investigated as alternative treatments to conventional antibiotics in infectious diseases, representing potential novel antimicrobials (Solórzano-Santos and Miranda-Novales, 2012).

1.4.2 Chemical composition of EOs

Characteristics of EOs such as fragrance, density, texture, colour, ability to penetrate cells and bioavailability are regulated by several chemical components (Yap *et al.*, 2014).

The number of components within EOs are affected by many factors, such as genetic variation, variety of the plant part being used, geographical location, surrounding weather and storage after harvest; in addition to that the method of extraction affects the EO composition (Raut and Karuppayil, 2014; Calo, 2015).

EOs are of low molecular weight volatile oils (generally < 500 Daltons) (Raut and Karuppayil, 2014), when freshly extracted they are colourless, while over time they darken due to oxidation that causes chemical alterations to the EOs structure and properties. Therefore, they should preferably be stored in firmly-stoppered glass containers, and kept in cool and dry conditions (Rassem *et al.*, 2016).

EOs are very complicated natural compounds of hydrocarbons and oxygenated components, with two main groups; 1) terpene origin compounds and 2) aromatic compounds (Wińska *et al.*, 2019, Bajpai *et al.*, 2012). The hydrocarbon components are mainly terpenoids while the oxygenated components (aromatic compounds) are alcohols, acyclic esters or lactones, ethers, aldehydes, ketones, lactones, phenols and phenol ethers (Stefanakis *et al.*, 2013).

1.4.2.1 Terpenes

The major components of most EOs are terpene compounds, which have been classified according to their structural and functional properties. The structural unit of terpenes is isoprene, a five carbon-containing unit (Bajpai *et al.*, 2012). Terpenes exist in a number of forms: mono- (C10), sesqui- (C15), hemi- (C5), di- (C20), tri- (C30) and tetraterpenes (C40) (Llana-Ruiz-Cabello *et al.*, 2015). Among the most common terpenes are *p*-cymene, limonene, terpinene, sabinene and pinene. *In vitro* tests show that terpenes when used alone are ineffective as antimicrobial agents (Hyldgaard *et al.*, 2012).

1.4.2.2 Terpenoids

Terpene molecules with added oxygen or with methyl groups removed by specific enzymes are called terpenoids. The most common and well-characterised terpenoids are thymol, carvacrol, linalool, menthol, geraniol, linalyl acetate and citronellal. For most terpenoids the antibacterial effect is related to their functional groups, and in this respect the important elements are the hydroxyl group of phenolic terpenoids, and the presence of delocalised electrons (Hyldgaard *et al.*, 2012).

1.4.2.3 Phenylpropenes

Phenylpropenes are composed of a six-carbon aromatic phenol groups and a three-carbon propene tails from cinnamic acid. Only a small number of EOs are phenylpropenes compounds, with the most studied are eugenol, isoeugenol, vanillin, safrole and cinnamaldehyde. The majority of their antimicrobial activity is linked to their free hydroxyl groups, but it can also depend upon the type and number of substitutions,

on the aromatic ring, as well as the microbial strain and conditions used to test the EO (Hyldgaard *et al.*, 2012) (Figure 1.1).

Carvacrol

Carvacrol is the main component of the EOs from oregano (*Origanum sp.*) and thyme (*Thymus sp.*) (Char *et al.*, 2016). With a phenolic structure, 2-methyl-5-(1-methylethyl)-phenol, carvacrol is biosynthesised from γ-terpinene through *p*-cymene. It is found in odorous plants, and is often extracted from the Labiatae family, including *Organium*, *Satureja*, *Thymbra*, *Thymus* and *Corydothymus* (Magi *et al.*, 2015).

The two main phenols, carvacrol and thymol, constitute about 78-85 % of the EO from oregano and are accountable for its antimicrobial activity. Wherein, γ-terpinene and *p*-cymene are minor constituents of oregano EOs, and participate in the antibacterial action of the oil (Fournomiti *et al.*, 2015).

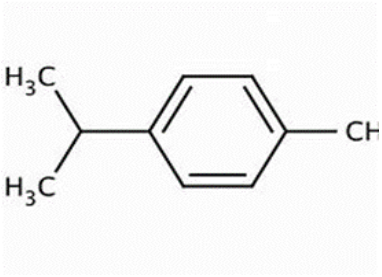
Cuminaldehyde

Cumin (*Cuminum cyminum L*) is an aromatic plant that comes from the *Apiaceae* family and was originally found in the Mediterranean region and Southwest Asia and used as food flavouring (Khan *et al.*, 2017; Pajohi *et al.*, 2011).

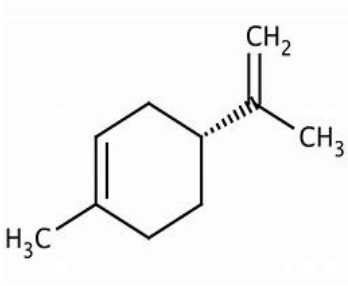
The fruits of cumin are seeds contain 2-3.5 % volatile oil that is yellow coloured when fresh. The major components of this oil are phenolic compounds, in particular cuminaldehyde, para-cymene and terpenoids (Saha *et al.*, 2016; Singh *et al.*, 2017). Cuminaldehyde is considered the most important component in the EO from cumin due to its bioactivity (Farvardin *et al.*, 2017).

A. Terpenes

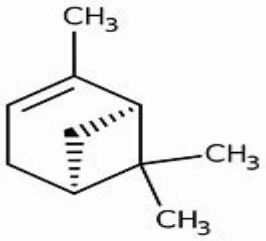
p-Cymene



Limonene

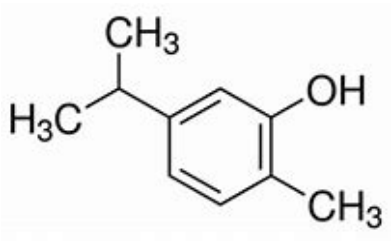


Pinene

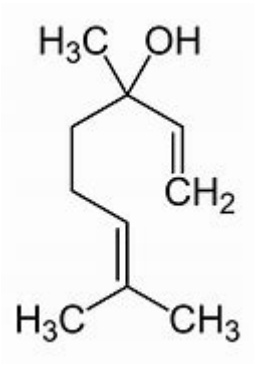


B. Terpenoids

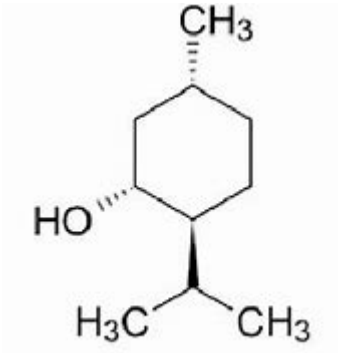
Carvacrol



Linalool

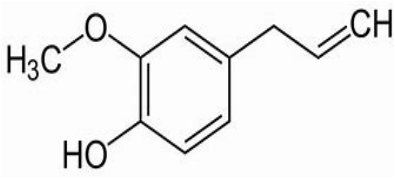


Menthol

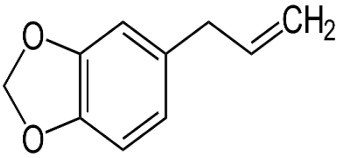


C. Phenylpropenes

Eugenol



Safrole



Cinnamaldehyde

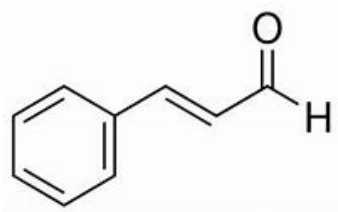


Figure 1.1. Chemical structure of selected EO components. A. The structural unit of terpene is isoprene, a five carbon-containing unit. **B**. Terpenoids are terpene molecules with added oxygen or without methyl groups. **C**. Phenylpropenes are six-carbon aromatic phenol groups and a three-carbon propene tails.

1.4.3 EOs as antimicrobials

The antibacterial activity of EOs is related to inhibition or interaction with multiple targets in the bacterial cell (Yap *et al.*, 2014), and can be measured conducting varied detecting assays, such as the broth dilution method (Balouiri *et al*., 2016; Weinstein, 2018). As reviewed by Rao *et al*. (2019) EOs with the highest antibacterial activity contain aldehydes or phenols as major components, such as cinnamaldehyde, citral, carvacrol, eugenol or thymol. In contrast, EOs containing ketones or esters have less antimicrobial action, such as β-myrcene, α-thujone or geranyl acetate (Bassolé and Juliani, 2012).

Gram-positive bacteria are usually more sensitive to EOs than Gram-negative bacteria, due to differences in the structures of their bacterial cell walls. Hydrophobic molecules can easily penetrate the Gram-positive bacterial cell wall, causing damage to its structure and also to the cytoplasm. Gram-negative bacteria have a more complex cell wall, with a 2-3 nm thick peptidoglycan layer, representing 20 % of the cell's dry weight, surrounded by the outer membrane (OM) that contains hydrophilic lipopolysaccharides; EOs (hydrophobic compounds) cannot easily penetrate this complex cell wall (Calo, 2015).

The outer membrane (OM) has many porins which act as transmembrane channels and allow small hydrophilic solutes to pass through, elucidating why Gram-negative bacteria are more resistant to hydrophobic molecules and toxic drugs to a definite point, since the OM is not completely impermeable to hydrophobic molecules (Nazzaro *et al.*, 2013).

For instance, S-(+)-linalool, the major component of coriander EO shows higher bactericidal effects against Gram-negative bacteria (*Acinetobacter baumannii*, *E. coli*, *K.*

pneumoniae, *Salmonella typhimurium*) compared with Gram-positive bacteria (*Bacillus cereus*, *S. aureus*, *E. faecalis*). These differences in activity are due to the ability of S-(+)-linalool to disrupt the negatively-charged Gram-negative bacteria outer membrane, whilst not being able to penetrate the thick Gram-positive cell wall (Aelenei *et al.*, 2016).

Lemon grass in contrast, the EO of *Cymbopogon citratus*, has shown antimicrobial activity towards Gram-positive bacteria; including *S. aureus*, *E. faecalis* and *B. subtilis* (Mangalagiri *et al*., 2021).

EOs have been established for their importance as antimicrobials against AMR microorganisms responsible for nosocomial infections. The antimicrobial activity of the EOs of *Citrus aurantium*, *Citrus x limon*, *Eucalyptus globulus*, *Melaleuca alternifolia* and *Citrus aurantium* alone and in combination with each other/with conventional antibiotics, has been investigated against VRE, MRSA and extended-spectrum β-lactamase-producing *Escherichia coli* (ESBL). The investigated EOs displayed antimicrobial actions at different extents against all strains included, and synergistic effects when in combinations (Iseppi *et al*., 2021).

Fourteen EOs and their major compounds have been investigated for their antimicrobial actions against *Haemophilus influenza*, *Streptococcus pneumonia*, *Streptococcus pyogenes* and *S. aureus* (Inouye *et al.*, 2001). Where cinnamon bark, lemongrass, and thyme EOs exhibited the highest antibacterial activities with minimum inhibitory dose (MID) of < 12.5 mg/l inhibiting all strains investigated, and weak activity of lemongrass oil against *E. coli* with MID of 100 mg/l. Among the aldehyde constituents of EOs, cinnamaldehyde from cinnamon bark oil had the greatest antimicrobial effects with MID

of < 6.25 mg/l against all strains tested. The highest activity among terpenes alcohols was assessed for geraniol with MIDs ranging from 6.25 to 12.5 mg/l (Inouye *et al.*, 2001).

In another study the antibacterial activity of the EOs from *Eucalyptus globulus* leaves against *E. coli* and *S. aureus* was assessed using agar disc diffusion and broth dilution methods (Bachir and Benali, 2012). The results showed that with this EO the inhibition zone diameter varied from 8 to 26 mm. With 100 % concentration of *Eucalyptus globulus* EO the largest inhibition zone was obtained against *E. coli*, and with 25 % concentration the smallest inhibition zone was for *S. aureus* (Bachir and Benali, 2012).

In addition, 27 EOs were tested against *S. aureus*, *E. coli* and *P. aeruginosa* using agar dilution method. *S. aureus* strains were highly susceptible towards black pepper and tea tree EOs at 0.21 mg/ml and towards copaiba at 26.52 mg/ml. *E. coli* was sensitive towards cinnamon (*Cinnamum cassia*) and clove (*Syzygium aromaticum*) EOs at 2.0 mg/ml, while *P. aeruginosa* was sensitive to *Syzygium aromaticum* EO at 8.29 mg/ml (Murbach *et al.*, 2014).

EOs of oregano, thyme, clove, lavender, clary sage and arborvitae have also shown variation in antimicrobial and antifungal properties against pathogenic bacteria, such as *E. coli*, *S. typhimurium*, *Yersinia enterocolitica*, *S. aureus*, *Listeria monocytogenes* and *E. faecalis*, as well as environmental bacteria such as *B. cereus*, *Arthrobacter protophormiae* and *Pseudomonas fragi* (Puškárová *et al.*, 2017). The antibacterial activities of the six EOs were assessed using the disc diffusion method. With inhibition zones of 26-54 mm of EOs from *Origanum vulgare* and *Thymus vulgaris* were highly effective against all tested bacteria; oregano, MIC 0.025 %, MBC 0.025-0.05 %; thyme,

MIC 0.025-0.125 %, MBC 0.05-0.125 %; while, clove and arborvitae, MIC 0.05-0.125 %, MBC 0.125-0.5 %; were less effective than oregano and thyme, with greatest inhibition against *Y. enterocolitica* (Puškárová *et al.*, 2017).

The antimicrobial activity of EOs extracted from star anise (*Illicium verum Hook*), basil (*Ocimum basilicum*), origanum (*Origanum vulgare*), clary sage (*Salvia sclarea*) and thymus (*Thymus vulgaris*) have been investigated against multidrug-resistant *E. coli* and *Enterococcus spp*. using both the agar disc diffusion method and broth micro-dilution method. *Verum* and *Salvia sclarea* EOs had no antibacterial action against *Enterococcus* isolates (Ebani *et al.*, 2018). In contrast a moderate inhibition of growth was observed towards some *E. coli* strains. EOs of *Origanum vulgare*, with MIC of 1.183 mg/ml, and *Thymus vulgaris*, with MIC of 1.171-2.342 mg/ml, showed higher inhibition of growth against *Enterococcus spp*., and more effects have been shown against *E. coli* strains with MICs of 0.293-1.183 mg/ml and 0.146-0.585 mg/ml, respectively (Ebani *et al*., 2018).

The antimicrobial, physical and chemical characteristics of the chemical compounds of carvacrol and cuminaldehyde the main constituents of the EO combination being investigated here are well documented (Char *et al.*, 2016; Khan *et al.*, 2017). The combination of carvacrol, cuminaldehyde and vancomycin reduced the growth of *Enterococcus* (VSE) and (VRE) after 24 hours (VSE by 5.83 Log_{10} CFU/ml fold, VRE by 5.07 Log_{10} CFU/ml fold) (Owen and Laird, 2019).

1.4.4 Antimicrobial activities of carvacrol

Carvacrol antimicrobial activities are revealed in a variety of actions. It causes damage to the bacterial membrane increasing membrane fluidity and permeability and reduces

ATP synthesis. This in turn reduces other energy-dependent cell processes, and enzymes and toxins synthesis (Hyldgaard *et al.*, 2012; Magi *et al.*, 2015).

Carvacrol has shown synergistic effect against *E. coli* and *S. typhinurium* when in combination with either cinnamaldehyde or rosemary oil. Whilst the combinations of carvacrol with either thyme, basil, marjoram or lemon balm have shown additive effect (Bassolé and Juliani, 2012).

Combining carvacrol with some hydrocarbons monoterpenes, such as α-penine, camphene, myrcene, α-terpinene and *p*-cymene, which typically show low antimicrobial activities, resulted in synergistic effect (de Azeredo *et al.*, 2011; Ultee *et al.*, 2000). This is believed to be due to hydrocarbons facilitate carvacrol penetration into the cell due to their capacity to interact with the cell membrane (de Azeredo *et al*., 2011).

A study in Italy had evaluated the antibacterial activity of EOs from *Origanum vulgare*, *Thymus vulgaris*, *Lavandula angustifolia*, *Mentha piperita* and *Melaleuca alternifolia* against 32 erythromycin-resistant Group A *streptococci* isolates. The highest antimicrobial activity was demonstrated with EOs from *Thymus* and *Origanum* with MICs from 256 to 512 µg/ml. Carvacrol with MICs ranging from 64 to 256 µg/ml, showed higher activity against Group A *streptococci*. Different combinations of carvacrol with erythromycin were tested using checkerboard assay and exhibited a 2-2048 fold reduction of the erythromycin MIC to what it was when being alone (Magi *et al.*, 2015).

Moreover, the antimicrobial effects of carvacrol and thymol have been evaluated both separately and in combination, against pathogenic *E. coli*, *Clostridium perfringens* and *Salmonella* strains. *E. coli* was affected more by thymol (MIC of 187.5 µg/ml and MBC of

375 µg/ml). In contrast, carvacrol had a greater effect on *Salmonella enteritidis* (MIC of 187.5 µg/ml and MBC of 750 µg/ml); whereas thymol showed the highest MIC and MBC against *S. enteritidis* (750 and 1500 µg/ml, respectively). The same antibacterial action of thymol and carvacrol was shown against *C. perfringens*, *S. typhimurium* and *S. pullorum* at MIC of 375 µg/ml and MBC of 750 µg/ml. An additive antibacterial effect between thymol and carvacrol was demonstrated at FICI between 0.5 and 1.0 (Du *et al.*, 2015).

1.4.5 Antimicrobial activities of cuminaldehyde

Cuminaldehyde (4-isopropyl benzaldehyde) is a monoterpene and aromatic volatile compound, and is considered to be the antimicrobial component of the EOs of *Eucalyptus*, *Myrrh*, *Cuminum cyminum*, *Carumcarvi* and *Cinnamomum cassia* (Singh *et al.*, 2017; Tsai *et al.*, 2016). Cuminaldehyde is known for its medical properties such as antibacterial, anti-fungal, antiplatelet, anti-diabetic, anti-Parkinson's and digestive effect (Singh *et al.*, 2017).

A study in methicillin-resistant *S. aureus* to evaluate the antimicrobial effects of cumin and its bioactive components in particular cuminaldehyde, has reported that cumin functions as an inhibitor of LmrS multidrug efflux pump. LmrS is lincomycin multidrug resistance protein of *S. aureus* and a member of the major facilitator superfamily. At high concentrations of cumin (5 and 25 mg/ml) and of cuminaldehyde (12.5 µg/ml), these components have inhibited the growth of bacterial cells lacking LmrS such as *E. coli* KAM32. Assuming that cumin and its constituents have multifactorial effects, including inhibition of LmrS efflux pump, disruption of the cell membrane and

dissipation of the proton motive force that is produced by respiration during metabolism (Kakarla *et al.*, 2017).

It has been found also that exposing *K. pneumonia* to cuminaldehyde affected the growth of the strain and led to cell elongation, capsule expression repression, and inhibition of urease activity (Derakhshan *et al.*, 2010; Singh *et al*., 2017). Cuminaldehyde minimised the extent of biofilm formation in *K. pneumonia*, while not affecting the integrity of plasmid DNA (R-plasmid), with no degradation observed (Derakhshan *et al.*, 2010).

The substantial antimicrobial activity of EO from *Cuminum cyminum* has been assessed using the agar disc diffusion method to determine its MIC and MBC values against 24 bacterial isolates, including *Streptococcus epidermidis*, *S. aureus*, *Micrococcus luteus*, *B. cereus*, *E. coli*, *Listeria monocytogenes, E. faecalis*, *P. aeruginosa* and *S. typhimirium*. For Gram-positive bacteria these studies yielded an MIC of 0.078 mg/ml, while against Gram-negative bacteria the MIC ranged from 0.078-0.15 mg/ml. Low concentrations of cumin EO were sufficient to eliminate the growth of *M. luteus,* MBC 0.15 mg/ml, and to stop the growth of *E. coli* and *E. faecalis,* MBC of 0.625 mg/ml (Hajlaoui *et al.*, 2010).

Variable MIC values of *Cuminum cyminum* EO against different bacteria have been reported; 3.4 mg/ml against *Salmonella typhi* and *E. coli*, while lower activity was noticed against *Salmonella paratyphi*, *Psedomonas fluorescens* and *S. aureus,* where MICs were at 14, 12.2 and 29.7 mg/ml, respectively (Naveed *et al.*, 2013).

Furthermore, using the agar disc diffusion method, the antimicrobial effect of EOs of cumin (*Cuminum cyminum*), black pepper (*Piper nigrum*), coriander (*Coriandrum*

sativum) and cardamom (*Elettaria cardamomum*), have been evaluated against *E. coli, Salmonella spp, S. aureus* and *Pseudomonas vulgaris*. All investigated organisms were sensitive to all EOs, with higher sensitivity of Gram-positive bacteria with inhibition zones diameters between 8-10 mm, and MIC was 60- > 600 ppm (parts per million). Lower activity was shown against Gram-negative bacteria, with inhibition zone diameters between 8-12.5 mm and MICs of 6-60 and > 600 ppm (Teneva *et al.*, 2016).

1.4.6 The interaction of essential oil components

The antibacterial activities of EOs are dependent on several factors such as the EOs components, quantity and the interaction between them. This interaction can be defined in three ways: additive, antagonist or synergetic (Faleiro, 2011)

When the resultant effect of the ingredients is equal to the sum of the individual effect of each component this refers to additive effect. Synergism is identified when the sum of subjects' activities is higher than the individual actions. Finally, when the influence of components reduces each other this indicates antagonist action (Faleiro, 2011). Examples of the interaction of some of the EOs components are shown in (Table 1.1).

Table 1.1. The antibacterial interactions of combinations of EOs and their components against different microorganisms

Pair combinations	Organism	Method	Interaction	Reference
Thymol/carvacrol	*S. aureus*	Broth dilution	Additive	(Lambert *et al.*, 2001)
	P. aeruginosa			(Pei *et al.*, 2009)
	E. coli	Checkerboard	Synergism	
	B. cereus	Checkerboard	Antagonism	(Rivas *et al.*, 2010)
	E. coli			(Zhou *et al.*, 2007)
	S. aureus,	Mixture	Additive	
	P. aeruginosa			
		Checkerboard	Additive	

Pair combinations	Organism	Method	Interaction	Reference
Thymol/eugenol	*E. coli*	Checkerboard	Synergism	(Pei *et al.*, 2009)
Carvacrol/eugenol	*E. coli* *S. aureus, B. cereus, E. coli*	Checkerboard Checkerboard	Synergism Antagonism	(Pei *et al.*, 2009)
Carvacrol/linalool Eugenol/linalool Eugenol/menthol Cinnamaldehyde/ Eugenol	*Listeria monocytogenes, Enterobacter aerogenes, E. coli, P. aeruginosa Staphylococcus spp., Micrococcus spp., Bacillus spp., and Enterobacter spp.*	Checkerboard Mixture	Synergism Additive	(Bassolé *et al.*, 2010) (Moleyar and Narasimham, 1992)
Cinnamaldehyde/ Carvacrol	*E. coli, S. Typhinurium*	Mixture	Synergism	(Zhou *et al.*, 2007)
Cinnamaldehyde/thymol	*E. coli,* *S. Typhinurium*	Checkerboard Mixture	Synergism Synergism	(Pei *et al.*, 2009) (Zhou *et al.*, 2007)
Cinnamaldehyde/ Eugenol	*Staphylococcus spp., Micrococcus spp., Bacillus spp., and Enterobacter spp.*	Mixture	Additive	(Moleyar and Narasimham, 1992)
1,8-Cineole/aromadendrene	*Methicillin-resistant S. aureus (MRSA) and vancomycin-resistant Enterococci (VRE) Enterococcus faecalis*	Checkerboard	Additive	(Mulyaningsih *et al.*, 2010)
α-Pinene/limonene	*Saccharomyces cerevisiae*	Checkerboard	Synergism, Additive	(Tserennadmid *et al.*, 2011)
Oregano/basil	*E. coli* *Bacillus subtilis* *S. aureus*	Checkerboard	Additive Additive Synergism	(Lv *et al.*, 2011)
Oregano/bergamont	*B. subtilis* *S. aureus*	Checkerboard	Additive Synergism	(Lv *et al.*, 2011)
α-Pinene/linalool Linalool/terpinen-4-ol *O. vulgare/T. vulgaris*	*L. monocytogenes* *Y. enterocolitica*	Mixture	Synergism	(Bassolé *et al.*, 2010)
Cinnamon oil/ thyme oil	*B. sublitis* *B. cereus* *S. aureus* *E. coli* *S. typhimurium*	Checkerboard	Additive	(Lv *et al.*, 2011)
Cinnamon oil/ clove oil	*B. sublitis* *B. cereus* *S. aureus* *E. coli* *S. typhimurium*	Checkerboard	Additive Indifferent Indifferent	
Basil/oregano *Thyme/oregano*	*E. coli*	Checkerboard	Additive	(Gutierrez *et al.*, 2008)

Pair combinations	Organism	Method	Interaction	Reference
Thyme/oregano	*S. aureus* *S. typhimurium*	Checkerboard	Synergism	(Stojković *et al.*, 2013)
Cumin/coriander Seed oil	*B. cereus* *L. monocytogenes* *S. aureus* *E. coli*	Checkerboard Time-kill method	Synergism	(Bag and Chattopadhyay, 2015)
Cumin/coriander Seed oil	*S. typhimurium* *M. luteus*	Checkerboard Time-kill method	Additive	
Thyme/myrtle	*S. aureus* *E. coli*	Checkerboard	Synergism	(Sadiki *et al*., 2014)

Different studies have evaluated the interaction between EOs and their components. Pei *et al.* (2009) have assessed the antibacterial activity of eugenol, cinnamaldehyde, thymol and carvacrol, both alone and when combined, against *E. coli*, MICs were 1600, 400, 400, and 400 mg/l, respectively. When combinations were tested, such as cinnamaldehyde/eugenol, thymol/eugenol, carvacrol/eugenol, and thymol/carvacrol, the MICs were reduced to 400, 100, 100 and 100 mg/l, respectively.

Combinations of linalool or menthol with eugenol were assessed using checkerboard test (Bassolé *et al.*, 2010). The highest synergy was recorded for (FIC: 0.05) linalool and eugenol against *L. monocytogenes,* and (FIC: 0.04) against *E. aerogenes.* Combining menthol and eugenol was synergistic against *L. monocytogenes* (FIC: 0.06), and against *E. aerogenes* (FIC: 0.04) which indicates that combining a monoterpenoid phenol and a monoterpenoid alcohol is effective (Bassolé *et al*., 2010).

The synergistic effect of a combination of cinnamaldehyde and carvacrol against both *S. aureus* and *E. coli* was revealed with a reduction in MICs from 0.31 to 0.009 mg/ml for cinnamaldehyde and 0.31 to 0.078-0.010 mg/ml for carvacrol (Ye *et al.*, 2013).

In contrast, antagonistic and indifferent effects due to different mechanisms of action of components were displayed by some combinations. For instance combinations of lovage/thyme and basil/thyme EOs against *B. cereus*, *S. aureus*, *P. aeruginosa* and *E. coli* showed antagonistic effects, while combinations of EOs of parsley/lovage, lovage/basil and parsley/basil EOs showed indifferent effect (Semeniuc *et al.*, 2017).

The synergistic antimicrobial effects of the EOs and their components against a wide range of bacteria, and the synergistic effect between commercial antibiotics being well documented, for instance trimethoprim/sulfamethoxazole, amoxicillin/clavulanate and piperacillin/tazobactam. However, the synergistic interaction between available antibiotics and EOs and/or their components has not been fully researched (Boire *et al.*, 2013).

1.4.7 Mechanisms of antimicrobial action of EOs

The antimicrobial activity of EOs is not related to one component, but indeed it is thought to be dependent on the EO compositions and their targets in the bacterial cell (Calo, 2015).

The mechanisms of antibacterial actions of EOs and their components in general can be categorised into: degradation of the bacterial cell wall, disruption of the cytoplasmic membrane, increasing cell membrane permeability and leakage of the cytoplasmic contents, cellular elongation, depletion of proton motive force, disruption of active transport, and coagulation of cytoplasm (Jayasena and Jo, 2013; Langeveld *et al.*, 2014). Some examples are included in Table 1.2.

EO components like alcohols and aldehydes have specific antibacterial activities. Alcohols, particularly terpene alcohols, demonstrate significant bactericidal action against a variety of microorganisms, for instance the exposure to carvacrol inhibited biofilm formation in *S. aureus*, *S. enterica* and *S. typhimurium*, through number of mechanisms including protein denaturation, bacterial cell dehydration or dissolving the bacterial cell membranes (Boire *et al.*, 2013).

Cinnamaldehyde has shown to have three mechanisms of actions which are concentration dependent. At low concentrations this EO inhibits the enzymes involved in producing cytokines, at higher concentrations it inhibits ATPase, but it perturbs the membrane at a lethal concentration (Hyldgaard *et al*, 2012).

Fisher and Phillips (2009) have reported that adding the EO from orange (*Citrus sinensis*) to *E. faecium* and *E. faecalis* increased cell membrane permeability by two folds. This resulted in loss of the membrane potential and cell lysis, which caused by loss of intracellular ATP in addition to a decrease in the intracellular pH. Moreover, treating *E. faecium* with citrus EO vapours has been observed by Atomic Force Microscopy causing the degradation of the cell surface structures (Laird *et al.*, 2012).

Allium sativum EO affects *E. coli* through increasing permeability of the membrane (Hyldgaard *et al.*, 2012), while *Litsea cubeba* EO affects *E. coli* via destructing the outer and inner membrane (Li *et al.*, 2014), and *Origanum vulgare* EO causes the cell membrane of *E. coli* to become permeabilised (Dorman and Deans, 2000). *Cuminum cyminum* affects *E. coli* causing changes in cytoplasm (Hyldgaard *et al.*, 2012), and *Curcuma vulgare* inhibits ergosterol biosynthesis (Hu *et al.*, 2017).

Table 1.2. The antibacterial mechanisms of actions of EOs and their components

EO or component	Mode of action	References
Oregano	Reduction in lipase and coagulase activity, enzyme inhibition	(de Barros *et al.*, 2009)
Carvacrol	Membrane disruption, inhibition of ATPase activity, membrane destabilisation, leakage of cell ions, fluidisation of membrane lipids, reduction of proton motive force	(Di Pasqua *et al.*, 2007; Ultee *et al.*, 2002)
Cuminaldehyde	Mild changes in cytoplasm; destructive effects on cell envelope	(De *et al.*, 1999; Pajohi *et al.*, 2011)
Thymol	Membrane disruption with potential intracellular targets, citrate metabolic pathway disruption	(Trombetta *et al.*, 2005; Di Pasqua *et al.*, 2007, 2010)
Cinnamic acid	Membrane disruption	(Chen *et al.*, 2011; Hemaiswarya and Doble, 2010)
Eugenol	Membrane disruption by inhibiting ATPase activity, possible efflux pump blocker, reduction of several virulence factors at sub inhibitory concentrations	(Bolla *et al.*, 2011; Di Pasqua *et al.*, 2007; Hemaiswarya and Doble, 2009; Qiu *et al.*, 2010)
γ-terpinene	Membrane disruption	(Oyedemi *et al.*, 2009)
Cinnamaldehyde	Inhibited histidine decarboxylase. Membrane depolarised and permeabilised. Cytoplasmic content leakage and coagulation. Respiration activity inhibition. Loss of membrane integrity And reduced intracellular PH	(Hyldgaard *et al.*, 2012)

1.4.8 EOs/antibiotics combinations

Returning to a pre-antibiotic era may become a reality in the next 30 years, due to multidrug-resistant microorganisms and the lack of new antibiotics in the pharmaceutical market. Combining existing antibiotics with EOs and/or their components might provide a solution to the problem of emerging antimicrobial resistant strains of bacteria, by providing alternative antibiotics to combat AMR (Boire *et al.*, 2013).

Combining EOs with conventional antibiotics has resulted in new formulations with antibacterial activities to reverse the AMR, and to modulate the antibiotic's action (Lahmar *et al.*, 2017). For instance, combining peppermint oil with piperacillin against *E. coli* reduced the MIC of piperacillin between 2 and 4 fold, from 1024 to 64 µg/ml (Yap *et al.*, 2013), and when combined with meropenem, peppermint oil minimised its MIC from 4 to 0.5 µg/ml (Yap *et al.*, 2013).

Likewise, the MIC of norfloxacin against four strains of MRSA was reduced by two components of grapefruit oil from above 100 down to 5-10 µg/ml (Langeveld *et al.*, 2014). Furthermore, in combination with tetracycline, EOs from *Salvia fruticose*, *Salvia officinalis* and *Salvia sclarea* reduced its MIC against tetracycline resistant *S. epidermidis*, with FICIs ranging from 0.122 to 0.37 demonstrating synergism (Chovanová *et al*., 2015). Examples of conventional antibiotics MICs reduction when in combination with EOs are shown in Table 1.3.

The synergism observed when EOs are combined with commercial antibiotics has resulted in a group of objectives, such as; the combination therapy is expected to produce greater efficacy than the monotherapy (Table 1.4, 1.5). The dosage of the drug is expected to reduce in combination with equal or increased level of efficacy (Table 1.3). The development of drug resistance may be reduced or delayed via the drugs combination as a result of multiple pathways inhibition. And finally, the undesired side effects are expected to minimise in the combination therapy (Rather *et al.*, 2013; Bag and Chattopadhyay, 2015).

In addition to the synergistic effect, antagonism between EOs components and antimicrobials can occur, and it is suggested this happens when; 1) in bacteriostatic (inhibition of bacterial growth) and bactericidal (killing the bacteria) antimicrobial combinations, 2) the site of action of the antimicrobials is the same, 3) interaction of antimicrobials with each other (Hyldgaard *et al.*, 2012).

Table 1.3. MICs reduction in combination of EOs and commercial antibiotics

EO	Antibiotic	Microorganism	Antibiotic MIC fold-reduction	Reference
Carvacrol	Ampicillin Penicillin Tetracycline Bacitracin Erythromycin Novobiocin	*S. typhimurium*	4-8	(Palaniappan and Holley, 2010)
Carvacrol	Ampicillin Penicillin Bacitracin	*S. aureus*	4-8	
		E. coli	4-8	
Carvacrol	Erythromycin	*Streptococcus pyogenes*	4-8	
Cinnamaldehyde	Ampicillin Penicillin Tetracycline Bacitracin Erythromycin	*S. typhimurium*	4-8	
Cinnamaldehyde	Ampicillin Penicillin Bacitracin	*S. aureus*	4-8	
Cinnamaldehyde	Ampicillin Penicillin Tetracycline Bacitracin Erythromycin	*E. coli*	4-8	
Cinnamaldehyde	Erythromycin	*S. pyogenes*	4-8	
Eugenol	Ampicillin Penicillin Tetracycline Bacitracin	*S. typhimurium*	4-8	
Eugenol	Ampicillin Penicillin Bacitracin	*S. aureus*	4-8	

EO	Antibiotic	Microorganism	Antibiotic MIC fold-reduction	Reference
Carvacrol	Ciprofloxacin	*E. coli* *Salmonella spp.* *K. pneumoniae* *S. aureus* *B. cereus*	4	(Fadli *et al.*, 2012)
Carvacrol Thymol Eugenol	Nalidixic Acid	*S. typhimurium*	4-8 2-8 2-8	(Miladi *et al.*, 2017)
Eugenol	Ampicillin	*S. mutans* *S. sanguinis* *S. anginosus*	4-8	(Moon *et al.*, 2011)
Clove oil	Ampicillin	*S. mutans* *S. sanguinis* *S. anginosus*	≥ 4	
Pitruranthos chloranthus Teucrium ramosissimum Pistacia lentiscus EOs	Ofloxacin Novobiocin Piperacillin	*E. coli*	2 64 No reduction	(Lahmar *et al.*, 2017)
Pitruranthos chloranthus Teucrium ramosissimum	Amoxicillin Piperacillin	MRSA	32	
Pistacia lentiscus EOs	Tetracycline Oxacillin			

Table 1.4. EO components and antibiotics interactions

EO	Antibiotics	Microorganism	Antimicrobial interaction	References
Rosemary	Gentamicin Tetracycline	*S. aureus*	Synergistic	(Barreto *et al.*, 2014)
	Chloramphenicol Cefepime Tetracycline	*E. coli*	Synergistic	
	Ciprofloxacin	*S. aureus*	Antagonistic	
Salvia officianlis Salvia sclarea Salvia fruticose	Tetracycline	*S. epidermidis*	Synergistic	(Chovanová et al., 2015)
Cuminaldehyde	Ciprofloxacin	*K. pneumoniae*	Coaction	(Derakhshan *et al.*, 2010)
Cravacol Or Thymol Or Eugenol	Ampicillin Penicillin Tetracycline Erythromycin	*S. typhimurium*	Synergistic	(Palaniappan and Holley, 2010)

Eugenol	Tetracycline	*E. coli*		
	Penicillin	*S. aureus*		
Thymol	Ampicillin			
Cinnamaldehyde	Bacitracin			
Thymol Carvacrol	Erythromycin	*S. pyogenes*		
Clove oil	Gentamicin	*S. mutans* *S. sanguinis* *P. gingivalis*	Synergistic	(Moon *et al.*, 2011)
Eugenol	Gentamicin	*S. sanguinis* *S. anginosus* *P. intermedia* *P. gingivalis*	Synergistic	
		S. mutans	Additive	
Cinnamon bark	Piperacillin	*E. coli*	Synergistic	(Yap *et al.*, 2013)
Lavender				
Peppermint				
Tea tree			No interaction	
Cinnamon bark	Ampicillin	*E. coli*	No interaction	
Lavender				
Peppermint				
Tea tree				
Cinnamon bark	Carbencillin	*E. coli*	No interaction	
Lavender				
Peppermint				
Tea tree				
Cinnamon bark	Cefazolin	*Ecoli*	No interaction	
Lavender				
Peppermint				
Tea tree				
Cinnamon bark	Meropenem	*E. coli*	No interaction	(Yap *et al.*, 2013)
Lavender			No interaction	
Peppermint			Synergistic	
Tea tree			No interaction	
Thymus willdenowii boiss	Tobramycin	*E. coli* *K. pneumonia* *E. aerogenes* *P. mirabilis* *Sallmonella enteritidis*	Antagonistic	(Moussaoui and Alaoui, 2016)
	Ticarcillin	*E. coli* *S. enteritidis* *E. aerogenes* *P. aeruginosa*	Synergistic	
	Imipenem	*K. pneumonia* *E. aerogenes* *P. mirabilis*	Antagonistic	
	Imipenem	*E. coli* *P. aeruginosa* *S. enteritidis*	Synergistic	

Table 1.5. List of EOs-antibiotic combinations against a panel of microorganisms

Pair combinations	Microorganisms	Methods	Interaction	References
Eremanthus erythropappus/ ampicillin	*S. aureus*	Time-kill assay	Synergistic	(Rodrigues *et al.*, 2009)
Oregano/ fluoroquinolones Oregano/ doxycycline Oregano/ lincomycin Oregano/ maquindox	*E. coli*	Broth microdilution Checkerboard assay	Synergistic	(Si *et al.*, 2008)
Piperacillin/cinnamon Piperacillin/lavender Meropenem/peppermint	*E. coli*	Checkerboard assay	Synergistic	(Yap *et al.*, 2013)
Ceftriaxone/leaf extract	*P. aeruginosa*	Agar dilution	Synergistic	(Sahu *et al.*, 2014)
Pelargonium graveolens/ norfloxacin	*S. aureus, B. cereus*	Agar dilution Checkerboard assay	Synergistic	(Rosato *et al.*, 2007)
Coriander oil/ciprofloxacin Coriander oil/gentamicin Coriander oil/tetracycline Coriander oil/piperacillin Coriander oil/cefperazone	*A. baumannii*	Checkerboard assay	Synergistic additive	(Duarte *et al.*, 2012)
Lantana montevidensis/ aminoglycosides	*E. coli*	Broth microdilution Checkerboard assay	Synergistic	(de Sousa *et al.*, 2013)
Eugenol/ vancomycin Eugenol/ -lactams	*E. coli, E. aerogenes, P. vulgaris, P. aeruginosa, S. typhimurium*	Broth microdilution Checkerboard assay	Synergistic	(Hemaiswarya and Doble, 2009)
Croton zehntneri/ gentamicin	*S. aureus, P. aeruginosa*	Disc diffusion test (indirect contact of EO)	-	(Rodrigues *et al.*, 2009)
Rosmarinus officinalis/ ciprofloxacin	*K. pneumoniae*	Broth microdilution Checkerboard assay	Synergistic	(van Vuuren *et al.*, 2009)
Eucalyptus/ chlorhexidine digluconate	*Staphylococcus epidermidis*	Broth microdilution Checkerboard assay	Synergistic	(Karpanen *et al.*, 2008)
Zataria multiflora/ vancomycin	*S. aureus (MRSA and MSSA)*	Broth microdilution Checkerboard assay	Synergistic	(Mahboubi and Ghazian, 2010)
Citrus limon/ amikacin Cinnamomum zeylanicum/ amikacin	*Acinetobacter spp*	Broth microdilution Checkerboard	Synergistic	(Guerra *et al.*, 2012)

1.5 Methods of screening the antimicrobial activity of EOs

Drug discovery, epidemiology and prediction of therapeutic outcome can be assessed by antimicrobial susceptibility testing. Several methodologies have been used to assess the antimicrobial interaction between EOs and/or their components in combination with conventional antibiotics (Bassolé and Juliani, 2012; Aelenei *et al.*, 2016), in addition to guidelines on European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI)(CLSI, 2018) as reviewed by *Rao et al*. (2019).

1.5.1 Agar disc-diffusion method

Agar disc-diffusion method is the standard method for routine antimicrobial susceptibility testing in many clinical microbiology laboratories. Agar plates are inoculated with a standardised inoculum of the test microorganism. The test compound is applied to filter paper discs of 6 mm diameter and placed on top of the inoculated agar (Balouiri *et al*., 2016; Patil *et al*., 2016). The petri dishes are then incubated for a defined period of time under standard conditions of temperature and oxygen. The inhibition of growth of the tested microbe is due to the diffusion of the antimicrobial agent into the agar and described by inhibition zones with different diameters. Bacteria are categorised as susceptible, intermediate or resistant according to the inhibition zone diameters which are approved by the CLSI (CLSI, 2018; Balouiri *et al*., 2016; Patil *et al*., 2016; Matuschek *et al*., 2014).

1.5.2 Broth dilution method

Broth micro-dilution or macro-dilution procedures include antimicrobial agents prepared in two-fold serial dilutions with concentration in µg/ml in a liquid growth medium, and divided in tubes with minimum volume of 2 ml (macro-dilution) or using 96 well-plate with smaller volumes of 200-250 µl (micro-dilution). The bacterial inoculum is prepared in the medium and diluted in accordance with standardised microbial suspension adjusted to 0.5 McFarland scale, to be added to each tube or well. The inoculated 96-well microtitration plate or tubes are incubated at the defined conditions after shaking or vortex mixing. The MIC is defined as the lowest concentration of the antimicrobial agent that inhibits the organism growth. The minimum bactericidal concentration (MBC) is the lowest concentration of antibiotic needed to kill 99.9 % of the bacterial inoculum following incubation for 18-24 hours. This is ascertained by sub-culturing samples from wells (micro-dilution) or tubes (macro-dilution) on agar plates, and incubating for 18-24 hours; where no growth is seen on plates this correlates to the MBC (Balouiri *et al*., 2016; Weinstein, 2018).

1.5.3 Time-kill test

Time-kill assay is a procedure for establishing the dynamic interaction between the antimicrobial agent and the microbial strain, in order to determine the bactericidal effect of that antimicrobial agent. It can be used to determine time-dependency or concentration-dependency of the antimicrobial action (Balouiri *et al.*, 2016).

The combined antimicrobial action of two agents can be evaluated by the time-kill method by measuring the sub-inhibitory concentration effect of one agent on the killing

ability of the other agent (Bassolé and Juliani, 2012). The incubation is performed under appropriate conditions and for different time intervals (for instance: 0, 4, 6, 8, 10, 12 and 24 hours). The bactericidal effect is generally assessed by a lethality percentage of 90 % for 6 hours, which is equal to lethality of 99.9 % for 24 hours (Balouiri *et al.*, 2016).

1.5.4 Checkerboard assay

Checkerboard assay is the most common test to assess synergy between EOs alone and combined with antibiotics. It is based on the fractional inhibitory concentration index (FICI) to express the antimicrobial interactions. The FIC of a factor is the MIC in combination with another agent divided by the MIC with the same effect when used alone.

$$FICI = FIC_1 + FIC_2$$

$$= \frac{MIC_1 in\ combination}{MIC_1\ alone} + \frac{MIC_2 in\ combination}{MIC_2\ alone}$$

The antimicrobial interactions on the basis of FICI have different interpretations:

1) FICI ≤ 0.5 or < 1 is considered a synergistic interaction.
2) 0.5 < FICI ≤ 1, FICI = 1 is interpreted as additive interaction.
3) 1 < FICI ≤ 2, 1 < FICI ≤ 4, 5 < FICI ≤ 4 is indifference effect.
4) FICI > 1, 2 or 4 is interpreted as an antagonistic effect (Aelenei *et al.*, 2016).

1.6 Scope of work

With increasing bacterial resistance to antibiotics becoming a global health threat, novel antimicrobial strategies need to be established. EOs represent a source of novel antimicrobials with new mechanisms of action and may offer solutions for treating AMR. EOs with antibacterial activities have raised a new research area and a promising treatment for the future. When used in combination, EOs may reduce the likelihood of AMR. Their antimicrobial mechanisms of action are not well understood and need to be fully established before being considered for drug development.

The Infectious Disease Research Group at De Montfort University has assessed the antimicrobial action of a formulation of EO components of carvacrol and cuminaldehyde together with the conventional antibiotic, vancomycin. This formulation has been shown to be effective against both vancomycin-sensitive (VSE) and vancomycin-resistant *E. faecium* (VRE) (Owen and Laird, 2019). The mechanism of action of the synergistic EO components, carvacrol and cuminaldehyde, in combination with vancomycin is yet to be determined and this study aims to address this.

This thesis focuses on determining the role of transport channels in the mechanism of action of the formulation of EO components (1.9 mM carvacrol and 4.20 mM cuminaldehyde) with 0.031 mg/l vancomycin against *E. faecium* (VSE and VRE). The formulation has been proved to reverse the resistance towards vancomycin in both isolates (VSE by 5.83 Log_{10} CFU/ml fold, VRE by 5.07 Log_{10} CFU/ml) (Owen and Laird, 2019).

1.7 Aim and objectives

Although EOs have multiple targets in the bacterial cell, there is limited published research on the antimicrobial mechanism of action of EOs alone or in combination with conventional antibiotics, and little is known about the involvement of transport channels in the mechanism of action of these natural products.

The overall aim of this study is to establish whether transport channels are involved in the antimicrobial mechanism of action of a novel formulation of combining plant extracts (carvacrol and cuminaldehyde) with conventional antibiotic (vancomycin), that has shown to be effective against *E. faecium* (VSE and VRE), including clinical isolates.

Objectives

- To determine if the presumed involvement of bacterial transport channels in the mechanism of action of the formulation are similar to the transient receptor potential vanilloid 1 (TRPV1) channels found in mammalian cells.
- To assess previously identified genes (*bcr*, *ecfA1*, *ecsA-1*, *yloB* and *nhaC_2*) that have been differentially regulated when exposed to the formulation (EOs-vancomycin) in the microarray through conducting qPCR.
- To perform a time course study using qPCR in order to understand the effect of the formulation on the expression levels of the above-mentioned genes that are involved in transportation in the bacterial cell.
- To evaluate the involvement of calcium transporting channels in the mechanism of action of the formulation in the presence of externally added calcium.

- To establish the effect of an efflux pump inhibitor/calcium chelator, EDTA, on the transcriptional profile of *bcr* gene, that is involved in an efflux system in *E. faecium,* upon exposure to the formulation.
- To construct gene knockouts of *bcr* and *yloB* in vancomycin-resistant *E. faecium* and to analyse the effect of the formulation on the subsequent strains.

Chapter 2

2 Is the mechanism of action of EOs against *E. faecium* mediated by channels similar to TRPV1 mammalian channels?

2.1 Introduction

EO components (carvacrol and cuminaldehyde) showed antibacterial activity against *E. faecium* (VSE and VRE), individually and in combination (Owen and Laird, 2019). It was hypothesised that the antimicrobial mechanism of action of these EO components could involve channels; however, these channels are yet to be established. EOs have been found to display their action through channels, as shown by a study that investigated the effect of extracts of *Anthemis hyalina*, *Nigella sativa* and *Citrus sinensis* on the expression of TRP genes during coronavirus infection using HeLa-CEACAM1a cells (Ulasli *et al*., 2014). Capsaicin, an EO component with antimicrobial activities as well, has been found to affect TRPV1 mammalian channels as an agonist (Samanta *et al.*, 2018). Thus, it was hypothesised in the current study that the antimicrobial action of capsaicin against *E. faecium* might be happening through channels that are similar to TRPV1 mammalian channels; similarly, the other EO components (carvacrol and cuminaldehyde) could be functioning via these channels. The antagonists of TRPV1 channels, AMG517 and RR, were used to further investigate the potential involvement of TRPV1-like channels in the antimicrobial actions of capsaicin and consequently the EO components investigated here. Furthermore, considering that the similarity between the prokaryote channels and eukaryotes' is described, where it has been found that prokaryotic sodium channels are homologous to eukaryotic sodium channels but simpler, they are single-domain proteins that assemble to form functional

homotetramers (Ren *et al.*, 2001); wherein the eukaryotes' are pseudotetrameric structures assembled of complex multidomain proteins (Bagnéris *et al.*, 2015; Ren *et al.*, 2001).

Bacteria have many types of ion channels including; sodium channels, chloride channels, calcium-gated channels, potassium channels and ionotropic glutamate receptors (Prindle *et al.*, 2015). Ion channels and pumps are specific macromolecular transport systems, and they facilitate and control the passage of ions in both directions across the cell lipid membrane (Roux, 2017). Each channel has its own characteristics including permeability to specific ions, conductance, opening and closing mechanisms, and additional regulatory characteristics (Minke and Cook, 2002).

In 1997, David Julius and colleagues were the first to characterize the capsaicin receptor in mammalian cells, which was later termed the transient receptor potential vanilloid1 channel (TRPV1). The discovery of the superfamily of transient receptor potential (TRP) ion channels (28 mammalian members) has opened up a new field of research on the importance of these channels as drug targets (Huang *et al.*, 2017).

It has been reported that the chemical components of EOs were found to directly activate gamma aminobutyric acid receptors and the transient receptor potential (TRP) channels in the skin and digestive system, and the olfactory receptors in the olfactory system. EOs have been involved in different application routes depending on their chemical compounds, and used as inhaling, topically applying on the skin and drinking. Thereby, EOs improved the physiological and psychological health condition through activating/ desensitizing the corresponding receptors (Koyama et al., 2020).

2.1.1 TRP channels

The superfamily of mammalian TRP channels comprises 28 members grouped into seven main subfamilies. These subfamilies are categorized on the basis of amino acids sequence homology to; the TRPC (Canonical) family, the TRPV (Vanilloid) family, the TRPM (Melastain) family, the TRPP (Polycystin) family, the TRPML (Mucolipin) family, the TRPA (Ankyrin) family and the TRPN (NOMPC) family (Benemei *et al.*, 2015; Nilius and Owsianik, 2011). The majority of TRP channels are non-selective cation channels; nevertheless, a few of them are highly selective for calcium (Ca^{2+}), and some are for magnesium (Mg^{2+}) permeable (Nilius and Owsianik, 2011; Pedersen *et al.*, 2005).

2.1.2 TRPV1 channels

The mammalian TRPV family was named based on the activation of this group by a vanilloid-like molecule, capsaicin; and the first member to be discovered was TRPV1 (Samanta *et al.*, 2018). The physiological studies showed that the TRPV1 receptors are involved in sensory reactions, such as detection of pain or nociception, and the activation of these receptors mediates a painful burning sensation in response to diverse range of activators including high temperature and importantly here, capsaicin (Samanta *et al.*, 2018). TRPV1 expression was first identified in the pain-sensitive neurons of the dorsal root ganglion and trigeminal ganglion neurons, and also was identified in the spinal terminals and peripheral nerves (Gees *et al.*, 2010).

TRPV1, is a cation channel, with relatively high permeability for Ca^{+2}. It is activated by vanilloid compounds such as capsaicin and vanillin, noxious or high temperature (≥ 43°C) (Carnevale and Rohacs, 2016; Çiğ and Nazıroğlu, 2015), voltage and low pH (< 5.9) (Liao

et al., 2013). It can be blocked by bioactive substances, such as capsazepine, AMG517, ruthenium red and resiniferatoxin (Ren *et al.*, 2015).

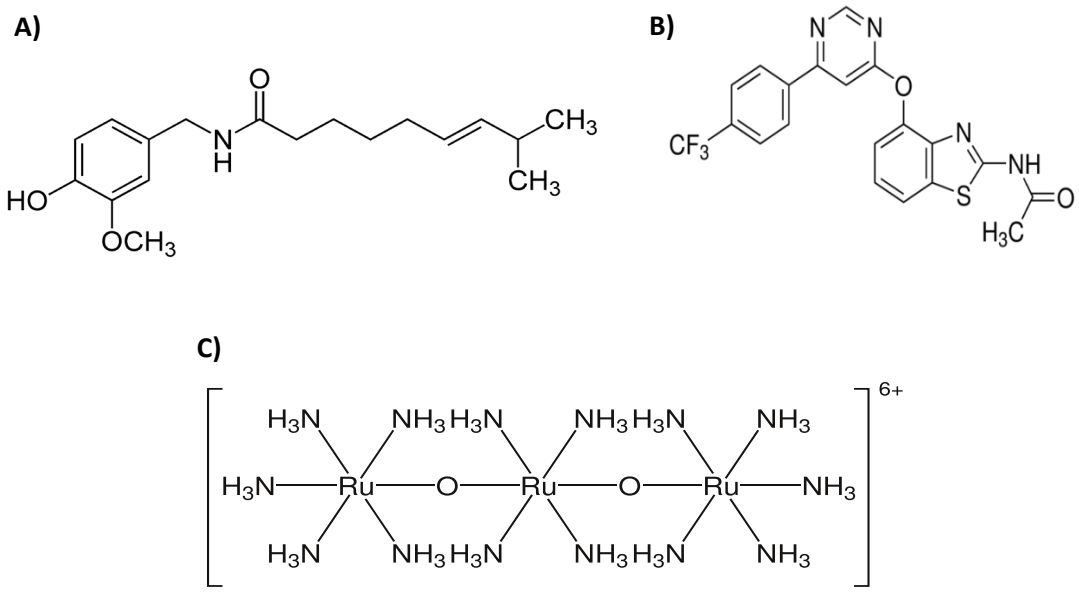


Figure 2.1. Chemical formula of comupounds used in the current study. A) capsaicin ($C_{18}H_{27}NO_3$) an alkaloid acts a TPRV1 channels agonist, B) AMG517 ($C_{20}H_{13}F_3N_4O_2S$) a drug acts as a selective antagonist of TRPV1 channels, and C) Ruthenium Red ($Cl_6O_2Ru_3.4H_2O.14NH_3$) an inorganic dye ammoniated ruthenium oxychloride acts as a non-selective blocker of TRPV1 channels. Adapted from Vriens *et al*., 2009.

2.1.3 TRPV1 channels activators

2.1.3.1 Capsaicin

The most studied agonist for TRPV1 channel is capsaicin (N-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methylnon-6-enamide) (Darre and Domene, 2015). Capsaicin, with the molecular formula of $C_{18}H_{27}NO_3$, is an alkaloid hydrophobic, colourless, odourless, crystalline component; with a melting point of 62-65˚C. Alcohol and other organic solvents are used to dissolve capsaicin in topical preparations and sprays (Sharma *et al.*, 2013; O'Neill *et al.*, 2012). Capsaicin comes from the genus capsicum, it is an essential oil component of chilli peppers (Chung and Campbell, 2016;

Darre and Domene, 2015). The pungent and spicy taste of hot chilli peppers is related to its chemical constituents belonging to the capsaicinoids group. The components of capsaicinoids family are primarily capsaicin 80-90 %, dihydrocapsaicin, nondihydrocapsaicin, homohydrocapsaicin, homodihydyocapsaicin and nonivamide (Rollyson *et al.*, 2014).

The action of capsaicin at TRPV1 channels is affected by different factors, including temperature, extracellular pH, membrane potential and the intracellular concentration of Ca^{2+} (Zheng, 2013). The continuous presence of an activating stimulus, such as capsaicin, can cause TRPV1 to undergo desensitization, and thereby capsaicin renders the neurons less sensitive to noxious stimuli. This action of capsaicin requires the availability of extracellular Ca^{2+} and activation of Ca^{2+}-calmodulin dependent protein kinase that promotes the channel phosphorylation (Brito *et al.*, 2014).

2.1.4 TRPV1 channels antagonists

A diseases potential treatment option is by acting on the TRPV1 channels in two ways; either by activating and desensitising TRPV1 by agonists (activators), or by using antagonists (blockers) drugs for TRPV1 receptor (Brito *et al.*, 2014).

Capsazepine was the first TRPV1 antagonist to be reported, through modifying the chemical backbone of capsaicin (Walpole *et al.*, 2002). In general, TRPV1 antagonists are categorised as competitive or non-competitive. The competitive antagonist binds to the agonist binding site to leave the TRPV1 channel in a locked, non-conductive state (Vriens *et al.*, 2009). Non-competitive TRPV1 channel antagonists act as open-channel blockers, that prevent channel opening by the agonist or via blocking its aqueous pore (Messeguer

et al., 2006). The first non-competitive antagonist of TRPV1 was ruthenium red (García-Martínez *et al.*, 2000).

It has been reported that the TRPV1 agonist capsaicin decreases the body temperature of humans and other species causing hypothermia (Brito *et al.*, 2014). In contrast, TRPV1 selective antagonists, such as AMG-0347 and AMG-517, have been observed to cause hyperthermia (Gavva *et al.*, 2007), by decreasing heat loss through the skin and increasing metabolic heat production (Brito *et al.*, 2014).

2.1.4.1 AMG517

In contrast to the agonists described above, TRPV1 receptors can be blocked by bioactive substances, such as ruthenium red (a non-competitive antagonist), and AMG517 (a competitive antagonist) (Heng *et al.*, 2014).

AMG517 is a selective and potent TRPV1 antagonist for all activation modes of TRPV1 (Abramov, 2016; Ren *et al*., 2015), with the formula N-(4-[6-(4-trifluoromethyl-phenyl)-pyrimidin-4-yloxy]-benzothiazol-2-yl)-acetamide. AMG517 has been assessed as a potential antagonist of TRPV1 activation *in vitro* by capsaicin, protons and heat, and *in vivo* to block flinch-induced by capsaicin in rats (Gavva *et al.*, 2007). AMG517 blocks calcium influx resulting from TRPV1 channel activation, and blocks downstream signal transduction pathways (Kort and Kym, 2012; Nash *et al.*, 2012).

2.1.4.2 Ruthenium red

The chemical formula of ruthenium red (RR) is $(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5]Cl_6$. RR is a synthetic crystalline inorganic hexavalent polycationic dye. It interacts strongly with fatty acids, phospholipids and acidic mucopolysaccharides (Malécot *et al.*, 1998). RR is a

non-selective TRP channels inhibitor, which binds with high potency to the channel pore (pore blocker) (García-Martínez *et al.*, 2000). It has no effect on capsaicin binding, but is thought to clog the channel pore due to the size of RR molecule (Szallasi and Appendino, 2004).

2.1.5 Antimicrobial activities of capsaicin

Constituents of chilli peppers including flavonoids and capsaicinoids have shown biological activities, such as antioxidant, anticancer, anti-inflammatory and antibacterial effects (Popelka *et al.*, 2017; Nascimento *et al.*, 2014). Capsaicin has shown bactericidal effects against food-borne pathogens (*Helicobacter pylori* and *Pseudomonas aeruginosa*) (Omolo, 2014). And at concentration of 50 µg/ml it had anti-virulence activity or growth inhibition against *Vibrio cholera* (Chatterjee *et al*., 2010). In addition, capsaicin in combination with ciprofloxacin showed bacteriostatic action against *Staphylococcus aureus* SA-1199 and SA-1199B. Capsaicin (MIC of 25 mg/l) has reduced the MIC of ciprofloxacin by 2-4 fold (0.12-8 mg/l) through acting as a NorA efflux pump inhibitor (Kalia *et al.*, 2012; Chatterjee *et al.*, 2010).

The mechanism of antimicrobial action of capsaicin was assessed by Marini *et al.* (2015) against *group A Streptococcus spp*. It was demonstrated that capsaicin has bactericidal activity through damaging the cell membrane and inhibiting the biofilm formed by the microorganisms (Marini *et al*., 2015).

2.1.6 Aim and objectives

The aim of the studies in this chapter was to investigate whether the antimicrobial mechanism of action of EO components (carvacrol and cuminaldehyde) against *E. faecium* may be associated with channels similar to TRPV1 channels found in mammalian cells, using capsaicin as a control EO component.

Objectives

- To determine the minimum inhibitory concentration (MIC) of capsaicin against *E. faecium*.
- To assess the antimicrobial effect of AMG517 and RR alone and in combination against *E. faecium*.
- To investigate the effect of RR and AMG517 on the action of capsaicin.
- To establish the antimicrobial effect of capsaicin, the EO components together (carvacrol and cuminaldehyde) against *E. faecium* all in the presence of AMG517.

2.2 Materials and methods

2.2.1 Microorganisms

The test organism was vancomycin-sensitive *Enterococcus faecium* (VSE) clinical isolate (Leicester Royal Infirmary, Leicester, UK) and was stored at – 80˚C on protect beads (Scientific Laboratory Supplies Limited organism, UK). Brain heart infusion (BHI) broth (53286-500G) and BHI agar (70138-500G) were purchased from Oxoid (Sigma Aldrich Ltd, UK). BHI broth used to grow the bacterial isolates at 37°C for 18-24 hours with shaking (100 rpm). Subcultures were carried out on BHI agar by plating 100 µl aliquots of the bacterial suspension and incubating the inoculated plates for 18-24 hours at 37˚C. All media were prepared following the manufacturer's instructions.

Working cultures were prepared by inoculating 10 ml BHI broth with two beads and growing the culture overnight at 37˚C with shaking (100 rpm), serial dilutions of the overnight growth were prepared by pipetting 100 µl aliquot in 9.9 ml BHI broth and were sub-cultured on BHI agar plates. The plates were incubated at 37˚C and were then stored at 4˚C. The working culture was sub-cultured every week for three weeks, thereafter was discarded, and a fresh culture was prepared.

2.2.2 Chemicals

The commercial EOs were purchased from Sigma Aldrich, UK, including cuminaldehyde (135178-5G) and carvacrol (282197-10G) and were stored at room temperature as recommended by the manufacturer. Capsaicin (M2028-50MG) was purchased from Sigma Aldrich, UK, AMG517 (5995-10MG) and RR (1439-100MG) were purchased from

Tocris, UK. Capsaicin and AMG517 were stored at 4˚C, and RR was kept at room temperature following the manufacturer recommendations.

2.2.3 Preparation of suspensions

Two to three colonies of the *E. faecium* VSE were picked up with a sterile wire loop from a working culture and grown overnight in 10 ml BHI broth at 37˚C with shaking (100 rpm). Growth was assessed by determining the optical density (OD) at wavelength of 600 nm (OD_{600}) in 10 ml broth using semi-micro wavelength cuvettes (BR759015-100EA, 1.5 ml) (BRAND, Scientific Laboratory Supplies Ltd, UK) and Spectramax plus 384 Spectrophotometer (Molecular Devices, USA). Aliquots of 100 µl were sampled from the overnight bacterial cultures and were inoculated into 9.9 ml of BHI broth in sterilised Duran bottles or Falcon tubes and vortexed. They were grown for 5-5.5 hours at 37 °C with shaking (100 rpm) to the mid exponential growth phase (OD_{600}= 0.2 ~ 1 x 10^8 CFU/ml). Thereafter to establish control growth or the effect of all treatment conditions, aliquots of 100 µl of the mid exponential phase bacterial suspensions were added to 9.9 ml BHI broth in universal bottles to give an inoculum concentration of 1 x 10^6 CFU/ml. Aliquots of 100 µl DMSO were added to each 9.9 ml of the bacterial suspensions alone, and in addition to all treatment conditions as detailed in the next paragraph, vortexed and incubated at 37°C with shaking (100 rpm) for the experimental durations. Thereafter, the inoculated bacterial suspensions were sub-cultured at the intended time points, where 100 µl aliquot out of each bacterial suspension was used to prepare serial dilutions in PBS as required. The serial dilutions were sub-cultured on BHI agar using spiral plater (Easy Spiral Pro, Interscience, France), incubated overnight at 37˚C, and

finally enumerated. The viable counts of the bacterial cells were assessed using the supplied schedule and evaluated into log using excel sheet.

Treatment conditions included EO components that were added to the bacterial suspensions at the following concentrations: 3.95 mM carvacrol; 134.41 mM cuminaldehyde; combination of 0.02 mM carvacrol and 16.80 mM cuminaldehyde. Treatment solutions were prepared in aliquots of 100 µl DMSO.

2.2.4 Determination of the MIC of capsaicin

In order to define the MIC of capsaicin against *E. faecium* (VSE) using broth dilution method (Horváth *et al.*, 2016; Kon and Rai, 2016), and in accordance with EUCAST guidelines for broth suspension method (Chryssanthou and Cuenca-Estrella, 2006). Serial dilutions of capsaicin were prepared in BHI broth with 1 % DMSO to give final concentrations ranging from 90-600 µg/ml. Aliquots of 1 ml of capsaicin were added to 9 ml of the bacterial suspension ˜ 1 X 10^6 CFU/ml in universal bottles. Samples were then incubated for 18-24 hours at 37˚C with shaking (100 rpm). The OD_{600} of each dilution was measured at zero and after 18-24 hours of incubation. The capsaicin concentration that visibly inhibited the bacterial growth, that is with lowest optical density by measuring absorbance at 600 nm wavelength using Spectramax plus, was considered the MIC. Controls were BHI broth alone, bacterial suspension in BHI broth and capsaicin stock solution.

2.2.5 Checkerboard assay

The antibacterial effects of AMG517 and RR and their synergistic interaction against *E. faecium* (VSE) were tested using the checkerboard method (El-Azizi, 2016), a two-

dimensional array of serial concentrations of AMG517 and RR was used. This assay was conducted following the EUCAST guidelines for broth suspension method of testing (EUCAST, 2021; Schön *et al*., 2021; Schön *et al*., 2020).

Serial dilutions with final concentrations of AMG517 (10-90 nM) and of RR (10-70 µM) were placed in 96 well plates (n= 4) (MIC9032-PK100) (SLS, UK). Aliquots (100 µl) of the bacterial suspension (1 x 10^7 CFU/ml) were added to all wells including the control wells, wells were inoculated with 100 µl of either AMG517, RR or both. Plates were incubated at 37°C and the optical density of all wells of each plate was measured at 600 nm wavelength at time zero and after 18-24 hours of incubation using Spectramax Plus 384 microplate reader and Softamax Pro version 6.4 software (Molecular Devices, USA). Control measurements were established from wells containing either BHI broth, bacterial suspension, AMG517 alone or RR alone.

The investigated concentration ranges of AMG517 and RR were decided in accordance to previous studies that investigated RR or AMG517 at different concentrations; therefore, broader ranges of concentrations of RR: 10-70 µM and of AMG517: 10-90 nM represent most of the studied concentrations were used in the current study (Bai *et al*., 2018; Sasaki *et al*., 2016; Cahusac, 2009; Gavva *et al*., 2007).

2.2.6 Growth curves

Growth curves were conducted with the individual EO components, both in combination (0.02 mM carvacrol and 16.80 mM cuminaldehyde), and capsaicin all in the presence of either 10, 30 or 90 nM of AMG517; bacterial suspension alone was used as a control growth curve. Aliquots of 100 µl of each component plus 100 µl of the bacterial

suspension (˜1 X 10^7 CFU/ml) were placed in the 96-well plate. Plates were sealed with permeable adhesive films (4ti-0516/96) (4 titude, SLS, UK) incubated at 37˚C in the Spectramax Plus 384 microplate reader, set up to measure hourly readings over a 24-hour period at 600 nm wavelength. The background absorbance of the components alone was also measured every hour for 24 hours, and the absorbance differences were considered to plot the growth curves.

2.2.7 Neutralisation method

The following method was adapted from BS EN 13237 (British Standards Institution., 2015), in order to eliminate the antimicrobial effect at the defined time of exposure, enabling accurate determination of the antimicrobial activity of an agent (Cousido *et al*., 2008). The neutraliser was prepared in distilled water, containing 1 g/l tryptone, 8.5 g/l sodium chloride, 30 g/l saponin, 1 g/l histidine, 30 g/l Polysorbate 80, 3 g/l asolectin from soya bean and 5 g/l sodium thiosulphate.

A 100 µl aliquot of 8 Log_{10} CFU/ml suspension of microorganism of interest, which had been incubated overnight, was added to 9.9 ml of BHI broth. A viability control of 100 µl of the test microorganism in 9.9 ml phosphate buffered saline (PBS) (slsBR014G) (SLS, UK) was prepared in tandem. Aliquots of 100 µl of the different combinations of bacterial suspension with either individual EO components (3.95 mM carvacrol, 134.41 mM cuminaldehyde), the EO components together, capsaicin or AMG517 were added to 900 µl neutraliser and vortexed. These samples were then incubated for 5 minutes at room temperature. The solutions were diluted in PBS as required and spread onto BHI

agar plates using Spiral Plater (Easy Spiral Pro, Interscience, France). Plates were enumerated after 18-24 hours of incubation at 37˚C.

2.2.8 Evaluating the toxicity and efficacy of the neutralising method

2.2.8.1 Neutraliser toxicity

Aliquots of 100 µl of three overnight bacterial suspensions (approximately 7 Log_{10} CFU/ml) were added to 900 µl of the neutraliser. After 5 minutes the solution was diluted in PBS as required, and spread onto BHI agar plates using spiral plater. The plates were incubated for 18-24 hours at 37˚C, and enumerated. The neutraliser is considered non-toxic when the mean of CFU/ml was greater than or equal to half times of the control.

2.2.8.2 Neutraliser efficacy

The intended concentrations of the EOs alone and in combination, capsaicin and AMG517 were prepared in BHI broth with 1 % DMSO and inoculated with bacteria. Aliquots of 100 µl of the tested combinations were added to 900 µl of the neutraliser and vortexed to homogenise the mixtures. After 5 minutes of contact, 100 µl aliquots of *E. faecium* suspensions were diluted in PBS as required, and plated onto BHI agar using Spiral plater and incubated for 18-24 hours at 37˚C, and then enumerated. The neutraliser is considered active against each agent when the mean of CFU/ml was greater than or equal to half times of the control.

2.2.9 Viable counts

Bacterial suspension of *E. faecium* at approximately 1 X 10^6 CFU/ml with 30 nM AMG517 and either 1.6 mM capsaicin, 3.95 mM carvacrol, 134.41 mM cuminaldehyde, or 0.02

mM carvacrol with 16.80 mM cuminaldehyde, were prepared in universal bottles. Samples were incubated at 37˚C with shaking (100 rpm), and 100 µl aliquots were taken at zero time point and 12, 13, 14 and 24 hours. The solutions were neutralised in 900 µl neutraliser for 5 minutes, diluted in PBS as required, and spiral plated onto BHI agar plates that were incubated for 18-24 hours at 37˚C, and then enumerated. Control was bacterial suspension alone.

2.2.10 Statistical analysis

Statistical analysis was conducted using SPSS software version 25 (IBM SPSS Statistics, USA) paired sample *t*-test. Significant differences in mean Log_{10} reductions were considered when $P \leq 0.05$. Controls were conducted in all experiments, and each experiment was carried out either twice in duplicate (n=4) or in triplicate on at least two separate occasions (n=6).

2.3 Results

Though EOs and their individual components have shown antimicrobial actions against wide range of bacteria, their mechanisms of action are yet not fully established. The study in this chapter focuses on investigating a similar of the mammalian TRPV1 channels in the bacterial cell that might be exploited by the formulation to display its antimicrobial action towards *E. faecium*, through using a component known to be a TRP channels agonist, capsaicin; and TRPV1 channels antagonists, RR and AMG517, that have been shown to reverse the effect of capsaicin at TRPV1 receptors.

2.3.1 Capsaicin MIC

Using the broth dilution method (section 2.2.4), capsaicin was shown to exert antibacterial activity against VSE with an MIC of 480 µg/ml (1.6 mM). The MIC was determined by the turbidity absorbance and visual observation (Figure 2.2).

The growth of VSE following incubation for 24 hours with a wide range (90-600 µg/ml) of capsaicin concentrations, showed that at 480 µg/ml capsaicin significantly inhibited *E. faecium* growth by approximately 0.91 absorbance unit in comparison to that of *E. faecium* VSE (0.187) without treatment, $P \leq 0.05$ (Figure 2.2).

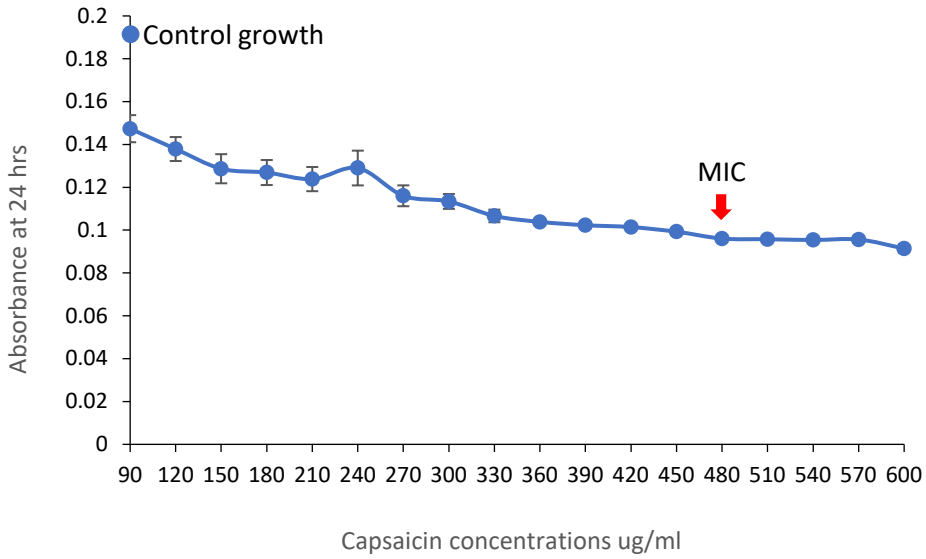


Figure 2.2. The minimum inhibitory concentration of capsaicin against *E. faecium* (VSE). The inhibition of growth from 0.187 Absorbance Unit of control growth is across a range of capsaicin concentrations. The minimum inhibitory concentration of capsaicin is 480 µg/ml that reduced 0.91 AU (n= 3, ±SEM).

2.3.2 Checkerboard test

AMG517 and RR have antagonist action at the TRPV1 channels in mammalians cells where they block the agonist action of capsaicin. Thus, AMG517 and RR were used for further investigations of the potential involvement of TRPV1 channel similar in the mechanism of action of either capsaicin, or EO components (carvacrol and cuminaldehyde) in combination towards *E. faecium* (VSE). AMG517 alone (10-80 nM) had no inhibitory effect on the growth of *E. faecium* (VSE). At the higher concentration of 90 nM there was a significant reduction of the bacterial growth, from 0.34 absorbance unit of average bacterial control growth to 0.24 absorbance unit (Table 2.1). RR showed growth inhibition of *E. faecium* at all tested concentrations (10-70 µM). As shown in Table 2.1, when AMG517 and RR are both present, the inhibitory effect of RR against *E.*

faecium was eliminated in the presence of higher concentrations of the AMG517 (70-90 nM). The reductions of absorbance units from 0.3 to 0.2 were noted at RR concentrations of 60 and 70 µM. The inhibitory effect of RR is reversed at high concentrations of AMG517 (70-90 nM), this may demonstrate that AMG517 might be able to bind to the same receptors that were occupied by RR, and thus the RR cannot use it and has no further inhibitory effect. Overall, the checkerboard assay demonstrated that AMG517 does not inhibit the growth of *E. faecium* and was therefore only carried forward as an antagonist in growth curves to test the theory of the presence in bacteria of channels similar to mammalian TRPV1 channels and of their potential involvement in the mechanism of action of capsaicin and EOs.

Table 2.1. Checkerboard assay of AMG517 and RR against *E. faecium* (VSE), average of 24 hours bacterial growth = 0.34; Green: below average growth; Yellow: large reduction in growth (> 0.1 absorbance unit) (n = 4; ±SEM)

Bacterial growth control	RR µM	AMG517 nM									
		0	10	20	30	40	50	60	70	80	90
0.33±0.05	**0**	0.33±0.02	0.33±0.02	0.32±0.02	0.33±0.02	0.32±0.02	0.37±0.02	0.39±0.01	0.37±0.02	0.30±0.02	0.24±0.01
0.34±0.02	**10**	0.24±0.02	0.21±0.02	0.25±0.02	0.38±0.01	0.16±0.02	0.23±0.02	0.21±0.02	0.33±0.02	0.34±0.01	0.41±0.02
0.33±0.08	**20**	0.29±0.01	0.25±0.02	0.25±0.01	0.23±0.01	0.22±0.03	0.2±0.01	0.14±0.01	0.41±0.02	0.37±0.02	0.4±0.01
0.30±0.05	**30**	0.25±0.01	0.23±0.02	0.27±0.01	0.26±0.03	0.2±0.02	0.2±0.02	0.29±0.06	0.34±0.02	0.39±0.01	0.43±0.02
0.38±0.06	**40**	0.23±0.01	0.24±0.01	0.22±0.01	0.27±0.01	0.27±0.03	0.23±0.03	0.21±0.01	0.32±0.01	0.35±0.03	0.45±0.01
0.34±0.01	**50**	0.24±0.02	0.22±0.02	0.23±0.01	0.32±0.02	0.16±0.03	0.19±0.02	0.2±0.01	0.34±0.03	0.45±0.01	0.45±0.02
0.35±0.06	**60**	0.19±0.02	0.24±0.01	0.27±0.01	0.3±0.03	0.28±0.03	0.28±0.02	0.28±0.04	0.3±0.02	0.47±0.02	0.5±0.04
0.36±0.03	**70**	0.2±0.04	0.19±0.03	0.32±0.02	0.24±0.03	0.26±0.01	0.37±0.02	0.34±0.03	0.31±0.05	0.54±0.01	0.5±0.02

2.3.3 *E. faecium* growth curves in the presence of AMG517

Growth curves were conducted to establish if AMG517, a known TRP mammalian channels antagonist, would reverse some of the antimicrobial effects of EO components, elucidating that bacteria may have similar channels to those found in the mammalian cells. Growth curves of *E. faecium* (VSE) were conducted with the individual EO components (carvacrol, cuminaldehyde), EO components together and capsaicin in the presence of either 10, 30 or 90 nM of AMG517.

2.3.3.1 *E. faecium* growth curves in the presence of AMG517 (10 nM)

AMG517 (10 nM) did not significantly inhibit the growth of *E. faecium* over 24 hours of incubation at 37°C (Figure 2.3). Similar results were established in the checkerboard test (Table 2.1). *E. faecium* growth curves demonstrated that the inhibitory effect of capsaicin, individual EO components (Appendix, Figures 9.1 and 9.2), or the EO components together was not reduced by 10 nM AMG517 over 24 hours of incubation (Figure 2.4).

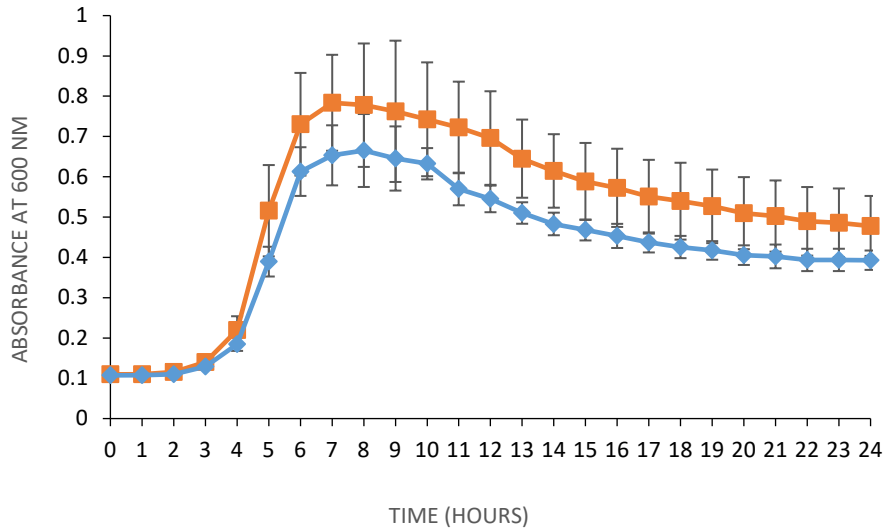


Figure 2.3. *E. faecium* (VSE) growth curve with AMG517 (10 nM). ■ *E. faecium* (VSE) control growth curve. ◆ *E. faecium* (VSE) growth curve with 10 nM AMG517, $P > 0.05$ (n= 4, ±SEM).

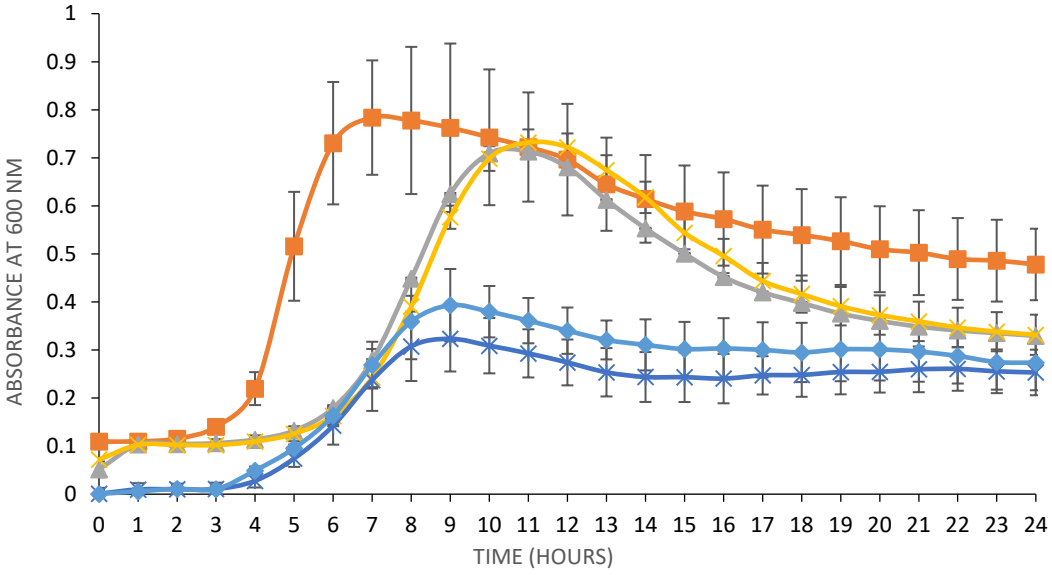


Figure 2.4. *E. faecium* (VSE) growth curves with either capsaicin or EO components together and in the presence of AMG517 (10 nM). ■ *E. faecium* (VSE) control growth curve. ✖ *E. faecium* (VSE) with (1.6 mM) capsaicin. ◆ *E. faecium* (VSE) with 1.6 mM capsaicin and 10 nM AMG517. ▲ *E. faecium* (VSE) with 0.02 mM carvacrol and 16.80 mM cuminaldehyde. ✖ *E. faecium* (VSE) with 0.02 mM carvacrol, 16.80 mM cuminaldehyde and 10 nM AMG517, $P > 0.05$ (n= 4, ±SEM).

Capsaicin alone caused significant inhibition to *E. faecium* growth at the established MIC (1.6 mM). The addition of 10 nM AMG517 did not counter the antimicrobial action of capsaicin; although a minor reduction in growth was observed in capsaicin's inhibitory effect in the presence of AMG517 between 6 and 16 hours. The EO components in combination led to a significant inhibitory effect on the growth of *E. faecium*. The addition of 10 nM AMG517 did not lead to any significant reversal of the inhibitory effect of the EO components in combination as shown in Figure 2.4.

2.3.3.2 *E. faecium* (VSE) growth curves in the presence of AMG517 (30 nM)

The previous experiments were repeated with 30 nM and 90 nM AMG517. AMG517 at concentration of 30 nM did not show significant inhibitory effect towards *E. faecium* (Figure 2.5). *E. faecium* growth curves showed that the presence of 30 nM AMG517 did not reverse the inhibitory effect of either capsaicin, EO components together (Figure 2.6), or individual EO components over 24 hours of incubation (Appendix, Figures 9.3 and 9.4).

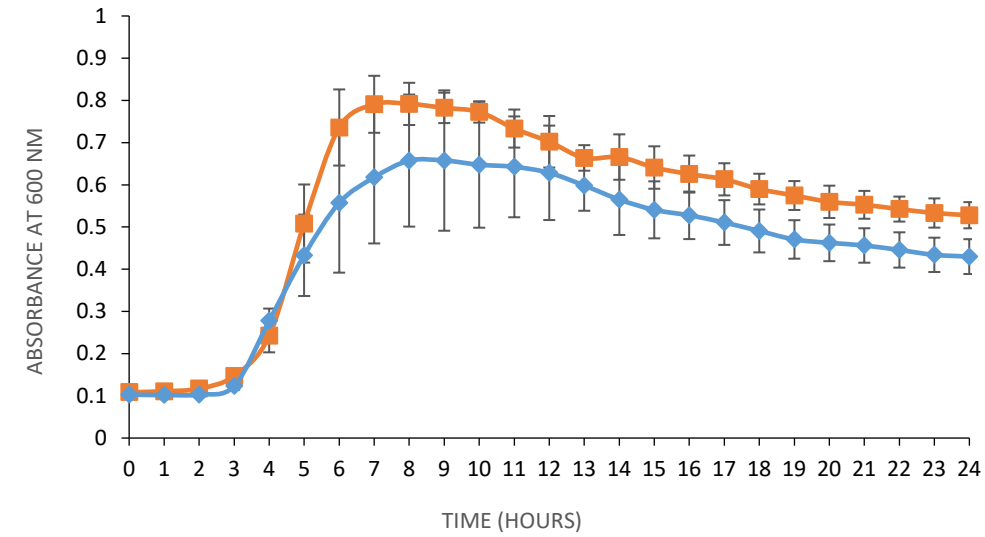


Figure 2.5. *E. faecium* (VSE) growth curve with AMG517 (30 nM). ■ *E. faecium* (VSE) control growth curve. ◆ *E. faecium* (VSE) with (30 nM) AMG517, $P > 0.05$ (n= 4, ±SEM).

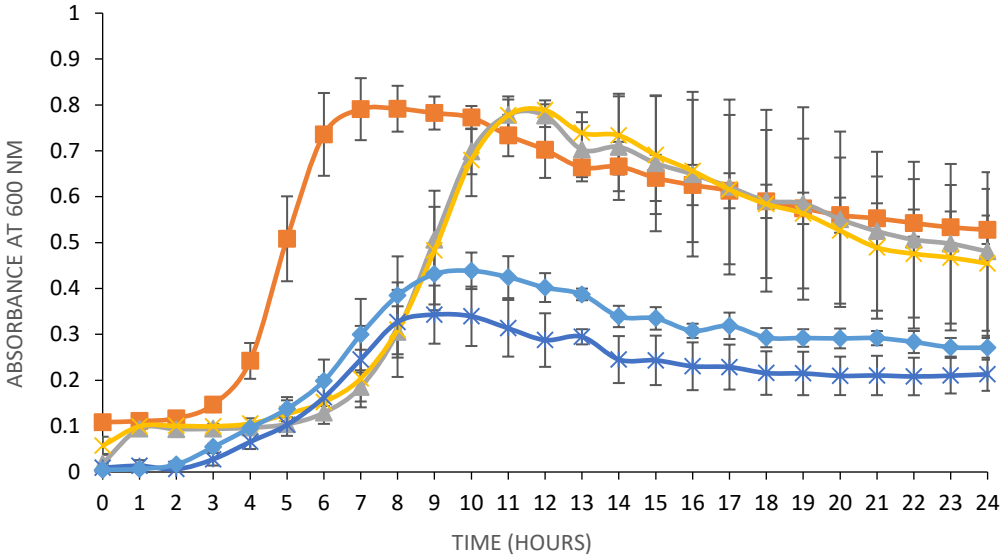


Figure 2.6. *E. faecium* (VSE) growth curves with either capsaicin or EO components together and in the presence of AMG517 (30 nM). ■ *E. faecium* (VSE) control growth curve. ✖ *E. faecium* (VSE) with 1.6 mM capsaicin. ◆ *E. faecium* (VSE) with 1.6 mM capsaicin and 30 nM AMG517. ▲ *E. faecium* (VSE) with 0.02 mM carvacrol and 16.80 mM cuminaldehyde. ✖ *E. faecium* (VSE) with 0.02 mM carvacrol, 16.80 mM cuminaldehyde and 30 nM AMG517, $P > 0.05$ (n= 4, ±SEM).

Capsaicin reduced the growth of *E. faecium* over the 24 hours of incubation; however, with the addition of 30 nM AMG517 there is some evidence of a small, but non-

significant, reduction in the inhibitory effect of capsaicin mainly between 12 and 14 hours. EO components together induced inhibition of the growth of *E. faecium*, the addition of 30 nM AMG517 did not cause significant reverse of the effect of the EO components together at any time point as shown in Figure 2.6. These definite time points were further investigated with viable count test.

2.3.3.3 *E. faecium* growth curves in the presence of AMG517 (90 nM)

The growth curves of *E. faecium* in the presence of 90 nM AMG517 showed that the TRPV1 antagonist significantly inhibited the bacterial growth (Figure 2.7), and no significant reduction in the inhibitory effect of either capsaicin (Figure 2.8) or carvacrol (Appendix, Figure 9.5) towards *E. faecium* when with 90 nM AMG517. The addition of AMG517 (90 nM) led to a slight increase in the inhibitory effect of cuminaldehyde toward the end of the incubation period (Appendix, Figure 9.6); in contrast to the data obtained with 10 nM and 30 nM AMG517 which demonstrated no inhibitory effect from cuminaldehyde (Appendix, Figures 9.2 and 9.4). The combination of EO components demonstrated inhibitory action; however, the addition of 90 nM AMG517 significantly increased this inhibitory effect, showing that AMG517 at 90 nM instead of reversing the inhibitory action of the combination of EO components led to an increased inhibition of *E. faecium* growth (Figure 2.8). Interestingly, when assessed alongside capsaicin, 90 nM AMG517 was not bactericidal. It did, however, lead to an increase in the reduction action of capsaicin. Nevertheless, AMG517 (90 nM) was unable to reverse the inhibitory effect of capsaicin on *E. faecium* (Figure 2.8).

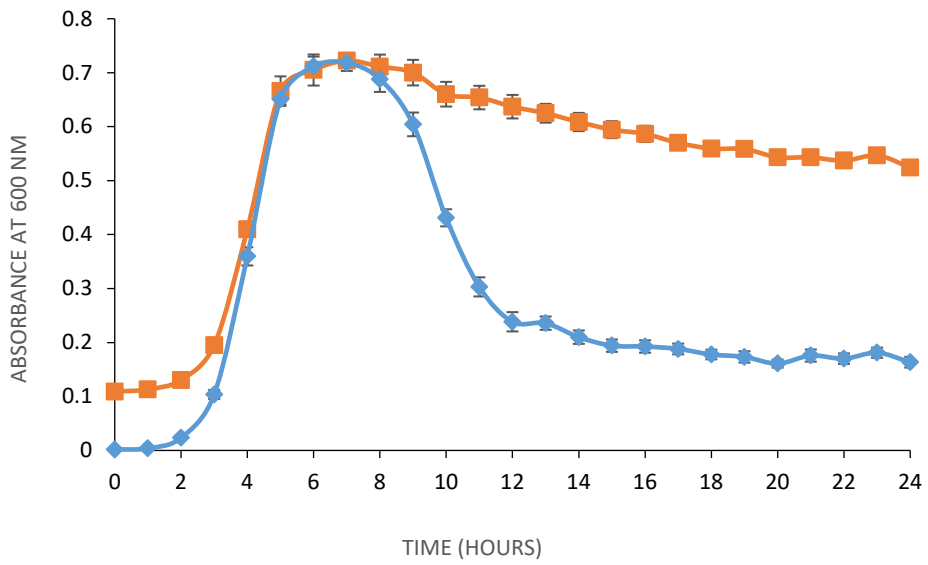


Figure 2.7. *E. faecium* (VSE) growth curve with AMG517 (90 nM). ■ *E. faecium* (VSE) control growth curve. ◆ *E. faecium* (VSE) growth curve with 90 nM AMG517 (n= 4, ±SEM).

AMG517 at 90 nM significantly reduced bacterial growth, with the inhibitory effect of AMG517 being observed immediately after the bacteria reached the exponential phase and continue for the duration of the incubation, 24 hours (Figure 2.7).

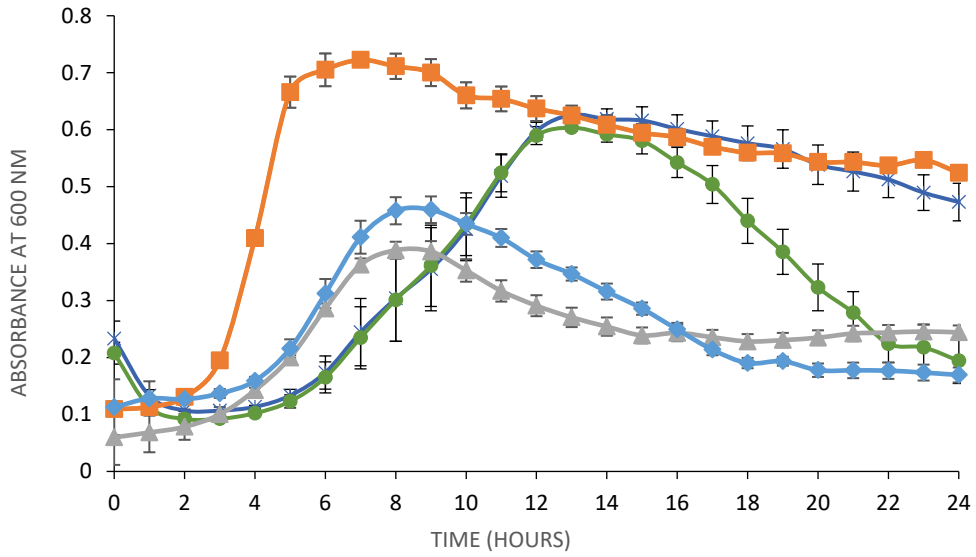


Figure 2.8. *E. faecium* (VSE) growth curves with either capsaicin or EO components together and in the presence of AMG517 (90 nM). ■ *E. faecium* (VSE) control growth curve. ▲ *E. faecium* (VSE) with 1.6 mM capsaicin. ◆ *E. faecium* (VSE) with 1.6 mM capsaicin and 90 nM AMG517. ✖ *E. faecium* (VSE) with 0.02 mM carvacrol and 16.80 mM cuminaldehyde. ● *E. faecium* (VSE) with 0.02 mM carvacrol, 16.80 mM cuminaldehyde and 90 nM AMG517, $P > 0.05$ (n= 4, ±SEM).

Capsaicin significantly inhibited the bacterial growth over the incubation period, adding AMG517 at concentration of 90 nM did not display significant alteration to the inhibitory effect of capsaicin. A slight reduction, yet non-significant effect of capsaicin was noted between 6 and 16 hours (Figure 2.8).

In order to fully assess the effect of 90 nM AMG517, it was further investigated by conducting viable counts at zero and 24 hours. The viable count results with 90 nM AMG517 showed that this concentration of AMG517 was toxic when added alongside with all EOs except capsaicin as bacterial growth was eliminated.

The results of the viable counts (data not shown) conducted in the presence of 90 nM AMG517 were not promising, as they did not elaborate the hypothesis of the present

work neither positively nor negatively, due to that AMG517 (90 nM) did not reverse the antimicrobial action of the EO components investigated instead it displayed antibacterial effect and increased the antibacterial action of the EO components investigated here; therefore, the results of these investigations were not shown.

Overall, the addition of 10 nM or 30 nM AMG517 did not reverse the action of either capsaicin or the EO components at any time point. What is known about AMG517 is that it is an antagonist at TRPV1 receptors in mammalian cells. Thus, it is thought to be an antagonist that reverses the action of capsaicin in bacteria, when that the antibacterial action of capsaicin involves channels in the bacteria similar to the mammalian TRPV1 channels.

2.3.4 Evaluating the toxicity and efficacy of the neutralising method

The neutraliser was found to be non-toxic as the mean of CFU/ml of the survived enumerated colonies with each treatment condition after being neutralised for 5 minutes at room temperature was greater than or equal to half times of the control. The neutraliser was also effective at quenching/terminating the activity of all treatment conditions used (Appendix, Table 9.1).

2.3.5 *E. faecium* viable counts with AMG517 (30 nM)

The viable count experiments were carried out at zero, 12, 13, 14 and 24 hours of incubation. The growth curves with AMG517 (30 nM) showed that there was some evidence of differences in growth, albeit not significant ($P > 0.05$), around 12-14 hours (Figure 2.6). Therefore, further viable count experiments were conducted to elucidate if 30 nM AMG517 has affected the action of any of the treatment conditions.

Table 2.2. Viable counts of *E. faecium* (VSE) in the presence of AMG517 (30 nM) at 0, 12, 13, 14 and 24 hours (n= 4, ±SEM)

Condition	Log_{10} CFU/ml				
	0 hr	12 hrs	13 hrs	14 hrs	24 hrs
E. faecium	4.27±0.08	5.80±0.1	5.82±0.09	5.76±0.07	5.63±0.13
E. faecium+ AMG517	4.14±0.08	5.91±0.11	5.65±0.06	5.88±0.11	5.49±0.08
E. faecium+ Capsaicin	4.26±0.05	3.64±0.07	3.48±0.08	3.23±0.08	2.12±0.09
E. faecium+ Capsaicin+ AMG517	4.18±0.02	3.43±0.15	3.22±0.07	2.98±0.15	1.99±0.22
E. faecium+ Carvacrol	4.02±0.06	3.20±0.09	2.90±0.10	2.82±0.08	2.65±0.15
E. faecium+Carvacrol+AMG517	3.99±0.02	3.25±0.09	3.20±0.12	3.08±0.08	2.72±0.13
E. faecium+Cuminaldehyde	3.97±0.08	3.34±0.13	3.12±0.07	2.87±0.02	2.70±0.08
E. facium+Cuminaldehyde+AMG517	3.89±0.04	3.69±.07	3.41±0.23	2.98±0.02	2.90±0.10
E. faecium +Carvacrol+Cuminaldehyde	3.93±0.20	3.73±0.10	3.40±0.09	3.38±0.02	2.980±0.13
E. faecium +Carvacrol+Cuminaldehyde+AMG517	4.03±0.20	3.83±0.09	3.64±0.16	3.59±0.17	2.80±0.09

The results of the conducted viable counts (Table 2.2) showed that there was no significant difference between the effect of capsaicin and its effect in the presence of AMG517 (30 nM) on the bacterial viability. It could be that there are related channels but these are not antagonised by AMG517, due to that the inhibitory effect of either carvacrol, cuminaldehyde, or both in combination when in the presence of 30 nM AMG517 there was an increase in the bacterial growth over the studied time points, but these were not significant (Table 2.2).

2.3.6 TRPV1 channels bioinformatics

To investigate whether there are any homologues of the TRPV1 channels from *Homo sapiens* in bacteria, some bioinformatics analysis was completed. The protein sequence of the channel (NCBI ref sequence NP_542437.2) was used in a BLASTP search. The protein sequence was analysed against all reference sequences in the bacterial species taxonomy identification (taxid). The initial search using the default parameters yielded

no significant results (Data not shown), and when the parameters were altered to using the PAM250 matrix which is less stringent to yield matches with less significance, there were still no significant matches (Data not shown). A specific search against *E. faecium* also resulted in no significant matches (Data not shown). Thus, there are no channels in bacteria with significant similarity to the TRPV1 channels found in *Homo sapiens*.

2.4 Discussion

Due to an increase in antibiotic resistance, novel antimicrobial compounds with mechanisms of action that are effective against antimicrobial resistant microorganisms are of particular interest (Simpkin *et al.*, 2017; Wikaningtyas and Sukandar, 2016). Natural products are sources of a wide group of different components with a variety of effects and different mechanisms of antimicrobial actions against bacteria. Efforts are being made to establish the potential role of plant extracts and their active components in combating antibiotic resistance in bacteria and to restore the efficacy of different antibiotics towards AMR microorganisms (Boire *et al*., 2013; Yap *et al.*, 2014; Yang *et al*., 2015). In the current study, the aim was to investigate if the antimicrobial mechanism of action of capsaicin towards *E. faecium* VSE is mediated by channels that might be similar to the TRPV1 mammalian channels. Which in turn could be potential to elaborate the involvement of the very similar channels in the mechanism of action of the EO components (carvacrol and cuminaldehyde).

The current study hypothesised that the mechanism of action of the combination (carvacrol and cuminaldehyde) exploits channels in the bacterial cell similar to TRPV1 mammalian channels. The antibacterial action of capsaicin was assessed, this EO component has antimicrobial properties and is an agonist of TRPV1 mammalian channels. Capsaicin was found to have an MIC of 1.6 mM against *E. faecium* (VSE) (Figure 2.2). Similarly, Popelka *et al.* (2017) reported antimicrobial action of capsaicin against *S. aureus* (CCM4223), *Sallmonella enterica* (CCM4420) and *Esherichia coli* (ATCC11338). Omolo (2014) also established that capsaicin was antimicrobial against *B. subtilis* with

an MIC of 25 µg/ml, while at concentrations of 200-300 µg/ml it inhibited the growth of *E. coli* (Molina *et al.*, 1999). Likewise, Marini *et al.* (2015) showed that capsaicin had MICs of 64 and 128 µg/ml against erythromycin-susceptible and erythromycin-resistant *Streptococcus pyogenes* (Group A Streptococci), respectively (Marini *et al*., 2015).

AMG517 and RR are TRP channel blockers that antagonist the agonist action of capsaicin. Therefore, the effects of AMG517 and RR against *E. faecium* were established (Table 2.1). The Checkerboard investigation (Table 2.1) showed that RR alone had inhibitory actions at concentrations above 10 µM. AMG517 had no additional inhibitory effect on *E. faecium* when in combination with RR. At low concentrations of AMG517 (10-30 nM) with RR (10-50 µM), they differentially reduced the growth of *E. faecium*. They were may be acting through the same pathways or competing for binding sites present in *E. faecium*, which may have been blocked preferentially by RR, a large trinuclear polyamine molecule. Although RR may have shown a higher affinity to this pathway; however, its action on bacteria is destructive at higher concentrations. In addition, it could be that RR might not be acting on many pathways because once AMG517 blocks the available pathways it becomes non-inhibitory (Table 2.1). The effects of AMG517 have been established by Gavva *et al.* (2007), where it was found that TRPV1 channels were not activated by AMG517 at concentrations up to 40 µM in cells expressing TRPV1; and thereby AMG517 was not a partial agonist. While with half-maximal inhibitory concentrations (IC_{50}) 1 to 2 nM AMG517 completely blocked the stimulating effect of capsaicin on TRPV1 channels (in rats) (Gavva *et al*., 2007).

The EO components together displayed variable degree of antimicrobial activity against the tested strain of *E. faecium* (VSE) in the presence of AMG517 (10 and 30 nM) (Figures 2.4 and 2.6). At concentrations of 10 and 30 nM AMG517 there was no significant difference in reversing the antibacterial effect of either capsaicin or EO components (Figures 2.4 and 2.6).

Viable count investigations of the effect of 30 nM AMG517 in reversing the inhibitory effect of capsaicin, showed that AMG517 (30 nM) did not significantly antagonist the activity of capsaicin on *E. faecium* (VSE) (Table 2.2); however, similar studies were not found in the literature before.

Assessing the antibacterial actions of the EO components in the presence of AMG517 at any concentration showed that AMG517 did not reverse the inhibitory effect of capsaicin or the EO components individually or together on *E. faecium*. These findings are not in support of the hypothesis that channels similar to TRPV1 channels found in the mammalian cells are present in the bacterial cells. In contrast, it might be that AMG517 affected different targets in the bacterial cell or changed the bacterial cell composition making it more sensitive to the antibacterial activity of EOs at higher concentration (90 nM) (Figure 2.8) (Appendix, Figures 9.5 and 9.6).

In conclusion, the conducted growth curves and the viable counts showed that the presence of AMG517 at concentrations of 10, 30 and 90 nM did not reverse the inhibitory effect of capsaicin on *E. faecium*; even though, it does in mammalian cells but not in bacteria. Thus so, the antimicrobial action of capsaicin towards *E. faecium* does not seem to involve channels similar to the mammalian TRPV1 channels; albeit, it was

expected to act through very similar channels that AMG517 is an antagonist for. Similarly, the antimicrobial mechanism of action of the EO components (carvacrol and cuminaldehyde) also did not appear to involve channels similar to TRPV1 channels, which is less expected. This conclusion is supported by the bioinformatic analysis, which showed that there are no channels in bacteria with significant similarity to that of TRPV1 channels.

2.5 Conclusion

A homologue of TRPV1 channels was not determined to be involved in the mechanism of action of the EO components (carvacrol and cuminaldehyde) on *E. faecium* in this study, the results were not promising and were not in line with the theory of the research. Therefore, a novel thought of the mechanism of action of the novel formulation was established relying on the microarray data. Microarray data has shown that genes related to channels; *bcr*, *ecfA-1*, *ecsA_1*, *nhaC_2* and *yloB*, were differentially regulated when exposed to an established formulation of combining EOs (carvacrol, cuminaldehyde) with vancomycin (Owen *et al*., 2020). These genes are involved in transport mechanisms, suggesting that there are channels may be involved in the mechanism of action of the formulation. Therefore, further investigations are required to establish if the mechanism of action of the formulation against *E. faecium spp.* are mediated by transport channels.

Chapter 3

3 Expression analysis of genes encoding transport channels in vancomycin-resistant *Enterococcus faecium* (VRE)

3.1 Introduction

Whole transcriptome analysis, using DNA microarrays was completed previously to establish the effect of the combination of EOs-vancomycin (carvacrol, cuminaldehyde and vancomycin), as well as their individual components, on *E. faecium* (VRE) for 60 minutes (Owen *et al.*, 2020). This formula has previously been established to be effective towards *E. faecium* (VRE) (Owen and Laird, 2019). The microarray data provides the basis for the present study as looking in more details at the changes in the gene expression levels can help to determine the mechanism of action of the formulation as a novel antimicrobial.

The microarray data identified numerous genes that were differently regulated when exposed to cuminaldehyde, carvacrol, vancomycin alone and the EO-vancomycin combination (Owen *et al.*, 2020). In response to carvacrol a total of 122 genes showed altered expressed with 13 upregulated and 109 downregulated (≥ 2-fold; $P \leq 0.05$). In response to cuminaldehyde 100 genes showed altered expression with 23 up-regulated and 76 down-regulated (≥ 2-fold; $P \leq 0.05$). Of these, sixty genes showed altered regulation in both conditions, carvacrol and cuminaldehyde. There were no-significant changes in gene expression in VRE when exposed to vancomycin alone. The application of the novel EOs-vancomycin combination produced change in the gene expression level of 15 genes (≥ 2-fold; $P \leq 0.05$) (Owen *et al.*, 2020). Genes involved in transportation in the bacterial cell were differentially expressed when exposed to the formulation, five

genes involved in transportation though not all were significantly altered, *bcr* at 1.16-fold, *ecfA1* at 2.17-fold, *ecsA_1* at -1.23-fold, *nhaC_2* at -1.42-fold and *yloB* at -1.24-fold, were chosen for further analysis here due to their importance in transportation in the bacterial cell.

3.1.1 Regulation in response to EOs and EO components

Bacteria respond to environmental stimuli (such as increased acidity or antimicrobials), and adapt to different conditions through switching on and off the expression of defined genes, i.e altering the gene expression levels; this leads to an increase or reduction in the expression levels of these genes, resulting in the production of more or less of certain proteins (De Bruijn, 2016). Bacteria can also adapt to stimuli by changing their morphology, producing biofilms, yielding spores or by moving away from the stress triggers via chemotaxis (De Bruijn, 2016).

Transcriptional regulation is the process that controls the expression of defined genes (induce or repress), under a particular condition and at a defined time point. It is essential for different biological processes within the cell, including cell development and differentiation, and the response and the adaptation to surrounding stresses (Ghedira, 2018).

The effects of EOs alone and in combination with conventional antibiotics on the expression level of bacterial genes have been established in Gram-positive and Gram-negative bacteria (van der Laan *et al.*, 2003).

A study using Gram-positive bacteria showed that the effect of *Salvia fruticosa* oil towards *Streptococcus epidermidis* involved the 37-fold decrease in *icaA* gene

expression, where *icaA* affects the ability of *S. epidermidis* to form the adherent slime that is associated with catheter infections; therefore, the ability of *S. epidermidis* to colonize and form biofilms was reduced (Chovanová *et al*., 2015). Moreover, *Salvia fruticosa* EO when combined with tetracycline against *S. epidermidis* caused a repression of tetracycline-resistant *tetK* gene by 2.2-fold, which in turn inhibited the formation of an efflux pump, potentially increasing its susceptibility to antibiotics, such as tetracycline, and improving its clinical efficacy. The growth curves showed that combining tetracycline with *S. fruticosa* resulted in a 3 Log_{10} fold reduction in the bacterial count after 360 minutes in comparison to tetracycline alone (Chovanová *et al*., 2015) (Table 3.1).

A global proteomic analysis evaluated the effect of vanillin towards *E. coli* has found that in response to vanillin 147 proteins were significantly altered, this provides the first cellular response to this aldehyde in details. Importantly, the study showed that efflux systems are potentially involved in the antimicrobial action of vanillin. Where in response to vanillin, AcrD encodes RND efflux pump was upregulated, and AaeAB which is aromatic acid efflux system was upregulated. In addition to up-regulation of *copA* gene that allowed copper ions to inhibit *E. coli* growth in the presence of vanillin (Pattrick *et al*., 2019).

Another study conducting qPCR has shown that the combination of *Salvia sclarea* EO and oxacillin has a synergistic effect against methicillin-resistant *S. epidermidis* (MRSE) in inhibiting the expression of the *mecA* gene significantly (Lambert, 2005). Applying the EO alone has expressed inhibition effect on the resistant genes, such as *mec*A*, mec* $\mathcal{I}$,

*mecR*1, *bla*I, *blaR*1. And when adding EO to oxacillin the induction of expression of *mecA* was reduced in comparison to untreated control (data not shown by the author). Where *mecA* is responsible for resistance to *B*- lactam antibiotics, and its expression is adjusted through either its cognate regulators Mec I (DNA binding repressor protein) and MecR1 (sensor/signal transducer), or respectively by Bla I and BlaR1 (similar *B*-lactamase regulators structurally and functionally) (Chovanová *et al.*, 2016) (Table 3.1).

A methicillin-resistant *S. aureus* (MRSA) strain (USA300) was exposed to the effect of lemongrass oil (citral), tea tree oil and willow bark extract (Ko *et al.*, 2016). The study revealed that nine transcripts were at least two-fold down regulated when treated with citral in comparison to non-treated cells including; *efb*, *agrC* and *tetR*, which are related to bacterial virulence through regulating virulence factors (Ko *et al.*, 2016). In response to citral treatment, there were eight up-regulated genes including; the expression of the gene that encodes VraX protein that was up-regulated by 2.3-fold change. This could be explained that due to the lipophilic nature of EOs, the cells in response to EO exposure have induced a stress response to combat cell membrane damage by over-expression of the *vraX* gene (Christensen and Anderson, 2017) (Table 3.1).

The growth of *Listeria monocytogenes* upon exposure to EO of *Baccharis psiadiodes* has been established by RNA sequencing and qPCR assays. The study showed that there are 333 genes were down regulated; and 273 genes were up regulated (Pieta *et al.*, 2017). Out of the down regulated genes were the virulence genes, such as *fur*, *hly* that is essential during the infection process for bacterial survival in the phagocytes, *actA*, and

agrA. While up-regulation has been noticed with the stress response genes including; *deg*U, *crp*, *fri*, *isc*R and *sod* (Pieta *et al.*, 2017) (Table 3.1).

Escherichia coli O157:H7 in response to cinnamon EO, which contains 60 % of trans-cinnamaldehyde, showed a reduction in *stx*2 gene expression, and an increase in the expression of oxidative stress response genes including; *oxyR*, *soxR* and *rpoS*. There was also a significant inhibition in the expression of *luxS* gene (Frazzon *et al.*, 2018) (Table 3.1).

Similarly, the antimicrobial action of cinnamon oil, cinnamaldehyde, was established against *E. coli*, *P. aeruginosa*, *E. faecalis*, *S. aureus*, *Salmonella spp.* and *Vibrio parahaemolyticus* (Nabavi *et al.*, 2015). Previously, the mods of antimicrobial actions of cinnamaldehyde were determined as; repression of active transport, loss of metabolites, inhibition of the proton motive force, and disturbing the synthesis of DNA, RNA, lipids, proteins and polysaccharides (Denyer, 1995). However, subjecting bacteria to cinnamaldehyde has caused down-regulation of virulence genes to different degrees, *S. aureus sed* gene by 6.6-fold, *E. coli stx1* gene by 5.2-fold, *Avibacterium paragallinarum hpg-2* gene by 2.7-fold, *Pasteurella multocida ptfA* gene by 2.4-fold, *Mycoplasma gallisepticum mgc2* gene by 1.2-fold, and *Ornithobacterium rhingtracheale adk* gene by 1.17-fold (Erfan and Marouf, 2019) (Table 3.1).

Out of eight different commercial EOs, a study reported that seven showed antimicrobial actions towards all tested strains of MRSA. The estimated EOs in the study were β-pinene, carvacrol, dimethyl trisulfide, linalool, limonene, menthol, monoterpene hydrocarbons and thymol. The effects of EO blend were estimated on virulence related

genes (*ica-9* and *ica-15*), the main findings of the study showed that the expression level of *ica-9* was down-regulated by 17.83-fold and *ica-15* was also down-regulated by 4.94-fold when exposed to EO blend (carvone 67.14 %, dimethyl trisulfide 18.87 % and thymol 14.00 %) (Gharaibeh *et al.*, 2020) (Table 3.1).

Table 3.1. The importance of the above-mentioned genes

Gene	The gene function	EO/stress agent	Regulation	Fold change	Reference
icaA	affects the ability of *S. epidermidis* to form the adherent slime	*Salvia fruticosa*	Downregulated	-37	(Chovanová *et al*., 2015)
tetK	responsible for tetracycline-resistant	*Salvia fruticosa*	Downregulated	-2.2	(Chovanová et al., 2015)
mecA	facilitates bacterial antibiotic resistance in particular to methicillin, penicillin, and other penicillin like antibiotics	*Salvia sclarea*	Downregulated	Data not shown	(Lambert, 2005) (Chovanová *et al.*, 2016)
mec I	encodes DNA binding repressor protein				
bla and *blaR*1	encode *B*-lactamse regulators structurally and functionally				
Efb	encodes virulence factor that protects bacteria by transferring the fibrinogen to the bacterial surface and forming a shield	Lemongrass oil (citral)	Downregulated	≥2	(Ko *et al.*, 2016)
agrC	an accessory gene regulator protein C				
tetR	encodes transcriptional regulator				
vraX	vancomycin-resistance associated gene	Citral	Upregulated	2.3	(Christensen and Anderson, 2017)
Fur	encodes a regulator of ferric iron uptake, involved in bacterial virulence and survival in the host	Baccharis psiadiodes oil	Downregulated	Data not shown	(Pieta *et al.*, 2017)
Hly	regulates the listeriolysin O				
actA	responsible for polymerisation of action				

	tails that move the microorganism toward neighbour cells				
*deg*U	encodes a regulator of the expression of flagellar and chemotaxis genes, major regulator of lysozyme resistance				
Crp	encodes the cyclic AMP receptor protein, controls the metabolism of amino acids and sugar, transport actions, and protein folding				
Fri	encodes an iron-binding ferritin-like protein				
*isc*R	a regulator for governing physiological processes during growth and stress responses				
Sod	encodes Sod, superoxide dismutase, that is involved in protection against superoxides and reactive oxygen species				
acpP	involved in fatty acid biosynthesis/metabolism	Lemongrass EO	Upregulated	Data not shown	(Hadjilouka *et al.*, 2017)
plcA	encodes phosphatidylinositol phospholipase C		Downregulated		
plcB	encodes phosphatidylcholine phospholipase C				
inlB, *inlC*, and *lmo*2470	encode the internalins proteins that are included in the interaction process between the host and the pathogen				
Hly	encodes listeriolysin O which is a cholesterol-binding, pore-forming toxin crucial for the bacterial escape from phagosomes				
*stx*2	responsible for Shiga toxin Stx production that is an essential virulence factor responsible for the pathogenicity	Cinnamon EO (trans-cinnamaldehyde)	Downregulated	Data not shown	(Lee *et al.*, 2016) (Frazzon *et al.*, 2018)
oxyR	encodes OxyR that is a positive regulator of catalases which remove toxic byproducts of		Upregulated		(Wang *et al.*, 2009) (Frazzon *et al.*, 2018)

	metabolism and reduce oxidative stress				
soxR	which mediate the survival of *E. coli* under the effects of superoxide radicals				
rpoS	encodes RpoS protein that plays key role in cell survival while in the stationary phase through conferring cell to be resistant to different stressors via biofilm forming				(Sheldon *et al.*, 2012) (Frazzon *et al.*, 2018)
luxS	produces LuxS protein a virulence factor through mediating quorum sensing mechanism in the biofilm		Downregulated		(Han *et al.*, 2013) (Frazzon *et al.*, 2018)
katA	encodes catalase	Clove EO	Upregulated	51	(Kovács *et al.*, 2016)
groEL	encodes molecular chaperone			20	
groES	encodes a cochaperonin			4	
dnaK	encodes another chaperon			4	
flhB	encodes flagella synthesis protein FlhB		Downregulated	7	
gale	involved in the lipooligosaccharide biosynthesis			7	
porA	a major outer membrane protein gene with strong antigenic capacity			3	
ptfA	Fimbrial adhesion	Cinnamaldehyde oil	Downregulated	2.4	(Erfan and Marouf, 2019)
Sed	Enterotoxin			6.6	
stx1	Enterotoxin			5.2	
mgc2	Cytadhesin			1.2	
hpg-2	Hemagglutinin			2.7	
Adk	Adenylate kinase			1.17	
ica-9	Virulence gene	EO blend	Downregulated	17.83	(Gharaibeh *et al.*, 2020)
ica-15				4.94	

Several studies have shown significant effects of EOs and their main components on the transcriptional levels of genes involved in the pathogenicity of bacteria; including virulence and antibiotic resistance as elucidated above. Therefore, the mechanisms of action of these EO components alone and in combinations need to be fully established

for them to be considered in creating novel antimicrobial formulations, and for that the responsible genes could be exploited as targets by these novel antimicrobial formulations in order to overcome the global dilemma of antimicrobial resistance.

3.1.2 Transport channels

The study in this chapter focuses on five genes from the microarray data due to their altered expression levels upon exposure to the formulation, and to their importance in encoding transport channels. Previously in Chapter 2, transport channels were potentially hypothesised to be involved in the antimicrobial mechanism of action of the formulation.

Bacteria increase or decrease the expression of genes that encode channels, in order to induce or inhibit the transportation of different substances through these channels; thus bacteria can resist the effects of antimicrobials and toxic compounds either through preventing them from entering the bacterial cells or efflux them to the surrounding area (De Bruijn, 2016).

Solute transporters in living cells can be categorised into two main groups; passive and active transporters. In the case of passive transporters, solutes transport across the membrane does not require biological energy and solutes are driven by the solute gradient from a high solute concentration toward a low concentration. Whereas, active transport uses biological energy to transport solutes that accumulate on one side of the membrane across the membrane. The active solute transporters are divided into two groups primary and secondary transporters that differ in the mean of energy source (Kumar *et al*., 2020). The primary active transporters (ATP binding cassette, ABC) use the

energy of adenosine triphosphate (ATP) hydrolysis to mediate solutes transportation across the membrane from low solute concentration to a high solute concentration. The secondary solute transporters, such as major facilitator superfamily (MFS), use the energy contained within ion gradients to move the solutes against solute gradient by moving the ions down their concentration gradients across the membrane (Kumar *et al.*, 2020).

3.1.2.1 Major facilitator superfamily (MFS)

The major facilitator superfamily is the largest family of secondary active solute transporters in bacteria, including uniporters, symporters and antiporters (Madej, 2014; Yan, 2013). The MFS constitutes of many families and sub-families distributed in all species from bacteria to humans. Thousands of solute transporters form the MFS and share many similarities including their protein sequences, type of energisation (secondary active), two-dimensional and three-dimensional structures, and in their mechanisms of transporting ions and solutes through the membrane (Kumar *et al.*, 2020).

The proteins of the MFS mediate the movements of wide variety of small molecules, including oligosaccharides, ions, lipids, peptides, amino acids, simple sugars, nucleotides and antibiotics in both sides across the membrane (Reddy *et al.*, 2012). MFS uses the ionic (H^+) gradient, protein motive force, as energy source to perform the transportation action (Saier *et al.*, 2016).

The members of MFS also mediate drug and multidrug resistance, and represent good targets for understanding the modulation and the efficacy restoration of the clinically

important agents, both at the level of gene expression and of inhibition of the drug transportation across the bacterial membrane (Kumar *et al.*, 2013).

The *bcr* gene in *E. faecium* NCTC12202 encodes a major facilitator superfamily transporter. Limited published data about *bcr* gene is available in the literature, but it is known that *bcr* genes are responsible for antimicrobial resistance and they are located on a plasmid that is transferred at high rate within *Enterococcus spp*. *bcrD* gene was first identified in *E. faecalis* (Charlebois *et al.*, 2012) and encodes an undecaprenol kinase that is involved in producing exopolysaccharide molecules. Therefore, bacitracin resistance was found to be associated with different mutations that inhibit the synthesis of exopolysaccharides (Manson *et al.*, 2004).

A study in New Zealand sequenced the genomes of 231 strains of VRE (75 human clinical strains and 156 poultry strains) for better understanding of the human and animal associated VRE strains. The study reported that multiple antimicrobial resistance plasmids are carried by an abundant VRE clone, such as both of tetracycline resistance determinants and the bacitracin resistance *bcr* operon are carried on plasmid pAR01.1. The products of a *bcr* operon resulted in bacitracin resistance, including a response regulator (*bcrR*), *bcrA* and *bcrB encoded transporters*, and putative undecaprenyl diphosphatase (*bcrD*) (Rushton-Green *et al.*, 2019).

3.1.2.2 ATP-binding cassette superfamily (ABC)

ABC transporter superfamily is one of the largest superfamilies in prokaryotes and are associated with importing nutrients and metals and exporting antibiotics and toxins demonstrating fundamental rule in cell viability, virulence and pathogenicity (El-Awady

et al., 2017; Jamshidi *et al.*, 2016). This superfamily of transporters uses the free energy from ATP hydrolysis to transfer molecules across the cell membrane. ABC transporters are categorised as primary transporters, and they transfer the molecules against the concentration gradients through either importing or exporting them, such as amino acids, sugars, drugs and proteins (Wilkens, 2015).

The ABC systems are not only involved in transmembrane transport, but they also couple the energy of ATP hydrolysis to a variety of non-transport-related biological functions, such as translational regulation and DNA elongation and repair. ABC systems are categorised into three groups; importers that mediate the uptake of nutrients, exporters that are responsible for the secretion of different molecules and the last group is involved in translating mRNA and repairing DNA (Davidson *et al.*, 2008).

The *ecsA_1* gene encodes ATP-binding protein, ABC transporter; it is presumed that for bacteria to survive in different environment circumstances, it requires numerous ABC systems to adapt to the surrounded conditions (Garmory and Titball, 2004). Where environment adaptation could result in increased or decreased expression level of *escA_1,* the transcriptomics are exploited by bacteria as either importer or exporter in order to resist the effects of the unfavoured surrounded circumstances (Garmory and Titball, 2004); however, there is limited published research about *ecsA_1*.

The *ecfA-1* gene encodes energy-coupling factor transporter. Energy-coupling factor (ECF) transporters are ABC transporters. ECF transporters provide the required energy to move a number of various substances (Bousis *et al*., 2019; Zhang, 2013), and was first

used to define transporters for the uptake of vitamins in lactic acid bacteria in the 1970s (Henderson and Zevely, 1978).

3.1.2.3 Sodium/proton antiporters

The antiporters are described as one of the three distinct categories of the MFS transporters that transport two or more substrates through the membrane in opposite directions (Abdel-Motaal *et al.*, 2018). The basic cellular function of the secondary transmembrane transporters, sodium/proton (Na^+/H^+) antiporters, is to protect the cells against the osmotic change in the surrounding environment, through extruding Na^+, lithium (Li^+) and potassium (K^+) in exchange for external H^+; thereby making the cell membranes less permeable to ions, which in turn affects the interactions between the ion transport and drug efflux genes. As a result, when exposing bacterial cells to pore-forming environmental stimuli (osmotic challenges), these cells will respond by overexpressing the genes that are responsible for resistance (Cohen, 2014).

Na^+/H^+ antiporters are described as integral membrane proteins present in all species. In bacteria, Na^+/H^+ antiporters function through using the proton motive force to eject Na^+ when present in the cytoplasm at toxic concentrations, or in an alkaline environment to sustain a lower cytoplasmic pH (Prágai *et al.*, 2001).

The *nhaC* gene encodes Na^+/H^+ antiporters (Wang *et al.*, 2000), and was found to have a repressive effect on bacterial growth and the synthesis of Alkaline phosphatases (APases) in *B. subtilis* in the presence of sodium. A study about the effect of phosphate starvation in *B. subtilis* found that the genes of the Pho regulon were either expressed or de-repressed, and some of these genes are involved in encoding APases. The

operation of Pho regulon is influenced by a set of genes, one such gene is *nhaC*. The study found that the effect of NhaC on the bacterial growth, the synthesis of APases, and its own expression, was dependent on the external Na^+ concentration (Prágai *et al.*, 2001). Similarly, the *nhaC_2* gene encodes putative Na^+/H^+ antiporters in *Enterococcus* genus (Ramsey *et al.*, 2014), and in *E. faecium* NCTC12202, with limited published data about this gene in *Enterococcus spp*.

3.1.2.4 Calcium-translocation P-type ATPase

The superfamily of P-type ATPase is widely distributed in bacteria, archaea and eukaryotes, and composes of integral membrane proteins that transport ions, including H^+, Na^+/K^+, Ca^{2+} and even phospholipids, across the cell membranes using the energy derived from ATP hydrolysis (Smith *et al*., 2014). These proteins are localised in prokaryotes to the cytoplasmic membranes (Qiu *et al.*, 2005).

The primary function of P-type ATPases in prokaryotes, has been suggested to protect these cells from excessive environmental stress conditions such as calcium fluctuations (Chan *et al.*, 2010). It has been found that in all cellular organisms, calcium transporting ATPases (Ca^{2+} pumps) are essential to maintain calcium homeostasis in the cells. The P-type ATPases superfamily is divided based on phylogenetic analyses and substrate specificity, into five major groups (I-V) (Bonza *et al.*, 2010).

Ca^{2+} has a critical role in the physiology of the bacteria, where Ca^{2+} is essential for enzymatic activity, gene regulation and conservation of the structural integrity of the cell walls of the bacteria. Due to bacteria, keep a tight intracellular Ca^{2+} concentrations in nanomolar range, Ca^{2+} efflux is carried through active pumps belong to the family II

of P-type ATPase antiporters, while the Ca^{2+} uptake is happening through channels. In the case of Ca^{2+} deficient, the energy-dependent Ca^{2+} uptake is considered important for bacterial survival (Gupta *et al.*, 2017).

yloB gene encodes calcium-translocating P-type ATPase in *E. faecium* NCTC12202. A study using the genomic sequence of *B. subtilis* found that the open reading frame designated *yloB* gene encodes a putative protein of P-type ion-motive ATPases. This protein was found to be expressed by the sporulating *B. subtilis* cells but not the vegetative ones using Western blotting (Raeymaekers *et al.*, 2002).

Looking at the importance of the five genes (*bcr*, *ecfA1*, *esfA-1*, *nhaC_2*, *yloB*) detailed above and consequently their translated proteins during the life cycle of the bacterial cell, and the way they are expressed under the effect of the formula in the microarray, created a motivation for further analysis of their transcriptional profile in a time course study in addition to evaluation of the microarrays data, for greater understanding of their involvement in the mechanism of action of the novel formulation of combining EOs with vancomycin.

3.1.3 Aim and objectives

The study in this chapter focuses on five genes (*bcr*, *ecfA-1*, *ecsA_1*, *nhaC_2* and *yloB*) due to their role in the bacterial cell in encoding proteins that are related to transportation actions and potentially their relation to antibiotic resistance. The current study aims to provide an in-depth analysis of the effect of EOs-vancomycin combination on these genes in VRE, that will provide information on the role of transport channels in the mechanism of action of their antimicrobial effect.

Objectives

- To confirm the microarray data by using qPCR to analyse the regulation of the five genes in the conditions used in the microarray (1 % DMSO control, 60 minutes incubation period, with 3.95 mM carvacrol, 537.65 mM cuminaldehyde, 256 mg/l vancomycin, and combination of 1.98 mM carvacrol, 4.20 mM cuminadlehyde and 0.031 mg/l vancomycin).
- To analyse the regulation of these five genes in the presence of 0.49 mM carvacrol and 8.40 mM cuminaldehyde combined.
- To determine the expression levels of the five genes at five time points (10, 30, 60, 120 and 360 minutes) in response to carvacrol, cuminaldehyde, carvacrol and cuminaldehyde together, vancomycin and the novel EO-vancomycin formulation.

3.2 Material and Methods

3.2.1 Growth and sampling conditions

Methods were described as previously explained in (Sections 2.2.1-2.2.3) unless stated below. The vancomycin-resistant *E. faecium* (VRE) NCTC12202 strain was purchased from Public Health England culture collection and was stored at -80˚C on protect beads. Vancomycin was purchased from Fisher Scientific Ltd, UK (10014257-1G) and was prepared in sterile distilled water using a PTFE syringe filter (0.45 µm; Merck Millipore) and was stored for 2 days at 4°C and 1 month at -20˚C. All materials were freshly prepared or were used from stock solutions.

Different treatments were prepared in 100 µl DMSO and added to the *E. faecium* (VRE) suspensions at the following concentrations; carvacrol 3.95 mM, cuminaldehyde 537.65 mM, vancomycin 256 mg/l, combination of carvacrol 0.49 mM and cuminaldehyde 8.40 mM, and a combination of carvacrol 1.98 mM, cuminaldehyde 4.20 mM and vancomycin 0.031 mg/l.

3.2.2 Quantitative Real-time poly chain reaction

3.2.2.1 Primers design

The sequences of the five genes (*bcr*, *ecfA1*, *ecsA_1*, *nhaC_2*, and *yloB*) and two reference genes (*rpoD* and *dnaB_2*) were bioinformatically analysed using the National Centre for Biotechnology Information (NCBI) website. Template-specific primers were checked using the OligoAnalyzer and PrimerQuest tools on Integrated DNA Technologies (IDT) website, for melting temperature, GC % content, hairpin formation, self-dimer and hetero-dimer and were purchased from IDT (Table 3.2).

Table 3.2. qPCR primers used in the current study

Gene	Primers 5`-3` (Direction and Sequence)	Length	T_m˚C	Accession number
bcr	F: CATTGCTGGCACGCTTATTC	20	62	UFYT01000003.1:c704606-703398
	R: CCCGATAGTTGGCGTATAAAGA	22	62	
ecfA1	F: GGAGGACAACGTCAAGCTATT	21	62	UFYT01000003.1:2725746-2726552
	R: TTTGGAAGTAGCAGGGTCAAG	21	62	
ecsA_1	F: CAGGGCAAATCCATCCTACTT	21	62	UFYT01000003.1:c383826-382945
	R: CCTTCTTCATCGAGTGTTCCTT	22	62	
nhaC_2	F: GCGGAGGAATCGAAAGTATGA	21	62	UFYT01000003.1:612637-614055
	R: CGGTTGAGATCAAGCCAAATTC	22	62	
yloB	F: GTCGCCAGATCCAGAAATCA	20	62	UFYT01000003.1:321733-323448
	R: GGGAGACATAGATGATCCGAAAC	23	62	
rpoD (reference)	F: CGACCAAGAAGCGAAACA	18	60	UFYT01000003.1:1079216-1080325
	R: CATGCCTCGTCCTACATAAC	20	60	
dnaB_2 (reference)	F: TAGTCTAGGCTGCGGTATC	19	60	UFYT01000003.1:c1528434-1527061
	R: CGAGGTCCCAGATCACTAT	19	60	

T_m: melting temperature, F: Forward, R: Reverse, Accession number of primers as shown on NCBI

3.2.2.2 Genomic DNA extraction for primer efficiencies

gDNA was extracted from bacterial suspensions that were incubated overnight at 37˚C with shaking (100 rpm), using DNeasy Blood and Tissue kit (Qiagen Technologies, Hilden, Germany), following the manufacture instructions.

3.2.2.3 Ribonucleic acid (RNA) extraction

The treated bacterial suspensions were split into volumes of 1 ml and 2 ml of RNA protect bacteria reagent was added to prevent RNA degradation, vortexed for 5 seconds, and then incubated at room temperature for 5-10 minutes following manufacturer's instructions. These were then centrifuged for 10 minutes at room temperature and 5000 × *g*. The resulting pellets were then either used immediately or stored at -80˚C for later use.

The total mRNA was extracted from the treated and non-treated pellets using RNeasy Mini kit (74104) (Qiagen Technologies, Hilden, Germany) according to the manufacturer's guidelines. RNase free water (50 µl) was added to the spin column to elute the bound RNA by centrifugation for one minute at 12,000 rpm. Sterile Eppendorf tubes were used to collected the eluted RNA.

DNA decontamination treatment was carried out using Turbo DNA Free Kit (AM1907) (Thermo Fisher Scientific Ltd, UK) following the manufacturer's instructions, for further purification of RNA prior to quantification.

3.2.2.4 RNA quantity and purity

The quantity and purity of the extracted RNA samples were measured using a spectrophotometer NanoDrop 1000^{TM} (Thermo Scientific, USA), by measuring the absorbance at 260 nm (A_{260}) to determine the RNA quantity, and calculating the absorbance ratio (A_{260}/A_{280}) to estimate the RNA purity. Samples with A_{260}/A_{280} of ~ 2.0 (1.8-2.2) were accepted for purity; otherwise, further decontamination step was carried out using gDNA removal columns (10043831) (Biolabs, New England). NanoDrop®

instrument was calibrated using 1 µl nuclease free water, and 1 µl of the extracted RNA was used to measure the quantity and purity. It detects 2-12,000 ng/µl of RNA in the samples.

3.2.2.5 cDNA synthesis for qPCR

cDNA was manufactured using Tetro cDNA synthesis kit (BIO-65042) (Bioline Ltd, UK), and according to the manufacturer instructions. Aliquots of 10 µl of the 50 ng/µl extracted RNA were reverse transcribed in two reaction volumes of 20 µl in RNase free reaction tubes, with the addition of the cDNA synthesis master mix (10 µl), that was prepared as shown in Table 3.3. The listed parameters were used accordingly unless otherwise stated.

Control reactions were performed alongside the reactions to determine whether there was genomic DNA carry-over, by following the same conditions without adding the reverse transcriptase enzyme to the cDNA synthesis master mix, and reverse transcriptase was substituted with DEPC (diethylpyrocarbonate) treated water. Accordingly, the threshold cycle (Cq) values supposed to be produced by the NTC and no reverse transcribed control (NRTC) samples are zero indicating no genomic DNA was carried over; nevertheless, NTC and NRTC samples were accepted when 5-7 cycles higher than that of the corresponding samples.

Table 3.3. Preparing the cDNA synthesis mix per one reaction

Primer Random Hexamer	1 µl
10 mM dNTP mix	1 µl
5× RT buffer	4 µl
RiboSafe RNase Inhibitor	1 µl
Tetro Reverse Transcriptase (200u/µl)	1 µl
DEPC treated water	Up to 10 µl

cDNA was generated running the following reaction parameters; incubation for 10 minutes at 25˚C, and for 60 minutes at 45˚C. The reaction was terminated by incubating for 5 minutes at 85˚C and samples were chilled on ice for 10 minutes. Resulted cDNA samples were kept at -20˚C until use.

3.2.2.6 qPCR

qPCR was performed with SYBR Green Master Mix using SensiFAST™ SYBR Lo-Rox Kit (BIO-94005) (Bioline, Ltd, UK) to establish the expression levels of the genes in question. Template-specific primers were designed and ordered from IDT (Table 3.2).

The qPCR amplification was carried out in 96-well piko PCR plates (SPL0960) (Thermo Scientific, UK) in biological and technical triplicates, with reaction volumes detailed in Table 3.4. No template control (NTC) and no reverse transcriptase control (NRTC) were conducted in tandem with all reactions.

Table 3.4. qPCR reagents mix per test

Reagent	Volume per test
2× SensiFAST SYBR Lo-ROX	10 µl
10 µM forward primer	0.8 µl
10 µM reverse primer	0.8 µl
Template (5 ng/µl)	2 µl
Nuclease free water	as required to final volume of 20 µl

The cycling parameters protocol for qPCR was set up as following: initial denaturation at 95˚C for 2 minutes, 40 cycles of denaturation at 95˚C for 5 seconds, annealing at 62˚C for 10 seconds, and finally, extension at 72˚C for 20 seconds. Each PCR run was followed

by a dissociation at 60˚C for 30 seconds, and a melting curve analysis between 60-95˚C. The final cooling step was carried out at 20˚C for 10 seconds.

3.2.2.7 Relative gene expression level

PikoReal software version 2.2 (Thermo Scientific, Piko Real 96, Real-Time PCR System) was used to calculate the Cq after each qPCR run. The Cq values represent the number of the cycles at which the fluorescence was generated in accordance to the detectable level, where the low Cq values relate to high expression levels.

The Cq values were analysed by the comparative Cq (ΔΔCq) method and normalised using reference genes (ΔCq). The results were normalised to the endogenous control (ΔΔCq). Relative gene expression or expression fold change was calculated as $2^{-\Delta\Delta Cq}$ using Pfaffl method (Pfaffl, 2001) as follows;

$\Delta Cq = Cq_{\text{(gene of interest)}} - Cq_{\text{(reference)}}$

$\Delta\Delta Cq = \Delta Cq_{\text{(treated sample)}} - \Delta Cq_{\text{(control)}}$

Fold change = $2^{-\Delta\Delta Cq}$

Control samples contained bacteria subjected to 1 % DMSO in all experiments. Treated samples contained bacteria exposed to the specified conditions. The expression level of gene was considered upregulated when > 1, and downregulated when < 1, the exact fold change of down-regulation was calculated by dividing 1 to the resulted fold change to be demonstrated as minus.

3.2.3 Statistical analysis

Statistical analysis was carried out using Excel and Statistical Package for Social Sciences (SPSS) software version 25 (IBMS SPSS version 25). The Student's *t-test* was used to determine the significant differences between treated and non-treated (controls) samples. Statistical significance was accepted for $P \leq 0.05$. Standard deviation and standard deviation error were calculated using Excel and error bars were displayed on the figures.

The qPCR data were presented as technical means of Cq values. The differences in gene expression according to the studied conditions were assessed using a one-way ANOVA with Tukey's post-hoc test and were considered significant when $P \leq 0.05$. Fold change was assessed by following Pfaffl method and was considered significant with ≥ 2-fold in the expression level of the gene of interest with any of the treatment conditions at any time point of the time course study. Genes with significant expression level difference and significant fold change were considered altered. Conducted experiments were carried out in biological triplicate and technical triplicate.

3.3 Results

Validating the microarray data using qPCR has displayed consistent results in the mean of up-regulation and down-regulation of the five genes of interest as shown in the array (*bcr* at 1.16-fold, *ecfA1* at 2.17-fold, *ecsA_1* at -1.23-fold, *nhaC_2* at -1.42-fold, and *yloB* at -1.24-fold) upon exposure to the formulation. For more understanding of the involvement of the transport channels in the antimicrobial mechanism of action of the formulation to be exploited as a novel antimicrobial, a time course study of five time points including the array time point (60 minutes) was decided. The time points for the time course study were determined relying on previously published growth curves (Owen and Laird, 2019), where in addition to the time point of the array, four additional time points (10, 30, 120 and 360 minutes) were chosen to investigate the expression levels of the five genes. The five genes of interest in response to all treatment conditions (3.95 mM carvacrol, 537.65 mM cuminaldehyde, 0.49 mM carvacrol and 8.40 mM cuminaldehyde, 256 mg/l vancomycin, the formulation (1.98 mM carvacrol, 4.20 mM cuminaldehyde and 0.031 mg/l vancomycin)) displayed varied expression levels, in particular at the early time points of 10 and 30 minutes.

3.3.1 Growth curves

To elucidate how the individual EO components, EO components in combination and the EOs-vancomycin combination have inhibited the growth of *E. faecium* (VRE), growth curves were conducted by Owen and Laird (2019) and produced before the microarray was carried out. The effects of the previously mentioned combinations were evaluated at different time points 10, 30, 60, 120 and 360 minutes that are highlighted by asterisks

in Figure 3.1. Upon exposure to the formulation, a reduction in the bacterial growth from 6.17 Log_{10} CFU/ml to 5.94 Log_{10} CFU/ml was noted at the time point of 10 minutes, and from 5.99 Log_{10} CFU/ml to 5.92 Log_{10} CFU/ml at 30 minutes. However, at 60 minutes the bacterial growth was further reduced by 0.58 Log_{10} CFU/ml. A significant reduction in the growth was noted at 120 and 360 minutes, from 5.36 Log_{10} to 3.78 Log_{10} to 1.59 Log_{10} CFU/ml, respectively (Figure 3.1).

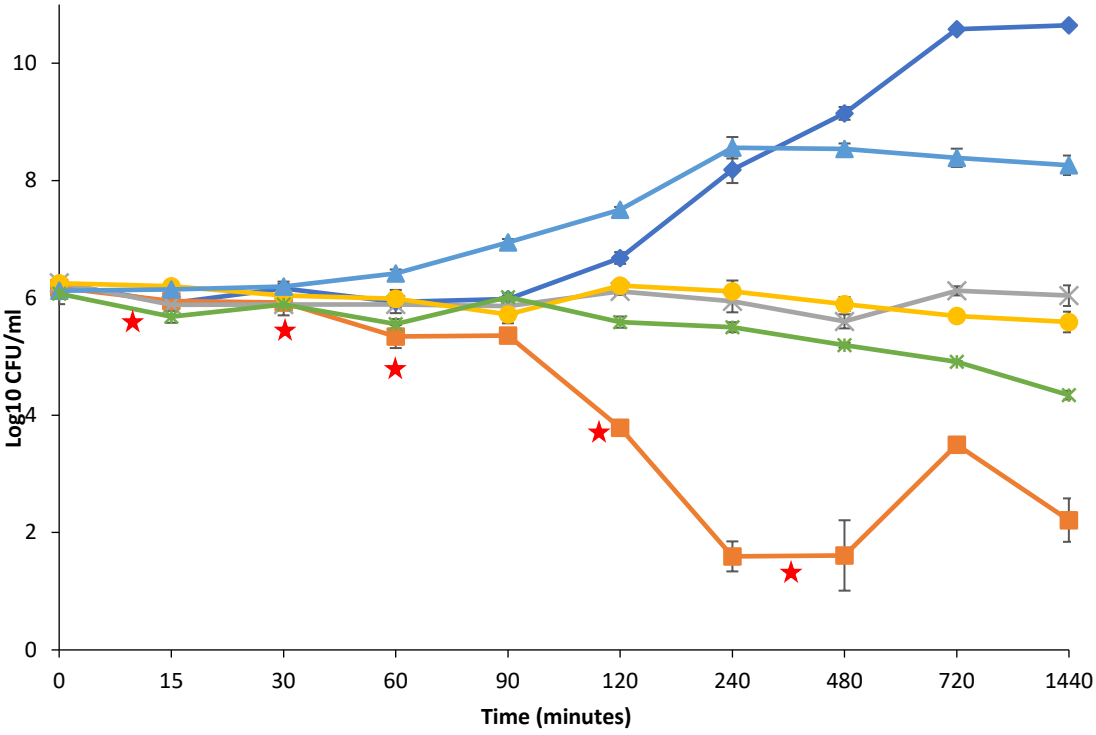


Figure 3.1. Growth curves of *E. faecium* (VRE) with all treatment conditions for 1440 minutes. ◆VRE control growth curve. ✖ VRE with 3.95 mM carvacrol. ●VRE with 537.65 mM cuminaldehyde. ✖VRE with 0.49 mM carvacrol and 8.40 mM cuminaldehyde. ▲VRE with 265 mg/l vancomycin. ■ VRE with the formulation (1.98 mM carvacrol, 4.20 mM cuminaldehyde and 0.031 mg/l vancomycin). The asterisks highlight the time points of interest for the time course study (n= 4, ±SEM). Adapted from Owen and Laird, 2019.

3.3.2 Quantitative Real-time PCR

3.3.2.1 qPCR primers design

Designing forward and reverse primers of the genes of interest was carried out in accordance to general criteria, including primer length that is typically between 18-24 nucleotides and melting temperature (T_m) of approximately 60-62˚C. The GC content in primer pairs should be 40-60 %, preferably no more 50 %, avoid multi A on 3'-end (Thornton and Basu, 2011), with secondary structure forming being checked using described tools for PCR primers designing on NCBI and IDT websites.

3.3.2.2 Reference genes stability analysis

Reference genes are non-regulated and exhibit minimal variability within the physiological conditions of the organisms, and are used as a reaction control and for normalising qPCR data, where their expression level is not affected by the experiment factors (Kozera and Rapacz, 2013, Everaert *et al*., 2011).

The two reference genes used in the present study are *rpoD* and *dnaB_2,* due to them being previously reported to be expressed stably in bacteria (Owen *et al.*, 2020; Rocha *et al.*, 2015). Determination the stability of the reference genes (*rpoD* and *dnaB_2*) was done using the cDNA sample panel of all samples at all treatment conditions and the geNorm version 3.5 (Sundaram *et al.*, 2019), which calculates the expression stability measure M of the reference gene as the average with all other tested reference genes. In the current study, the means of Cq values for the two reference genes (*rpoD* and *dnaB_2*) were almost identical with all samples (data not shown) with average

expression stability M was < 1 (Appendix, Figure 9.7), displaying the stability of the reference genes under all treatment conditions at different time points.

3.3.3 gDNA and cDNA concentrations

gDNA was extracted from bacterial cultures of *E. faecium* (VRE) were grown without any treatment as discussed in section 3.2.2. Serial dilutions of gDNA were prepared as following; 100, 50, 10, 1 and 0.1 ng/µl, to be used in evaluating the primers efficiencies.

Determination of the optimal cDNA dilution to be used in qPCR is a common and important optimisation step, in order to get reproducible and meaningful results. Where concentrated cDNA can interfere with the PCR reaction and acts as PCR inhibitors (França *et al.*, 2012). Several dilutions were prepared of the synthesised cDNA and were tested in the qPCR. The diluted cDNA at concentration of 10 ng/µl has shown no significant variation between the tested cDNA concentrations. Consequently, all the synthesised cDNA was diluted to the concentration of 5 ng/µl, and 2 µl were used in each qPCR reaction.

3.3.4 Validation of the microarray data running qPCR

qPCR was used to verify the array data, reported in Owen *et al*. (2020), in the expression level of the genes of interest (*bcr*, *ecfA1*, *ecsA_1*, *yloB*, and *nhaC_2*) (Table 3.6). Bacterial suspensions were treated with same conditions used in the array; 60 minutes incubation with carvacrol, cuminaldehyde, vancomycin and the formulation, and in addition to an extra condition the EO components together (carvacrol and cuminaldehyde), the samples were processed to be validated using qPCR assays (Section 3.2.2). The correlation was strongly positive between the oligonucleotide microarray data and qPCR

results, with more sensitivity in detection with the qPCR. The qPCR results were consistent with the array data in the mean of up or down-regulation with slight differences in the fold change. The qPCR results confirmed that at 60 minutes of incubation with the different treatment conditions, there were up and down-regulation to the five genes related to the function of each gene in the bacterial cell as discussed previously (Figure 3.2).

Table 3.6. The fold change of the five genes of interest in (**A**) the array data (Owen *et al*.,2020) and (**B**) the qPCR runs in the current study at 60 minutes in response to carvacrol, cuminaldehyde, vancomycin and the formulation (carvacrol, cuminaldehyde and vancomycin), and in response to another condition in the current study; carvacrol and cuminaldehyde together.

Gene	Description	Carvacrol		Cuminaldehyde		Vancomycin		Formulation		Carvacrol and cuminaldehyde
		Fold Change								
		A	B	A	B	A	B	A	B	B
bcr	Transporter, major facilitator family protein	-2.382	-1.41	-2.4377	-3.52	-1.2274	-1.30	1.1577	-1.53	-4.22
ecfA1	Energy coupling factor transporter ATP-binding protein	4.083	1.23	2.6357	2.10	1.3358	1.58	2.1715	2.13	1.60
ecsA-1	ABC transporter ATP-binding protein	-1.021	-1.64	2.395	2.16	-1.328	-1.21	-1.232	-1.14	-4.69
yloB	Calcium-translocating P-type ATPase	-2.223	-2.41	-1.4515	-2.07	-1.2172	-2.07	-1.2399	-1.8	-4.93
nhaC_2	Sodium/proton antiporter	-3.056	-2.36	-1.9222	-2.52	-1.2106	-2.35	-1.4203	-3.09	-4.53

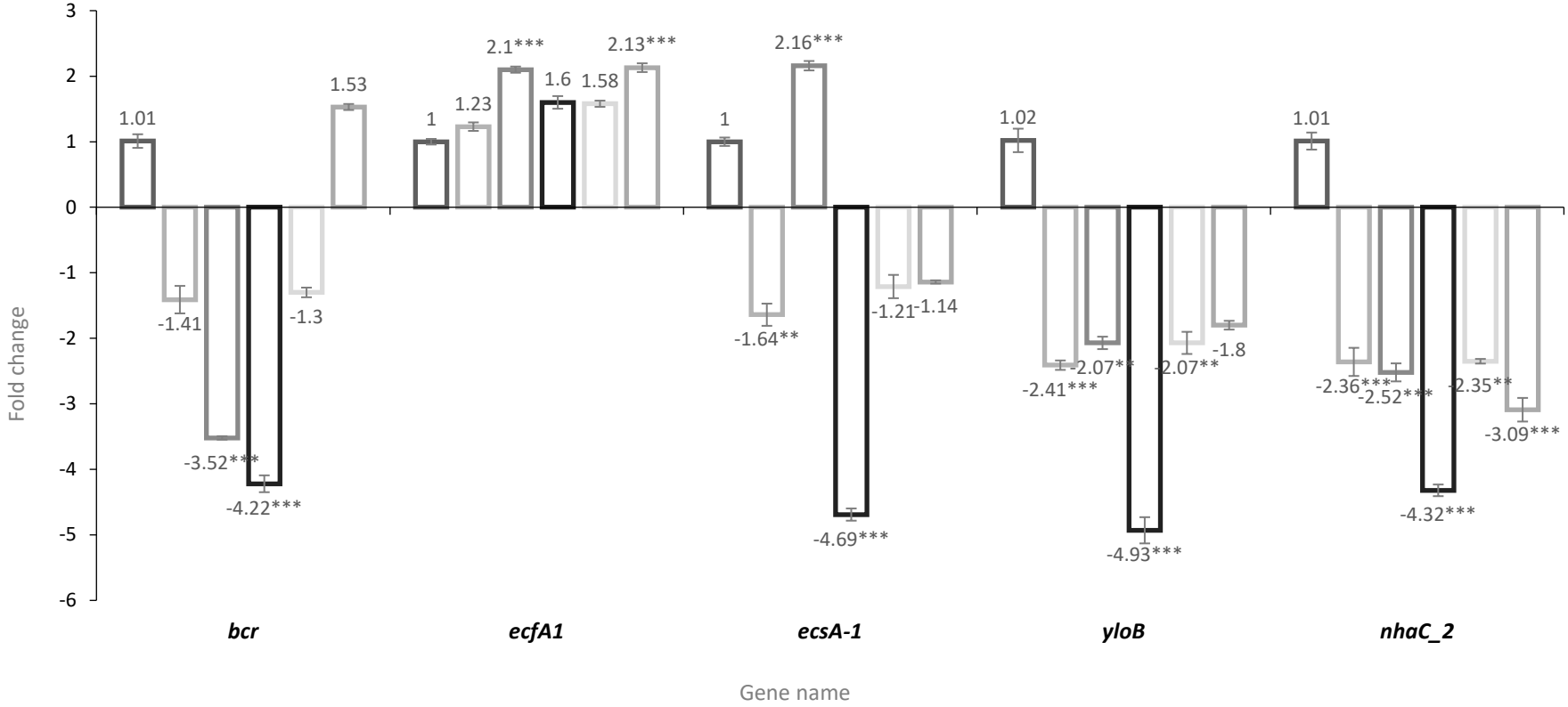


Figure 3.2. Changes in gene expression of the five genes of interest in response to EOs in formulation for 60 minutes. The fold change of *bcr*, *ecfA1*, *ecsA-1*, *yloB* and *nhaC_2* genes were measured using qPCR at 60 minutes under the following conditions;■control DMSO 1 %; ■ 3.95 mM carvacrol; ■ 537.65 mM cuminaldehyde; ■ a combination of 0.49 mM carvacrol and 8.40 mM cuminaldehyde; ■ 256 mg/l vancomycin; ■ a formulation of 1.98 mM carvacrol, 4.20 mM cuminaldehyde and 0.031 mg/l vancomycin. Fold change was calculated as $2^{-\Delta\Delta Cq}$ against reference genes *rpoD* and *dnaB_2,* *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$ (n= 3, ±SEM).

The results of this study at 60 minutes treatment confirmed the microarray data with no significant differences. The expression level of *bcr* gene, which is responsible for encoding transporter protein, was down regulated by 3.52-fold when being treated with cuminaldehyde alone in comparison to 2.44-fold in the array, and by 4.22-fold with EOs combination, while no significant change was noted with the other conditions of treatment in line with the array. *ecfA1* gene that encodes energy coupling factor transporter (ATP binding protein), there was an increase in the gene expression by 2.1-fold with cuminaldehyde alone and 2.13-fold with the EOs-vancomycin combination, that was similar to 2.64 and 2.8-fold change in the array, respectively, and no significant change was noted when being treated with the other conditions in resemblance to the array. Similarly, the *ecsA_1* gene, which is translated into ABC transporter superfamily ATP-binding protein, was up-regulated by 2.16-fold when with cuminaldehyde alone in comparison to 2.4-fold in the array, and down regulated by 4.69-fold with EOs combination. The gene that encodes the calcium-translocating P-types ATPase protein, *yloB*, was down regulated with all treatment conditions, by 2.42-fold with carvacrol, 2.07-fold with cuminaldehyde and 2.07-fold with vancomycin; and 2.22-fold, 1.5-fold and 1.2-fold in the array, respectively; and by 4.93-fold when being with carvacrol and cuminaldehyde. Likewise, the gene that is transcribed into sodium-proton antiporter, *nhaC_2* was down regulated with all treatment conditions by 2.36-fold with carvacrol, 2.52-fold with cuminaldehyde and 2.35-fold with vancomycin, in comparison to 3-fold, 2-fold and 1.2-fold in the array, respectively; by 4.32-fold with carvacrol and cuminaldehyde and by 3.09-fold with EO-vancomycin combination compared to 1.4-fold in the array (Figure 3.2). In comparison with the control DMSO 1 %, One-Way ANOVA

and *t-test* showed that there were significant differences in the expression levels of the genes when being exposed to cuminaldehyde, carvacrol and cuminaldehyde, and the formulation, $P \leq 0.05$. While in the case of exposure to carvacrol and to vancomycin, there were no significant differences, $P > 0.05$. Nevertheless, the overall P value was ≤ 0.05, which confirmed a significant difference between the applied conditions and the control. After validating the microarray data using qPCR, a time course study was carried out to establish the transcriptional profiles of the five genes of interest at two earlier times points (10 and 30 minutes), and two later times points (120 and 360 minutes). Although, the expression level of the five genes of interest had not been established when exposed to the effect of carvacrol and cuminaldehyde in combination by microarray, it was assessed by running qPCR assays. This was completed as it was hypothesised that by understanding how the EOs in combination alone might affect the expression levels of the five genes, it could help in elucidating how EOs participated in re-sensitising VRE to vancomycin.

3.3.5 Expression levels of the five genes over time

Time course experiments were a valuable addition to the single-time-point microarray study and help provide a deeper insight into the regulation of the bacterial response to the applied conditions.

A time course study was completed to understand the changing expression of the five analysed genes in response to all established EO conditions over a set time period at different points (Section 3.3.1). To build up a time course study, the microarray data was considered as base time point (60 minutes), then two time points before 60 minutes

were analysed in the study, 10 and 30 minutes, and a further two time points after 60 minutes were chosen, 120 and 360 minutes. The fold changes of the expression level of all genes of interest at all time points and all treatment conditions are detailed in Appendix, Tables 9.8-9.12.

The expression levels of *bcr* gene were differentially regulated under the effect of all treatment conditions over the time points of the time course study. At 10 minutes, *bcr* was significantly down regulated under the effect of the formulation by 13.5-fold, with carvacrol by 2.95-fold and with vancomycin by 2.53-fold ($P \leq 0.05$); however, no significant expression change was noted under the effect of cuminaldehyde and EOs combination ($P > 0.05$). Treating the bacteria with similar treatment conditions for 30 minutes has not implicated significant effect on the expression level of *bcr* gene ($P > 0.05$), except being upregulated by 2.16-fold when being exposed to the combination with significant change ($P \leq 0.05$). At 60 minutes, *bcr* gene was significantly down-regulated by 3.5-fold and 4-fold by cuminaldehyde and EOs combination ($P \leq 0.05$), respectively; but no significant effect was noted with carvacrol, vancomycin or the formulation ($P > 0.05$). Moving forward to the data of 120- and 360-minutes treatment incubation, it was found that *bcr* gene was upregulated by 4.89-fold and 3.29-fold with carvacrol, 12.26-fold and 1.04-fold with cuminaldehyde, 12.94-fold and 1.41-fold with EOs combination, 2.34-fold and 1.16-fold with vancomycin, and 15.03- and 15.71-fold with the formula, respectively; with significant effect with all treatment conditions at 120 minutes ($P \leq 0.05$). Wherein at 360 minutes, no significant effect was noted with cuminaldehyde, EOs combination or with vancomycin ($P > 0.05$), and significant effect with carvacrol and the formulation ($P \leq 0.05$) (Figure 3.3).

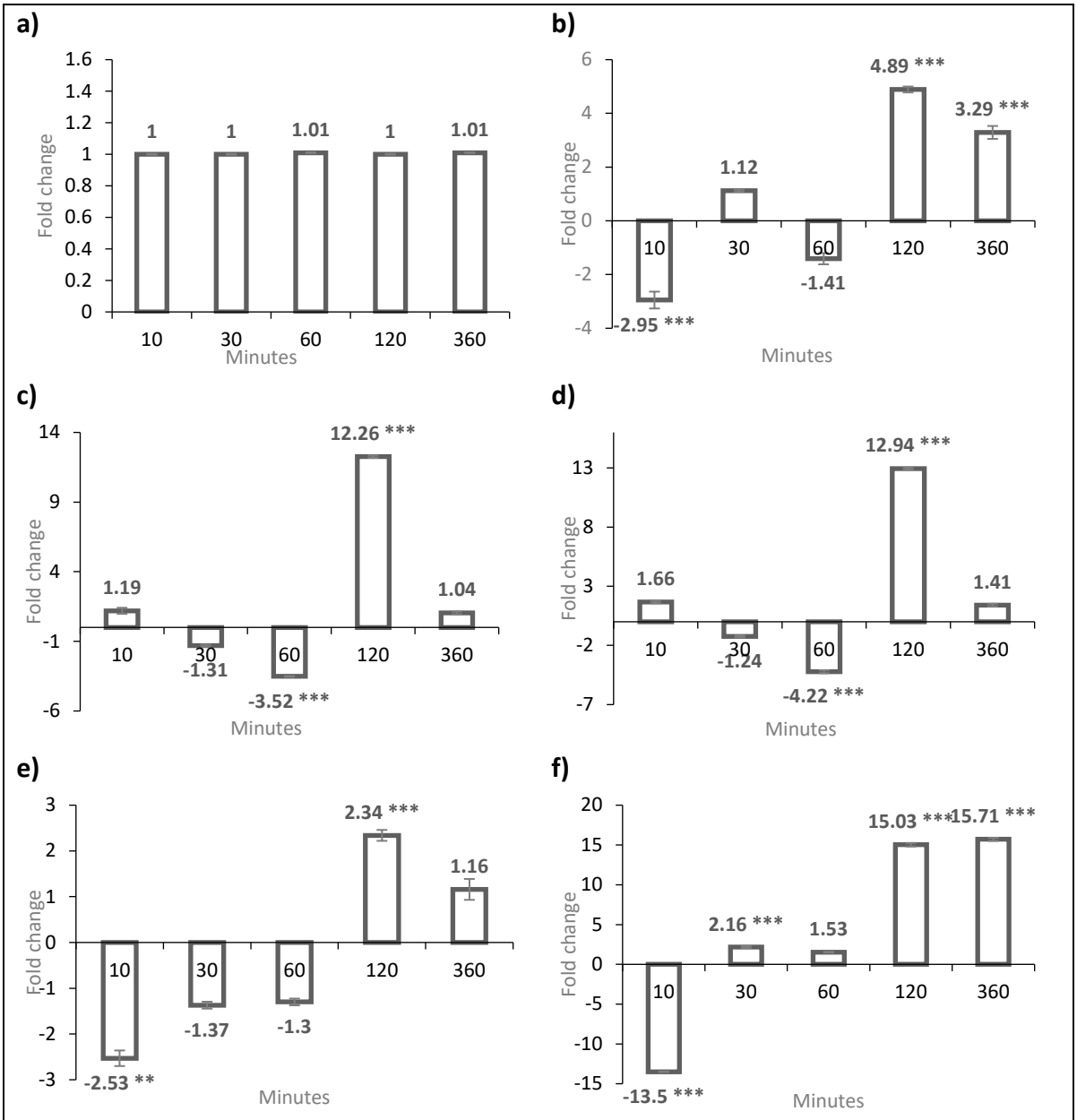


Figure 3.3. Changes in gene expression of *bcr* in response to EOs in formulation over time. The fold change of *bcr* gene was measured using qPCR over time (10, 30, 60, 120 and 360 minutes) under the following conditions; **a)** control DMSO 1 %; **b)** carvacrol 3.95 mM; **c)** cuminaldehyde 537.65 mM; **d)** a combination of carvacrol 0.49 mM and cuminaldehyde 8.40 mM; **e)** vancomycin 256 mg/l; **f)** a formulation of carvacrol 1.98 mM, cuminaldehyde 4.20 mM and vancomycin 0.031 mg/l. Fold change was calculated as $2^{-\Delta\Delta Cq}$ against reference genes *rpoD* and *dnaB_2*, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$ (n= 3, ±SEM).

The expression levels of *ecfA1* were not affected at the early stages of incubation with all treatment conditions, where no significant fold change was noted at 10 minutes ($P > 0.05$). Wherein, the expression level of *ecfA1* at 30 minutes was significantly down regulated by 3.89-fold with cuminaldehyde, 2.67-fold with EOs combination and 6.53-fold with vancomycin ($P \leq 0.05$), and no significant change with carvacrol or with the formulation ($P > 0.05$). At 60 minutes, the expression levels of *ecfA1* was significantly upregulated by 2.1-fold and 2.13-fold with cuminaldehyde and the formulation ($P \leq 0.05$), respectively, and no significant change was noted when being exposed to the effect of carvacrol, EOs combination or vancomycin for 60 minutes ($P > 0.05$). Furthermore, the expression levels of *ecfA1* continued to be significantly upregulated when being treated for 120 minutes by 4.96-fold with carvacrol, 9.7-fold with cuminaldehyde, 7.06-fold with EOs combination and 2.85-fold with the formulation ($P \leq 0.05$), and no significant change with vancomycin alone ($P > 0.05$). The expression level of *ecfA1* turned to be non-significantly changed under the effect of all treatment conditions at 360 minutes of incubation ($P > 0.05$), except with vancomycin was significantly down regulated by 3.99-fold ($P \leq 0.05$) (Figure 3.4).

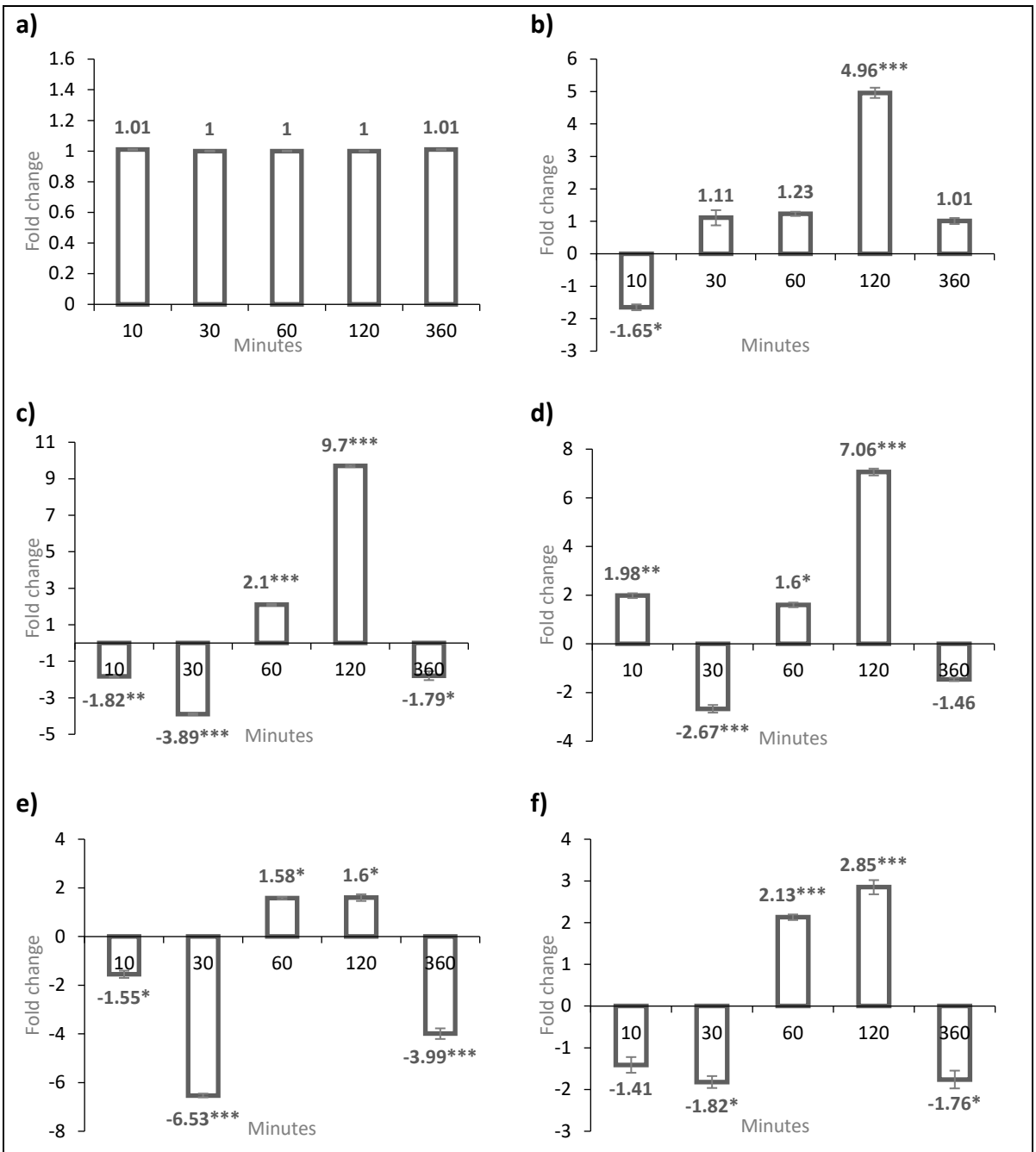


Figure 3.4. Changes in gene expression of *ecfA1* in response to EOs in formulation over time. The fold change of *ecfA1* was measured using qPCR over time (10, 30, 60, 120 and 360 minutes) under the following conditions; **a)** control DMSO 1 %; **b)** carvacrol 3.95 mM; **c)** cuminaldehyde537.65 mM; **d)** a combination of carvacrol 0.49 mM and cuminaldehyde 8.40 mM; **e)** vancomycin 256 mg/l; **f)** a formulation of carvacrol 1.98 mM, cuminaldehyde 4.20 mM and vancomycin 0.031 mg/l. Fold change was calculated as $2^{-\Delta\Delta Cq}$ against reference genes *rpoD* and *dnaB_2,* *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$ (n= 3, ±SEM).

Similarly, the expression levels of *ecsA_1* were also differentially regulated under the effects of all treatment conditions over the five time points. The transcriptional responses of *ecsA_1* at 10 minutes happened only under the effect of the formulation with significant fold change at 3.95 down-regulation, and with EOs combination by 2.04-fold up-regulation ($P \leq 0.05$). Wherein, at 30 minutes no significant transcriptional response was noted with any of all treatment conditions ($P > 0.05$). Moreover, at 60 minutes *ecsA_1* was significantly up regulated by 2.16-fold when with cuminaldehyde, and significantly down regulated by 4.69-fold under the effect of EOs combination ($P \leq 0.05$), and no significant change was noted with the other treatment conditions ($P > 0.05$). However, the expression level of *ecsA_1* was significantly up regulated at 120 minutes by 6.36, 5.09 and 2.11-fold with cuminaldehyde, EOs combination and vancomycin ($P \leq 0.05$), respectively; but no significant transcriptional response with carvacrol alone or with the formulation was noted ($P > 0.05$). Interesting and significant change to the expression level of *ecsA_1* was noted when being exposed to the formulation for 360 minutes that was upregulated by 25.87-fold, and by 2.25-fold with vancomycin ($P \leq 0.05$); however, no significant fold change was noted with the EOs components individually or in combination ($P > 0.05$) (Figure 3.5).

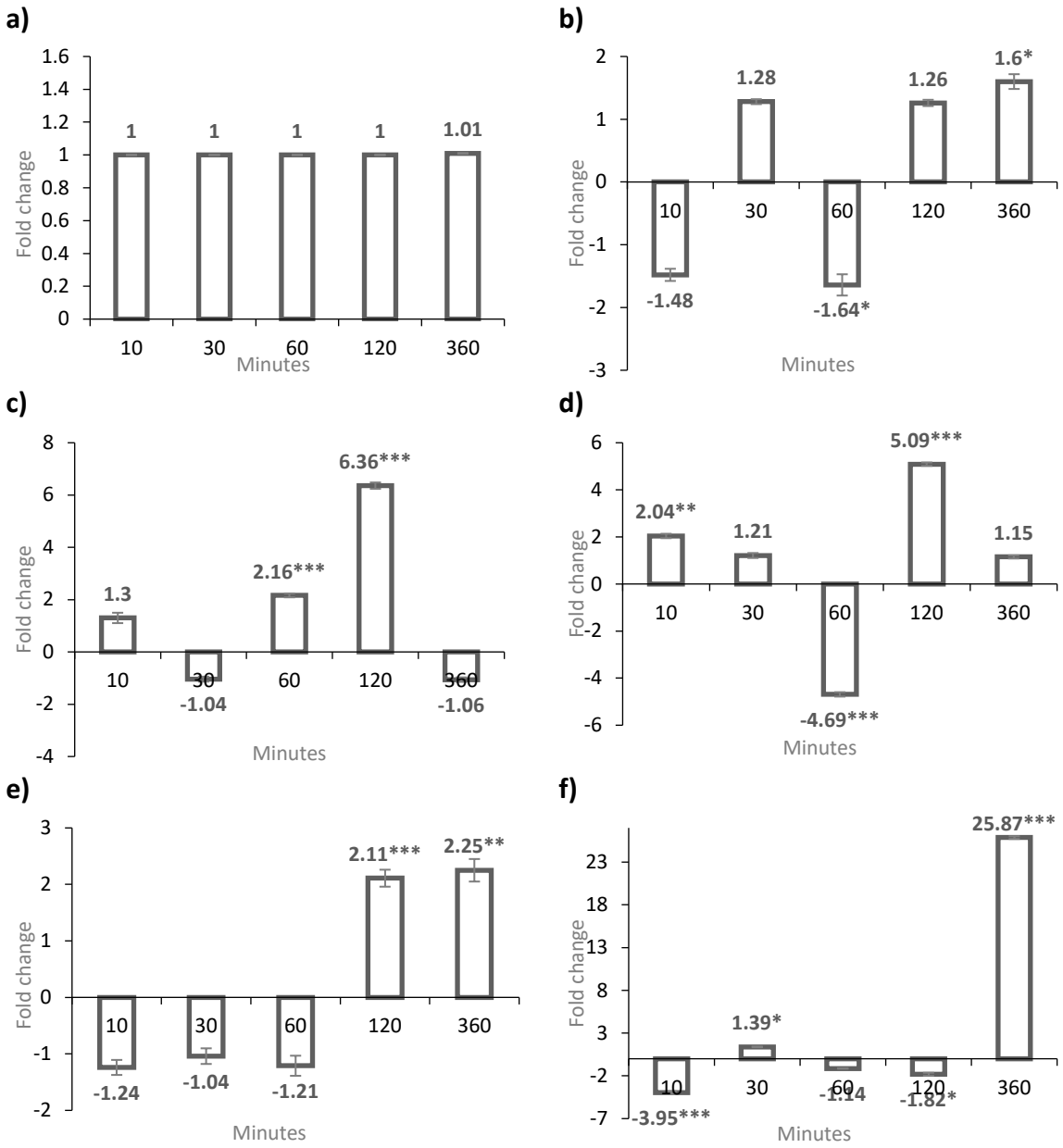


Figure 3.5. Changes in gene expression of *ecsA-1* in response to EOs in formulation over time. The fold change of *ecsA-1* was measured using qPCR over time (10, 30, 60, 120 and 360 minutes) under the following conditions; **a)** control DMSO 1 %; **b)** carvacrol 3.95 mM; **c)** cuminaldehyde 537.65 mM; **d)** a combination of carvacrol 0.49 mM and cuminaldehyde 8.40 mM; **e)** vancomycin 256 mg/l; **f)** a formulation of carvacrol 1.98 mM, cuminaldehyde 4.20 mM and vancomycin 0.031 mg/l. Fold change was calculated as $2^{-\Delta\Delta Cq}$ against reference genes *rpoD* and *dnaB_2,* *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$ (n= 3, ±SEM).

The expression level of *yloB* gene was significantly down regulated at 10 minutes by 2.13-fold with carvacrol and 5.67-fold with the formulation ($P \leq 0.05$), and no significant change was noted with the other treatment conditions ($P > 0.05$). Opposite to 10 minutes, the expression level at 30 minutes with the formulation was significantly up regulated by 3.68-fold ($P \leq 0.05$), and no further significant changes with the other treatment conditions ($P > 0.05$). However, the transcriptional response of *yloB* was significantly re-down regulated at 60 minutes by 2.41, 2.07, 4.93 and 2.07-fold with carvacrol, cuminaldehyde, EOs combination and vancomycin ($P \leq 0.05$), respectively; and no significant change was noted with the formulation ($P > 0.05$). Interestingly, no significant change in the expression levels of *yloB* gene at 120 and 360 minutes with the formulation was noted ($P > 0.05$), while it was significantly upregulated at 120 minutes by 2.81, 6.57, 7.48, and 2.36-fold with carvacrol, cuminaldehyde, EOs combination and vancomycin; and was significantly downregulated at 360 minutes by 4.1-fold with vancomycin ($P \leq 0.05$) (Figure 3.6).

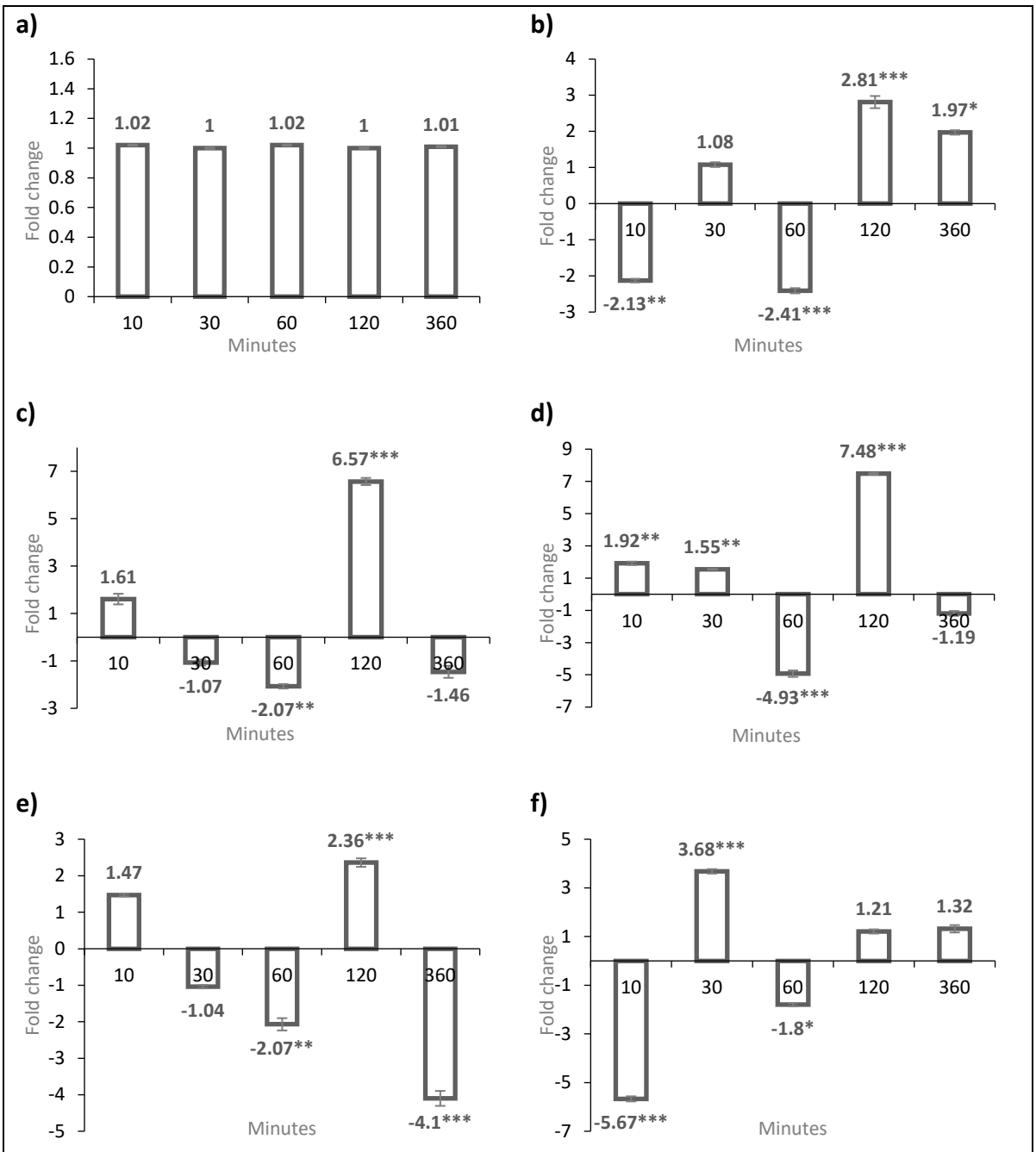


Figure 3.6. Changes in gene expression of *yloB* in response to EOs in formulation over time. The fold change of *yloB* was measured using qPCR over time (10, 30, 60, 120 and 360 minutes) under the following conditions; **a)** control DMSO 1 %; **b)** carvacrol 3.95 mM; **c)** cuminaldehyde537.65 mM; **d)** a combination of carvacrol 0.49 mM and cuminaldehyde 8.40 mM; **e)** vancomycin 256 mg/l; **f)** a formulation of carvacrol 1.98 mM, cuminaldehyde 4.20 mM and vancomycin 0.031 mg/l. Fold change was calculated as $2^{-\Delta\Delta Cq}$ against reference genes *rpoD* and *dnaB_2,* *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$ (n= 3, ±SEM).

At 10 minutes, the expression level of *nhaC_2* was significantly down regulated by 2.16-fold when exposed to carvacrol, and interestingly by 5.31 and 6.31-fold with vancomycin and the formulation ($P \leq 0.05$), respectively. No significant change was noted with neither cuminaldehyde nor EOs combination ($P > 0.05$). Similarly, the expression level of *nhaC_2* at 30 minutes did not change upon exposure to all treatment conditions ($P > 0.05$) except being significantly upregulated by 2.84-fold with the formulation ($P \leq 0.05$). Interestingly, the transcriptional response of *nhaC_2* at 60 minutes was significantly down regulated upon exposure to the five different treatment conditions by 2.36, 2.52, 4.32, 2.35 and 3.09-fold with carvacrol, cuminaldehyde, EOs combination, vancomycin and the formulation ($P \leq 0.05$), respectively. In contrast to 60 minutes, the expression level of *nhaC_2* was significantly up regulated at 120 minutes by 3.96, 15.5, 4.79, and 4.7-fold with carvacrol, cuminaldehyde, EOs combination and the formulation ($P \leq 0.05$), respectively; and no significant change with vancomycin ($P > 0.05$). Moreover, the transcriptional response of *nhaC_2* turned to be significantly re-down regulated at 360 minutes by 5.18, 2.1 and 3.13-fold with cuminaldehyde, EOs combination and vancomycin ($P \leq 0.05$), respectively; but no significant change in the expression level with neither carvacrol nor the formulation ($P > 0.05$) (Figure 3.7).

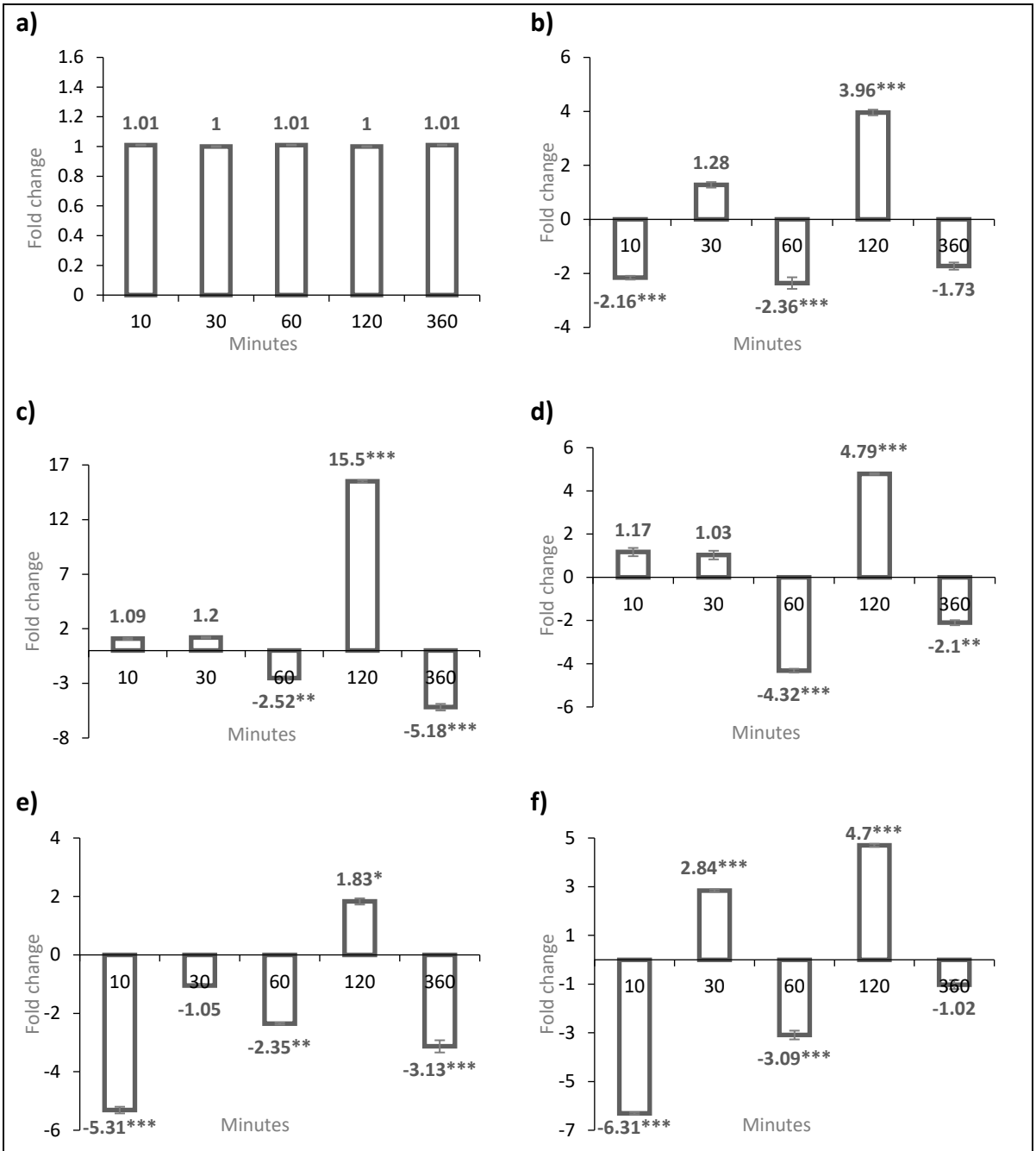


Figure 3.7. Changes in gene expression of *nhaC_2* in response to EOs in formulation over time. The fold change of *nhaC_2* was measured using qPCR over time (10, 30, 60, 120 and 360 minutes), under the following conditions; **a)** control DMSO 1 %; **b)** carvacrol 3.95 mM; **c)** cuminaldehyde 537.65 mM; **d)** a combination of carvacrol 0.49 mM and cuminaldehyde 8.40 mM; **e)** vancomycin 256 mg/l; **f)** a formulation of carvacrol 1.98 mM, cuminaldehyde 4.20 mM and vancomycin 0.031 mg/l. Fold change was calculated as $2^{-\Delta\Delta Cq}$ against reference genes *rpoD* and *dnaB_2,* *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$ (n= 3, ±SEM).

3.4 Discussion

Microarrays produce a wide range of comparative gene expression profiles and detect either expression or differential expression of thousands of genes of biological samples. Nevertheless, microarray data needs to be validated due to the possible lack of consistency within the technologies (Miron *et al.*, 2006). qPCR technique has become one of the most sensitive and reliable quantitative methods being used in laboratories for high-throughput analysis of gene expression levels. It is used worldwide to verify microarray data and further analyse gene expression (Yuan *et al.*, 2006).

Transcriptomic analysis carried out by Owen *et al.* (2020) shows that a wide range of *E. faecium* (VRE) genes displayed altered expression by exposure to EOs alone, and in combination with vancomycin. Some of these genes are involved in transportation in the bacterial cell and have been analysed in this chapter. Similar analyses have been demonstrated after exposing *S. enteritidis* to trans-cinnamaldehyde and eugenol, where almost 10 % of the genes were modulated (Kollanoor Johny *et al.*, 2017). Some of those genes are involved in multiple transport systems (*mglB*, *mglA*, and *mglC*: ABC superfamily, galactose transporters) and outer membrane proteins (*ompS1*: putative porin, *tsx*: nucleoside channel receptor) (Kollanoor Johny *et al.*, 2017).

Similar transcriptional profiles were established by Kun *et al*. (2019) by subjecting *S. typhimurium* strain S6 to acid stress, showing the differentially regulation of 1461 genes with up-regulation of 721 genes and down-regulation of 740 genes. The majority of these genes were clustered to transport, pathogenesis, regulatory and metabolism gene groups. Out of these, 28 genes associated with ABC transporters were up regulated upon

exposure to acid, such as *fhuB* (iron-hrdoxamate transporter), *fepB* (iron-enterobactin transporter), *znuA* and *znuC* (ABC superfamily high affinity Zn transport protein), and *yej* genes (microcin C ABC transporter permease YejB) inside host macrophage, elucidating the importance of these genes in encoding efflux pump and responsibility for antibiotic resistance (Kun *et al.*, 2019).

Owen *et al*. (2020) showed that transcriptome analysis of VRE treated with the formulation at 60 minutes, identified genes related to transportation in the bacterial cell were differentially regulated. Out of these genes, five genes were chosen to be validated using qPCR in the present study due to their relation to transportation systems in the bacterial cell. In order to understand what is happening at selected time points a time course study was conducted. Two time points earlier to 60 minutes (10 and 30 minutes), and two-time pointes later to 60 minutes (120 and 360 minutes) were chosen. Evaluating the expression levels of the bacterial genes at different time points, presents information on how these genes are regulated in response to the applied stress (Lowe *et al.*, 2017).

The results of the conducted growth curves showed that *E. faecium* (VRE) has grown in the presence of the individual EO components, EO components together and in combination with vancomycin (Figure 3.1), and with the time course study displaying up and down-regulation of the five genes of interest at the intended MICs in response to all treatment conditions over time (Figures 3.3-3.7).

Bacterial growth started to be reduced after 10 minutes of being treated with EOs-vancomycin combination. The gene expression levels were investigated thoroughly at

this time point of treatment to establish the action of the formulation. It was found that the formulation has induced a significant down-regulation of the genes that encode Na^+/H^+ antiporter (*nhaC_2*), calcium transporter (*yloB*) and in particular the major facilitator superfamily transporter (*bcr*). Thus so, it was thought that the formulation may be through these transportation ways was able to display its antimicrobial effect upon exposure for 10 minutes. Comparing the results at 10 minutes with those at 60 minutes, there were significant differences in the effect of the EOs-vancomycin combination on the expression levels of the genes of interest between the two time points (Figures 3.2-3.7), which could be demonstrating that the formulation action on the transcriptomic level started at the early stages and was reduced or increased over time in accordance to the function of each gene investigated here.

Interestingly, at 30 minutes vancomycin induced significant down-regulation of *ecfA1*, which requires more understanding in line with the mechanism of action of vancomycin, that may be vancomycin has required energy to display its antibacterial action. And when comparing it to the growth curves (Figure 3.1), it was noted that the bacterial growth was inhibited by vancomycin alone at this time point; however, this inhibitory effect of vancomycin was not carried on for further time point (Figure 3.4).

Up-regulation of *bcr*, *nhaC_2* and *yloB* was noted upon exposure to the formulation at 30 minutes, which could be explained that the bacterial cell is trying to combat the applied stress by the formulation through expressing genes related to efflux systems, to expel the treatment components outside the bacterial cell (Figures 3.3, 3.6 and 3.7).

However, at 120 minutes, there was up-regulation to all five genes investigated under the effect of all treatment conditions with significant differences in comparison with the control (Figures 3.3-3.7). This could be explained by that the bacterium is trying to survive under the applied stresses and restore the stable state through over expressing genes that are related to resistance mechanisms as such transport channels and efflux pumps. Thereby, the bacterial cell will be able to pump out the treatment conditions, and to restore the intracellular concentrations of the ions that were disturbed by the applied stresses and are essential for the bacterial life cycle stability.

An interesting response of the bacterial cell towards the formulation at 360 minutes was noted, where the bacteria significantly over-expressed the genes that are related to efflux pumping the combination, and those genes that are needed for providing the nutrients for the bacterial cell. This response could be explained by hypothesising that the bacterium at that time point was able to overcome the action of the formulation (EOs-vancomycin) (Figures 3.3 and 3.5).

Figure 3.8 summarizes the significant expression levels of genes (*bcr*, *ecsA-1*, *yloB* and *nhaC_2*) investigated in the current study in response to formulation at time points of 10, 30, 120 and 360 minutes, as detailed in Section 3.3.5 (Figures 3.3f, 3.4f, 3.5f, 3.6f and 3.7f).

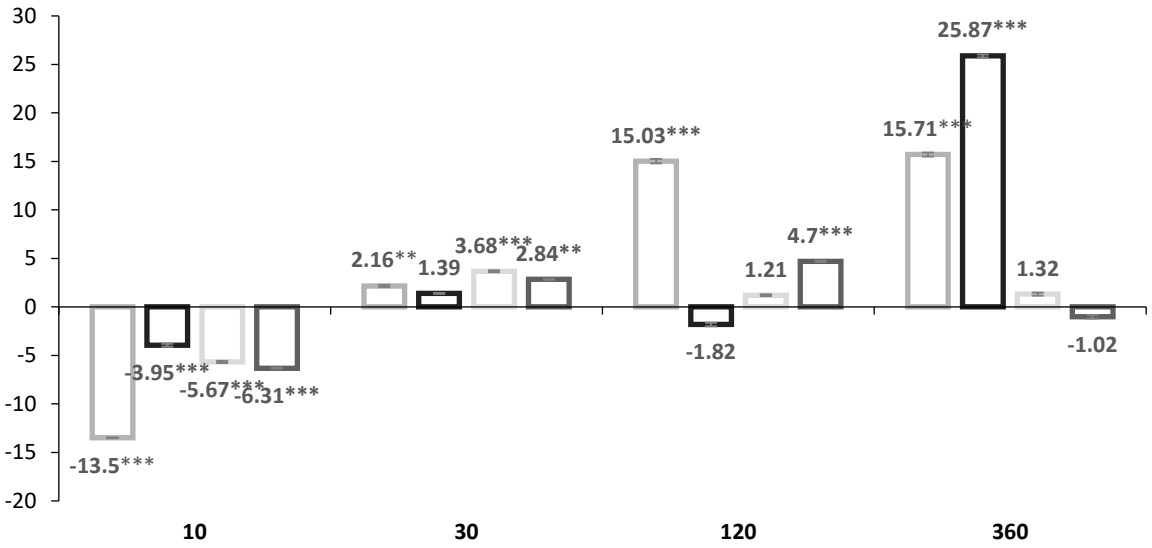


Figure 3.8. Changes in gene expression of *bcr*, *ecsA-1*, *yloB* and *nhaC_2* in response to EOs in formulation over time. The fold change of ■ *bcr*, ■ *ecsA-1*, ■ *yloB* and ■ *nhaC_2* genes were measured using qPCR at 10, 30, 120 and 360 minutes under the effect of the formulation of 1.98 mM carvacrol, 4.20 mM cuminaldehyde and 0.031 mg/l vancomycin. Fold change was calculated as $2^{-\Delta\Delta Cq}$ against reference genes *rpoD* and *dnaB_2,* *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$ (n= 3, ±SEM).

ecsA_1 genes encode transporters that are important virulence factors in bacteria due to their rule in uptake and efflux toxins and antimicrobial agents (Davidson and Chen, 2004). It was noted from the data of the current study that at 10 minutes treatment with the formulation there was down regulation by 4-fold change, that might be due to the importance of *ecsA_1* gene in virulence; and thus so, the formulation has overcome the function of this gene for a while. In contrast, the results at 120 minutes treatment showed that there was up-regulation with the components but not with the formulation. However, there was a significant effect by 25.87-fold up-regulation at 360 minutes with the formulation, that could be related to the thought that the bacterial cell was combating to overcome the antibacterial effect of the formulation by

overexpressing these genes to efflux pumping these components through channels and to uptake the nutrients from the surrounded area (Figure 3.5).

The superfamily of P-type ATPase composes of integral membrane proteins that transfer ions; the function proteins of calcium translocation P-type ATPase are encoded by *yloB* gene. These proteins are localised to the cytoplasmic membranes in the prokaryotes, and supposed to protect the bacterial cells from excessive environmental stress circumstances (Bonza *et al.*, 2010). The transcriptional profile of *yloB* gene was significantly fluctuating over the time points investigated, this could be due to the theory that the formulation has used channels to affect *E. faecium* (VRE) and these channels might be the calcium channels. And when the formulation first entered the bacterial cell through the calcium channels, it had to downregulate the expression of *yloB* to prevent the Ca^{2+} efflux; and thereby, the cell will not be in need for Ca^{2+} to be influx while the channels are being used by the formulation. Thus, this transcriptomics was expressed or repressed accordingly to overcome this effect of the formulation and maybe to either influx or efflux Ca^{2+} (Figure 3.6).

In response to pore-forming stimuli, bacterial cell overexpresses the intended genes in order to survive in the osmotic challenges of the surrounded environment. *nhaC_2* genes encode the Na^{+}/H^{+} antiporter, where in the case of increased cell membrane permeability to ions such as Na^{+}, the antiporters react appropriately to maintain low levels of intracellular concentrations of these ions (Cohen, 2014; Ito *et al.*, 2017). In the current study, the transcriptional response of *nhaC_2* gene was fluctuating throughout the time points under the effect of all treatment conditions, that could be explained that

the cell was trying to get rid of the increased intracellular concentrations of these components through expressing or repressing *nhaC_2* to antiport them against their gradient concentrations that are higher in the surrounded area (Figure 3.7).

Exposure to vancomycin alone has induced expression of *bcr* gene; this alteration mainly was not significant at all points of the time course study (Figure 3.3). This action in response to vancomycin was interesting as this transcriptomic is not related to cell wall precursors forming; wherein, the *van* gene clusters were not altered under the effect of the formulation at 60 minutes exposure as shown in the microarray data (Owen *et al*., 2020). This finding is in line with the results of a study about the acquired resistance to bacitracin in *E. faecalis* that is mediated by a novel regulatory protein BcrR, encoded by *bcrABD* operon. By conducting Northern blot analysis, the study found that vancomycin has no induction of the *bcrABD* expression, suggesting that the induction of *bcrABD* expression has no impact on the synthesis of cell wall precursors (Manson *et al.*, 2004).

Generally speaking, the time course study has elucidated that the main action of the formulation has happened at early stage of 10 minutes rather than later time points; with fluctuation of regulation over time. Moreover, the bacterial recovery response was at the highest at 360 minutes.

The current study shows that EO components alone and in combination have modulated the expression levels of genes that are related to antibiotic resistance either express or repress, and when being combined with vancomycin resulted in significant change of the expression levels of these genes; similar results have been reported by a mini review by Frazzon *et al.* (2018). The mini review stated that *tetK* gene of *S. epidermidis* was

repressed when exposed to a combination of *Salvia fruticose* EO and tetracycline. The resistant gene, *mecA*, in MRSE as well was significantly inhibited in response to combination of *Salvia sclarea* with oxacillin. An 8-fold to 155-fold down-regulation has been noted in the expression level of genes involved in virulence and biofilm formation (such as *csgA*, *csgB*, *fimA*, *ecpA* and *ecpR*) in *E. coli* (EHEC) upon exposure to clove oil (Frazzon *et al*., 2018).

3.5 Conclusion

Overall, the five genes of interest are involved in encoding proteins function with transportation through channels in the bacterial cell. Interestingly, it was noted that the main actions of either EOs alone or the formulation have happened at early stages of 10 and 30 minutes, wherein after that, the bacterial cell started to recover from the applied stresses by overexpressing the intended genes in order to restore the stable state. This could be explained by either the EOs alone or the formulation are trying to enter the bacterial cell; they lowered the expression levels of these genes to prevent the bacteria from pumping out these components to the surrounded environment. Even though, after certain time the bacteria was able to combat the applied stresses through over-expressing the relative genes. In addition, many potential factors could have affected the susceptibility of *E. faecium* (VRE) towards the formulation, including the alteration of the permeability of the bacterial membrane that was disturbed by the action of the EOs.

The results of the present study elucidate that the antimicrobial action of EOs-vancomycin combination is accompanied by up-regulation and down-regulation of

genes related to the pathogenicity capability of the microorganism. Moreover, this study displays that treating *E. faecium* NCTC12202 (VRE) with EOs (carvacrol and cuminaldehyde) alone and in combination with vancomycin for different time scales, has resulted in fold change in the expression levels of the genes of interest. These changes could be considered in formulating novel antimicrobials with new mechanisms of action with consideration of how these new antimicrobials will reach their targets, and when to release the effective components in order to reduce the pathogenicity and the virulence of the bacteria.

Chapter 4

4 The influence of calcium on the expression levels of *yloB,* involved in calcium transportation, in response to the formulation

4.1 Introduction

Findings in relation to *yloB* gene, responsible for calcium transportation, in chapter three showed that *yloB* was significantly upregulated and downregulated under the effect of the formulation (carvacrol, cuminaldehyde and vancomycin) over a period of time; suggesting the involvement of a calcium transportation system in the mechanism of action of the formulation. Therefore, the work in this chapter is to establish greater understanding of the mechanism of action of the formulation through assessing the effect of extracellular concentration of Ca^{2+} on *Enterococcus faecium* (VRE) constructing different detection methods, such as looking at the increase/decrease in bacterial growth, detecting the changes in the intracellular Ca^{2+} concentration through measuring the fluorescent signal, and establishing the transcriptomic profiles of *yloB* gene under the effect of all treatment conditions in the presence of extracellular concentration of Ca^{2+} over time.

Ca^{2+} was thought to include in the current study due to that divalent cations such as Mg^{2+} and Ca^{2+} participate in the stability of the bacterial cell wall and activate the formation of ionic bridge among the bacterial cells through interaction between biopolymers matrix and negatively charged cell membranes, i.e. it influences bacterial aggregation (Das *et al.*, 2014).

Moreover, significant transcriptomic variations of hundreds of genes have happened in bacterial species, such as *Escherichia coli*, *Pseudomonas aeruginosa and Bacillus subtilis*,

when with external concentration of Ca^{2+} (Bruni *et al*., 2017; Naseem *et al*., 2009). Genomic studies have shown up-regulation and down-regulation of hundreds of genes and proteins due to changes in the intracellular Ca^{2+} as well (Domínguez, 2018).

Ca^{2+} has been shown to indirectly be involved in different bacterial cellular actions, such as the mechanisms of transportation across membrane (channels, primary and secondary transporters), cell division and the differentiation processes of the cell (spore formation, heterocyst differentiation, virulence and chemotaxis), elucidating the importance of Ca^{2+} in the physiology of prokaryotes as a regulator (Domínguez, 2018; Duarte *et al.*, 2012; Shemarova and Nesterov, 2014). Moreover, different stages of bacterial infections and the interactions of host-pathogens have included Ca^{2+} signalling. In response to a number of stress signals such as cold and heat shock, bacteria respond by transient changes in the intracellular concentration of free Ca^{2+} [Ca^{2+}] (Domínguez *et al.*, 2015). The Ca^{2+} binding proteins, primary and secondary transporters, and ATP-dependent Ca^{2+} pumps regulate the free cytosolic Ca^{2+} to keep the intracellular concentration of Ca^{2+} in the nanomolar range and to prevent calcium phosphate toxicity (Domínguez, 2018).

Prokaryotes keep a tight intracellular concentration of [Ca^{2+}] which is well regulated, ranging from 100-300 nM. Calcium homeostasis maintain occurs through two mechanisms: secondary calcium/proton or calcium/sodium antiporters; however, *Streptococcus faecalis* was an exception due to it presents ATP-dependent extrusion of calcium using a primary calcium pump rather than a secondary calcium antiporter, in a way similar to that of eukaryotic cells (Houng *et al.*, 1986). It has been reported that in

bacterial cells, Ca^{2+} homeostasis involves ion channels, primary and secondary transporters and Ca^{2+} binding proteins (CaBP) (Dominguez, 2004).

4.1.1 Calcium detection

In all cell types, Ca^{2+} is an essential second-messenger signal. Changes in [Ca^{2+}] can be detected in all live cells at nanomolar levels with either dyes or biosensors. Using indicator dyes or genetically coded biosensors, which specifically bind to Ca^{2+}, it is possible to visualise the calcium gradients and their shifts (Kopp *et al.*, 2014).

Measuring calcium signals in cultured bacterial cells is applied by using calcium imaging. Calcium imaging techniques depend on the indicator dyes, which when bind to Ca^{2+} ions change their spectral properties. There are two categories of calcium indicator dyes, one is ratio-metric dyes including Fura-2 and Indo-1, and another is single-wavelength dyes such as Flu0-4 (Barreto-Chang and Dolmetsch, 2009).

In response to binding to calcium ions, the ratio-metric dyes change their excitation or their emission spectra which results in determination of the intracellular calcium concentration from the fluorescence emission or excitation ratio at various wavelengths. The ratio signal of the ratio-metric dyes over single wavelength is independent from the dye concentration, illumination intensity and optical path length, allowing independent determination of [Ca^{2+}] (Barreto-Chang and Dolmetsch, 2009).

Previously in chapter 2, an EO component, capsaicin, was included in the studies as a control. It was reported in the literature that the agonist action of capsaicin is enhanced by the existence of extracellular calcium (Brito *et al.*, 2014), and when TRPV1 channels are activated by capsaicin it resulted in flowing of calcium and sodium ions into the cell; potentially inducing desensitisation of TRPV1 channels (Yang and Zheng, 2017). Calcium

transportation in the bacterial cell is mediated by different genes, such as *yloB* in the current study that was differentially regulated in response to the formulation. Therefore, it was thought to establish the effect of externally added calcium on the antimicrobial action of the formulation on the regulation of *yloB*, through establishing the expression levels of *yloB* in response to capsaicin, to the EOs of interest individually and in formulation, all in the presence of calcium.

4.1.2 Aim and objectives

The investigations in this chapter aim to establish the involvement of a calcium transporting channel, *yloB*, in the mechanism of action of the formulation (carvacrol, cuminaldehyde and vancomycin).

Objectives

- To assess the growth of *E. faecium* (VRE) in the presence of external concentrations of calcium at 0.01, 0.1 and 1 mM.
- To establish the time-kill curves of *E. faecium* (VRE) with all treatment conditions (carvacrol, cuminaldehyde, EO components together, vancomycin and the formulation) and in the presence of calcium at 1 mM.
- To determine the time-kill curves of *E. faecium* (VRE) with capsaicin alone and in the presence of calcium at 1 mM.
- To evaluate the expression profiles of *yloB* gene in the presence of calcium at 1 mM, with capsaicin and with all treatment conditions over the time points of the time course study (Chapter 3) by qPCR.

4.2 Material and Methods

4.2.1 Bacterial growth and sampling

Methods were described previously (Sections 2.2.1-2.2.3 and 3.2.1) unless stated below. Calcium was purchased from Sigma Aldrich Ltd, UK (C1016-100G), and was prepared at concentrations of 0.01, 0.1 and 1 mM to be used in establishing growth curves. Ca^{2+} was included at a concentration of 1 mM to validate the expression of *yloB* gene with 1 mM Ca^{2+} alone, 1.6 mM capsaicin and all treatment conditions (carvacrol, cuminaldehyde, EO components together, vancomycin and the formulation) at defined time points (10, 30, 60 and 120 minutes). The time points were that when the expression of *yloB* gene was significantly regulated as shown in Figure 3.6. All materials were freshly prepared or were used from stock solutions.

4.2.2 Viable counts

Viable counts were conducted to establish the effect of 0.01, 0.1 or 1 mM Ca^{2+} on *E. faecium* growth at time points of 0, 10, 30, 60, 120 and 360 minutes. Aliquots of 100 µl of Ca^{2+} stock solutions at the intended concentrations were added to 9.9 ml of mid-exponential grown bacterial suspensions were sub-cultured in 9.9 ml of BHI broth to get ~ 10^6 CFU/ml. The inoculated bacterial suspensions were incubated at 37°C with shaking (100 rpm) for the experiment durations. Aliquots of 100 µl were transferred into 900 µl of neutraliser (Section 2.2.7), and were incubated for 5 minutes at room temperature. Serial dilutions were prepared in 9.9 ml PBS as required and were plated on BHI agar using spiral plater, and were enumerated after being incubated at 37°C for 18-24 hours.

4.2.3 Growth curves with calcium and capsaicin

To establish the effects of adding calcium on the inhibitory action of capsaicin on the *E. faecium* (VRE) growth curves, aliquots of 100 µl DMSO (1 %) were added to each 9.9 ml of ˜ 10^6 CFU/ml bacterial suspensions were grown to mid exponential phase with 1 mM Ca^{2+} and the following conditions and durations; 1 mM Ca^{2+} alone, 1.6 mM capsaicin alone, or capsaicin in combination with 1 mM Ca^{2+} for 5, 10, 30, 60, 120, 240, 360 minutes and 24 hours. Aliquots of 100 µl were transferred into 900 µl of neutraliser, and were incubated for 5 minutes at room temperature; thereafter, plated on BHI agar using Spiral plater. The inoculated plates were incubated overnight at 37˚C; then, enumerated.

4.2.4 Detection of intracellular Ca^{2+} changes in the presence of capsaicin

In order to monitor the intracellular Ca^{2+} changes in *E. faecium* exposed to capsaicin, a protocol of loading fluorescent indicator dye (Fura-2/AM) into the bacterial cells was applied (Clementi *et al.*, 2014). Probenecid and Powerload are required for effective loading into bacteria, where probenecid increases dye retention by blocking anion-transporters, and Powerload increases loading efficiency. Fura-2/AM monitors Ca^{2+} changes by measurement of the resulting alterations to fluorescence, detected using a fluorescence detection plate reader, SpectraMax M5 (MVD2364) (Molecular Devices, USA). Fura-2/AM (11524766-F1221), PowerLoad Concentrate (100 X) (10429993-P10020) and Probenecid (11529306-P36400) were purchased from Fisher scientific Ltd, UK, and were stored according to the manufacturer instructions. The fluorescence detection method was adapted from Clementi *et al.* (2014).

4.2.4.1 Preparation of bacterial suspension

To prepare the bacterial cells to be loaded with Fura-2/ AM, two colonies of *E. faecium* (VRE) were inoculated in 10 ml of BHI broth and incubated at 37˚C for 18-24 hrs at 100 rpm. Then, aliquot of 100 µl bacterial suspension was added to 9.9 ml of prewarmed BHI broth and incubated at 37˚C until the culture reached mid-exponential growth phase (OD_{600} = 0.2 ~ 10^8 CFU/ml). The bacterial cells were pelleted by centrifugation at 2400 x g for 10 minutes at room temperature, washed twice by removing supernatant and then resuspending the pellet in 10 ml of PBS, and centrifuging again.

4.2.4.2 Reagents and loading buffer preparation

A 5 mM stock of Fura-2/AM dye was prepared by adding 15.5 µl of DMSO in a tube containing 50 µg of the dye. The stock was stored at -20˚C and used within 3 months, as recommended. A stock of 100X probenecid was prepared by adding 1 ml of PBS to 77 mg, stored at -20˚C and used within 6 months. The 1 ml loading buffer was combined with 20 µl 100X Powerload and 2 µl of 5 mM Fura-2 was added. The solution was then vortexed to ensure homogeneity. An aliquot of 960 µl of PBS was added to the mixture followed by 20 µl of 100X probenecid. The mixture was vortexed again and wrapped in foil to be protected from light.

4.2.4.3 Loading *E. faecium* with Fura-2 dye

The mid-exponential growth phase was pelleted and washed as described previously, the supernatant was removed, and the pellet was resuspended in 5 ml of PBS to make a 2X cell suspension. One ml of this cell suspension was added to the same volume of 2X loading buffer containing Fura-2. The reagents were incubated in a water bath at 37˚C

for 75 minutes, protected from light. Following loading with the Ca^{2+} sensitive dye, the cells were washed by centrifuging at 2400 × g for 10 minutes, removing supernatant, and resuspending the cells in 2 ml PBS containing 1X probenecid. This was repeated three times before incubating for a further 30 minutes at 37˚C. The washing protocol was repeated and the cells were finally resuspended in 2 ml of PBS containing 1X probenecid.

4.2.4.4 Fluorescence Detection

The fluorescence detection plate-reader was prewarmed to 37˚C. A 200 µl aliquot of the Fura-2 loaded cells was added to each well of a 96-well plate (15214566) (Fisher Scientific Ltd, UK). The plate was sealed with a permeable barrier sheet to avoid evaporation and cross contamination. In order to establish baseline readings, fluorescence readings at an emission wavelength of 510 nm were taken every second for one-minute following excitation at wavelength of 340 nm (value 1) and 380 nm (value 2). The plate was ejected and the treatments of choice, calcium at 1 mM and capsaicin at 1.6 mM, were added to the wells (5 µl), pipetted up and down a few times to mix, and immediately placed back in the fluorescence detection plate-reader to be read on set up intervals for desired length of time, every 36 seconds for 10 minutes. The ratio of the fluorescence values was calculated for each time-point by dividing value 1 by value 2. The changes in fluorescent signals were used to qualitatively determine the changes in intracellular calcium concentrations.

4.2.5 qPCR

All methods were described previously (Section 3.2.2) unless stated below.

4.2.5.1 Validation of the expression level of *yloB* gene

qPCR was conducted to evaluate the expression level of *yloB* gene at 10 minutes in the presence of 1 mM Ca^{2+}, 1.6 mM capsaicin alone, and a combination of Ca^{2+} and capsaicin; and over defined time points with significant alterations in the expression level of *yloB* upon exposure to either the formulation or its individual components, all in the presence of 1 mM Ca^{2+}.

4.2.6 Statistical analysis

Methods were described previously (Section 2.2.7 and 3.2.3) unless stated below. The effects of the Ca^{2+} with either capsaicin, EO components, EOs combination, vancomycin, or the EOs-vancomycin formulation on the expression levels of *yloB* gene were carried out in biological triplicate and technical triplicate.

4.3 Results

The EOs included in the formulation differentially altered the expression levels of a wide range of genes as shown in the microarray (Owen *et al*., 2020), and most importantly in the current study those that are involved in transportation in the bacterial cell, with the time course study displaying significant regulation of *yloB*, involved in calcium transportation, in response to the formulation and its components over time. The expression level of *yloB* gene was differentially regulated in response to the formulation at 10 and 30 minutes and to all treatment conditions from 60 to 120 minutes (Figure 3.6). Due to this regulation profile of *yloB*, it was thought to establish the effect of externally added Ca^{2+} on the expression levels of *yloB* in response to selected treatment conditions (carvacrol, cuminaldehyde, EOs together, vancomycin or the formulation) at selected time points (10, 30, 60 or 120 minutes). The selected time points and treatment conditions showed significant alterations in the expression level of *yloB* when in the absence of Ca^{2+} as shown in Figure 3.6.

4.3.1 Bacterial growth curves with calcium

The effect of Ca^{2+} (0.01, 0.1 or 1 mM) was studied on bacterial cell growth at a number of time points (0, 10, 30, 60, 120 and 360 minutes), to find out how introducing extracellular concentration of calcium in the surrounding environment might affect the bacterial viability (Figure 4.1).

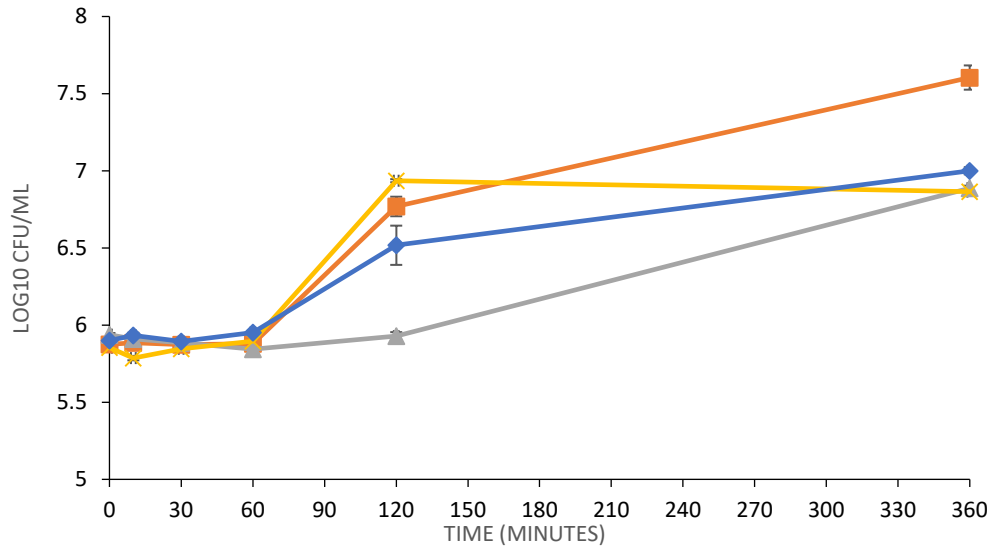


Figure 4.1. The effect of extracellular concentration of calcium at 0.01, 0.1 or 1 mM on the viability of *E. faecium* (VRE). ■ *E. faecium* (VRE) control growth curve. ▲ *E. faecium* (VRE) growth curve with 1 mM Ca^{2+}. ◆ *E. faecium* (VRE) growth curve with 0.1 mM Ca^{2+}. ✱ *E. faecium* (VRE) growth curve with 0.01 mM Ca^{2+} (n= 6, ±SEM).

The bacterial cell counts varied between 5.75 Log_{10} and 5.95 Log_{10} CFU/ml in the case of the control growth and in the presence of calcium at the intended concentrations without significant different in the viable count of the bacterial cells, $P > 0.05$ (Figure 4.1). Adding calcium in the concentration range 0.01-1 mM, did not have significant inhibitory effect on bacterial viability ($P > 0.05$) (Figure 4.1); therefore, the subsequent growth curves of VRE with the formulation and its individual components were conducted in the presence of 1 mM Ca^{2+}. Figure 4.2 shows a greater reduction in the bacterial growth when carvacrol, a combination of carvacrol and cuminaldehyde, and the formulation (carvacrol, cuminaldehyde, and vancomycin) are added with 1 mM Ca^{2+} in comparison to control bacterial growth (1 % DMSO) alone ($P \leq 0.05$). Where carvacrol in the presence of 1 mM Ca^{2+} inhibited the bacterial growth from 5.55 Log_{10} CFU/ml to

4 Log_{10} CFU/ml, ($P \leq 0.05$); similarly, the combination of carvacrol and cuminaldehyde when with 1 mM Ca^{2+} reduced the bacterial cell count from 5.66 to 3.42 Log_{10} CFU/ml, ($P \leq 0.05$); in addition, 1 mM Ca^{2+} increased the inhibition effect of the formulation by reducing the bacterial growth from 5.65 to 3.94 Log_{10} CFU/ml, ($P \leq 0.05$) (Figure 4.2). However, adding 1 mM Ca^{2+} to either cuminaldehyde or vancomycin has not induced greater reduction in the bacterial growth of VRE in comparison to the control (1 % DMSO) alone ($P > 0.05$) (Figure 4.2).

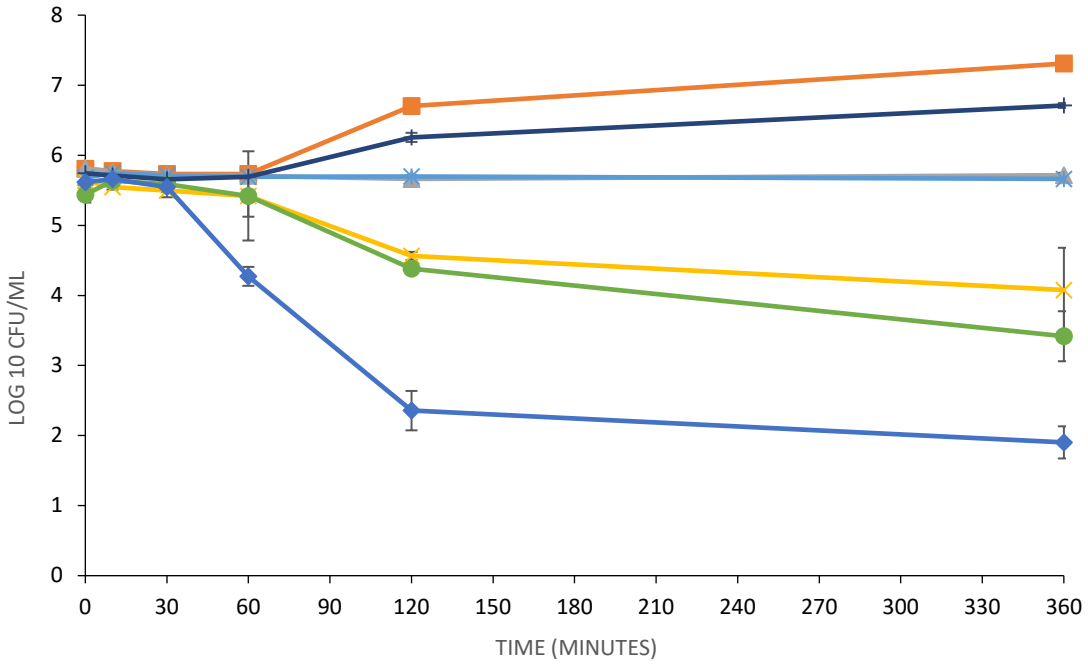


Figure 4.2. The effect of extracellular calcium (1 mM) on *E. faecium* (VRE) growth curves with all treatment conditions over time. ■ *E. faecium* (VRE) control growth curve. ▲ *E. faecium* (VRE) growth curve with 1 mM Ca^{2+}. ✖ *E. faecium* (VRE) growth curve with 3.95 mM carvacrol and 1 mM Ca^{2+}. ✖ *E. faecium* (VRE) growth curve with 537.65 mM cuminaldehyde and 1 mM Ca^{2+}. ● *E. faecium* (VRE) growth curve with carvacrol, cuminaldehyde and 1 mM Ca^{2+}. ▌ *E. faecium* (VRE) growth curve with 256 mg/l vancomycin and 1 mM Ca^{2+}. ◆ *E. faecium* (VRE) growth curve with the formulation (1.98 mM carvacrol, 4.20 mM cuminaldehyde and 0.031 mg/l vancomycin) and in the presence of 1 mM Ca^{2+} (n= 6, ±SEM).

Moreover, comparing the growth curves of *E. faecium* (VRE) in the presence of 1 mM Ca^{2+} (Figure 4.2) and with that in the absence of 1 mM Ca^{2+} (Figure 3.1), and with all treatment conditions, showed that the addition of Ca^{2+} significantly increased the inhibition effect of carvacrol from 5.60 to 4.08 Log_{10} CFU/ml, EO components together from 5.19 to 3.42 Log_{10} CFU/ml, and vancomycin from 8.54 to 5.71 Log_{10} CFU/ml ($P \leq 0.05$). Wherein the inhibition effect of either cuminaldehyde or the formulation has not been significantly reduced when with 1 mM Ca^{2+} in comparison to their effects when being added without Ca^{2+} ($P > 0.05$) (Figure 4.2).

4.3.2 Growth curves with calcium and capsaicin

The established MIC of capsaicin towards *E. faecium* (VSE) (Section 2.3.1) was found to be effective against *E. faecium* (VRE), which growth was significantly inhibited by 3 Log_{10} CFU/ml. Growth curves were carried out to ascertain the effect of capsaicin on bacterial viability over time in the presence and absence of Ca^{2+}. The bacterial viability was assessed over a range of time points from 5 minutes to 24 hours. It was noted that the growth inhibition effect of capsaicin either alone or in combination with Ca^{2+} started after 10 minutes of incubation and continued to 24 hours; however, the presence of 1 mM extracellular Ca^{2+} did not significantly affect the level of this inhibition (Figure 4.3).

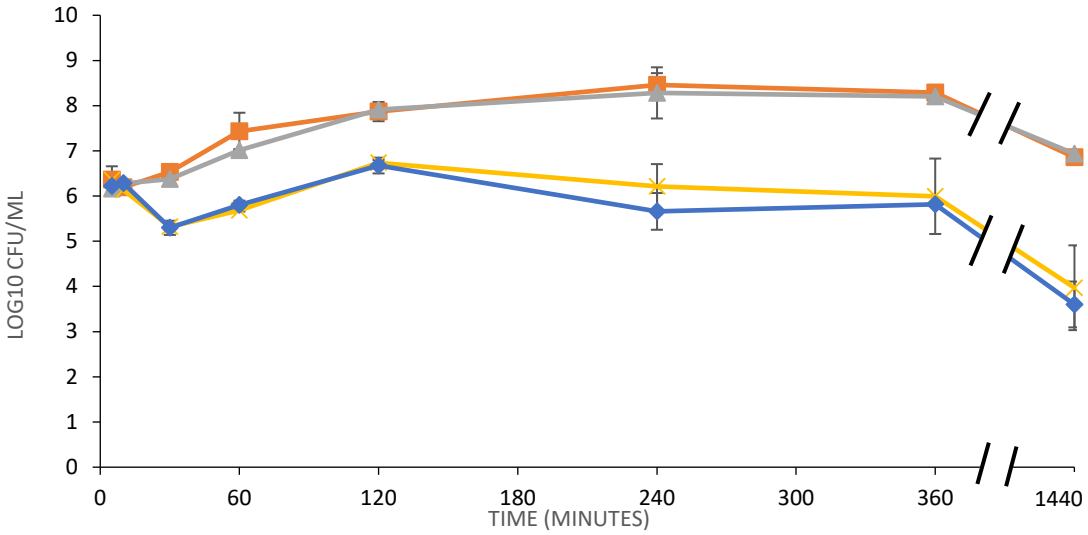


Figure 4.3. The effect of capsaicin alone on the viability of *E. faecium* (VRE) and in the presence of 1 mM Ca^{2+}. ■ *E. faecium* (VRE) control growth curve. ▲ *E. faecium* (VRE) growth curve with 1 mM Ca^{2+}. ✖ *E. faecium* (VRE) growth curve with 1.6 mM capsaicin. ◆ *E. faecium* (VRE) growth curve with 1.6 mM capsaicin in the presence of 1 mM Ca^{2+}. The assessed time points are; 5, 10, 30, 60, 120, 240, 360 and 1440 minutes (n= 6, ±SEM).

Comparing the growth curve of *E. faecium* under the effect of capsaicin to the control growth curves showed that capsaicin has significantly inhibited the growth of *E. faecium* cells just after 10 minutes to 24 hours of incubation ($P \leq 0.05$); however, the statistical analysis demonstrated that the growth of VRE was significantly inhibited by capsaicin both in the presence and absence of 1 mM extracellular Ca^{2+}. This was found to be the case at all time points (10, 30, 60, 120, 240, 360 minutes and 24 hours).

4.3.3 Detection of intracellular Ca^{2+} concentration in the presence of capsaicin

As detailed above, viability of VRE was not significantly reduced when cells were exposed to capsaicin in the presence of extracellular Ca^{2+} compared with Ca^{2+} free condition. Nevertheless, experiments were conducted to assess the changes in $[Ca^{2+}]$ of bacterial cells exposed to capsaicin in the presence of 1 mM extracellular Ca^{2+}. These experiments employed the ratiometric fluorescent Ca^{2+} indicator Fura-2.

The results demonstrated that applying capsaicin to loaded cells with Fura-2 in the presence of 1 mM Ca^{2+} has led to a significant increase in the intracellular Ca^{2+} concentration above control level ($P \leq 0.05$). The increase in [Ca^{2+}] was reflected on the fluorescent signal ratio that was significantly increased (data not shown).

4.3.4 Expression levels of *yloB* in the presence of calcium

Considering the growth curves data and results from the fluorescence detection experiments, there was a need to understand further if under the same conditions there is an alteration in the expression level of *yloB* gene. Therefore, further experiments were conducted to establish the transcriptomic profile of *yloB* gene upon exposure to 1 mM Ca^{2+}, 1.6 mM capsaicin and a combination of Ca^{2+} and capsaicin for 10 minutes. This time point was chosen because the inhibition effect of capsaicin, as shown in the growth curves (Figure 4.3), has started at 10 minutes and lasted over the 24 hours of incubation.

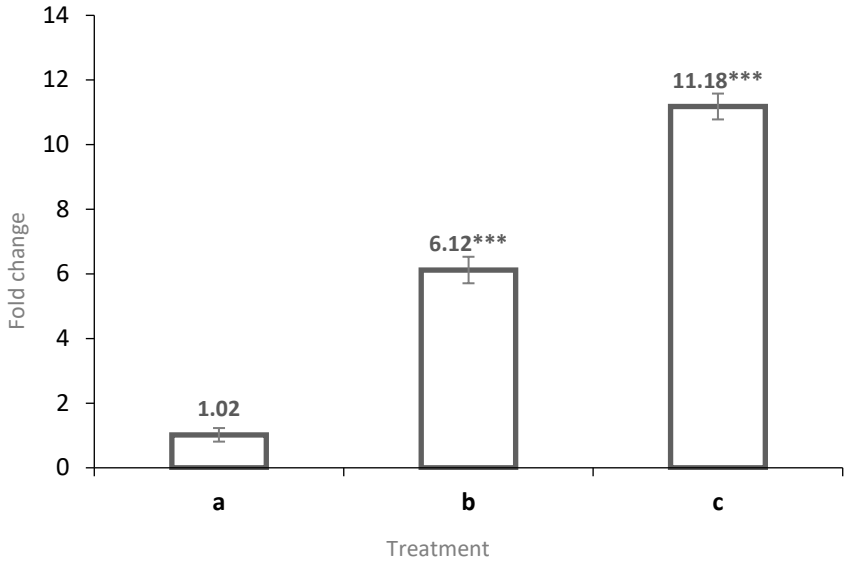


Figure 4.4. Changes in gene expression of *yloB* in response to capsaicin and in the presence of 1 mM Ca^{2+} at 10 minutes. The fold change of *yloB* gene was measured using qPCR at 10 minutes, under the following conditions; **a)** control DMSO 1 %; **b)** 1.6 mM capsaicin; **c)** 1.6 mM capsaicin in the presence of 1 mM Ca^{2+}. Fold change was calculated as $2^{-\Delta\Delta Cq}$ against reference genes *rpoD* and *dnaB_2,* *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$ (n= 3, ±SEM).

As shown in Figure 4.4, in Ca^{2+} free conditions capsaicin increased the expression level of *yloB* gene by 6.12-fold. In the presence of extracellular Ca^{2+} (1 mM) this increased to 11.18-fold. According to the literature, the bacterial cell keeps tight [Ca^{2+}] and to maintain calcium homeostasis the Ca^{2+} transportation systems are of great importance (Gupta *et al.*, 2017, Bonza *et al.*, 2010). Thus, the increased expression level of *yloB* gene could have induced more movement of calcium to the extracellular environment through encoding efflux pumps. In the presence of 1 mM calcium further transportation might have occurred and resulted in increasing the

internal concentration of calcium; with the bacteria responding by pumping the excessive calcium outside to combat its toxicity through up-regulation of the expression level of *yloB* gene.

The addition of calcium to capsaicin has almost doubled the expression level of *yloB* and has significantly increased the fluorescence ratio 340/380; whilst not affecting the viability of the bacterial cells. These observations might suggest that capsaicin has used the calcium channels to enter the cells prompting more calcium to enter the cell; consequently, the intracellular concentration of calcium would have increased, with the bacterial cell responding by an up-regulation in the expression of *yloB* gene, resulting in reducing the excessive intracellular concentration of calcium by pumping it out of the cell, thereby protecting themselves from the toxicity of increased intracellular concentration of calcium.

4.3.5 The expression levels of *yloB* gene in response to the formulation conditions in the presence of calcium

In accordance to the expression profile of *yloB* under the treatment conditions at defined time points of the time course study (Figure 3.6), the transcriptional profile of *yloB* across these time points with similar treatment conditions but with the addition of 1 mM Ca^{2+} should be considered. Treating *E. faecium* with EOs-vancomycin combination in the presence of 1 mM Ca^{2+} for 10 minutes led to a 50- fold down-regulation in the expression level of *yloB* gene (Figure 4.5).

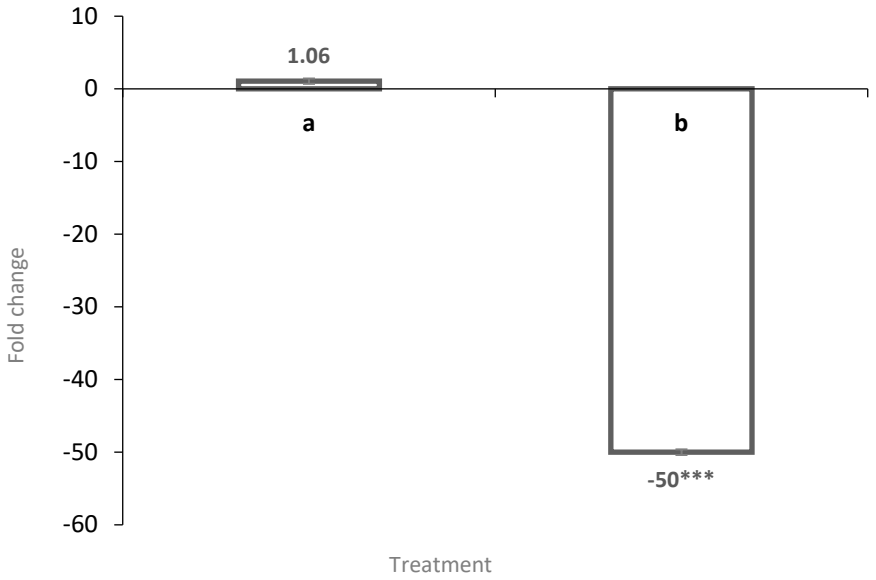


Figure 4.5. Changes in gene expression of *yloB* in response to the formulation and in the presence of 1 mM Ca^{2+} at 10 minutes. The fold change of *yloB* gene was measured using qPCR at 10 minutes under the following conditions; **a)** control DMSO 1 %; **b)** the formulation (1.98 mM carvacrol, 4.20 mM cuminaldehyde and 0.031 mg/l vancomycin) and in the presence of 1 mM Ca^{2+}. Fold change was calculated as $2^{-\Delta\Delta Cq}$ against reference genes *rpoD* and *dnaB_2,* *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$ (n= 3, ±SEM).

Under the effect of the formulation (carvacrol, cuminaldehyde and vancomycin) and in the presence of Ca^{2+} at concentration of 1 mM for 30 minutes, there was only a 4.4-fold decrease in the expression level of *yloB* gene, in contrast to the 3.68-fold up-regulation in the expression level of *yloB* observed in the absence of calcium (Figure 3.6). The current effect of the formulation on the expression of *yloB* was less in comparison to what has happened at 10 minutes, where there was a 50-fold down-regulation (Figure 4.5), which is possibly due to that the main action of the formulation is happening at the early stages of treatment (Figure 4.5). However, the action of the formulation continued to happen at 30 minutes when with 1 mM Ca^{2+} resulting in repressing of *yloB* (Figure 4.6).

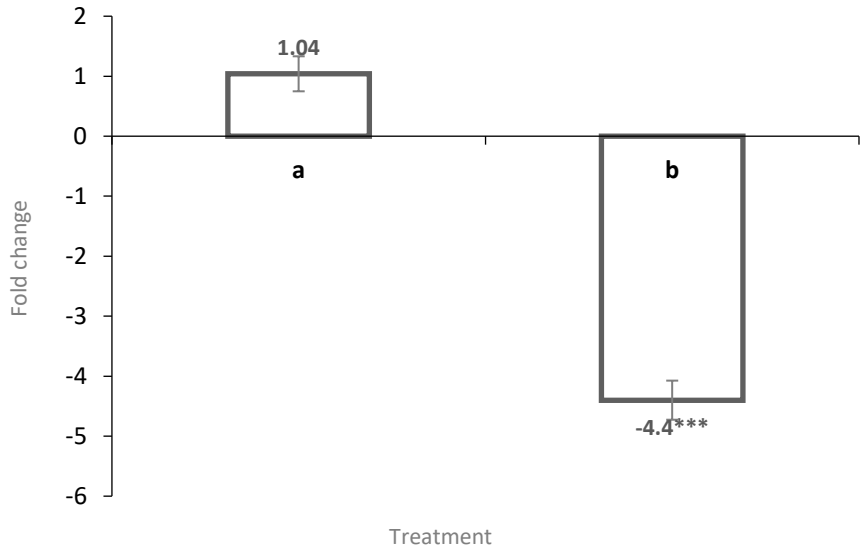


Figure 4.6. Changes in gene expression of *yloB* in response to the formulation and in the presence of 1 mM Ca^{2+} at 30 minutes. The fold change of *yloB* gene was measured using qPCR at 30 minutes under the following conditions; **a)** control DMSO 1 %; **b)** the formulation (1.98 mM carvacrol, 4.20 mM cuminaldehyde and 0.031 mg/l vancomycin) and in the presence of 1 mM Ca^{2+}. Fold change was calculated as $2^{-\Delta\Delta Cq}$ against reference genes *rpoD* and *dnaB_2,* *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$ (n= 3, ±SEM).

Moreover, the addition of Ca^{2+} at 1 mM to all treatment conditions for 60 minutes has altered the transcriptional behaviour of the bacterium by up-regulating the expression level of *yloB* by 4.36-fold with carvacrol, 6.24-fold with cuminaldehyde, 6.6-fold with the formulation and 2.1-fold change with the EO components combination. Interestingly under these conditions, vancomycin led to a down-regulation in the expression level of this gene by 2.4-fold (Figure 4.7).

In the absence of calcium, the transcriptional profile of *yloB* at 60 minutes was down-regulated under all treatment conditions (Figure 3.6), while in the presence of calcium the expression level of *yloB* gene was up-regulated with all treatment conditions except for vancomycin it was a 2.4-fold down-regulated (Figure 4.7). It is possible that the

existence of calcium has increased the action of the formulation, and since it is possible that the formulation is using the calcium channels to enter the bacterial cell more calcium has entered the cell from the surrounded area. In order to get rid of the toxic effect of the different treatment conditions together with the toxic effect of increased [Ca^{2+}], the bacterium responds by up-regulating the expression level of *yloB* gene. This enables bacterium recovering and efflux-pumping the stress factors. This combined with the growth curves where decreases in the bacterial viability has been observed at 60 minutes (Figures 4.2 and 4.7), suggesting that *yloB* or other calcium channels are involved.

Despite the bacterium trying to overcome the applied stresses (EO components, a combination of EOs, vancomycin and a formulation of EOs-vancomycin), the effect of vancomycin was to cause a down-regulation of transcript levels by 2.4- and 2.07-fold in the presence and absence of calcium, respectively (Figure 4.7; Figure 3.6). This demonstrates that the action of vancomycin was unaffected by changes to extracellular calcium concentration. Nevertheless, the antibacterial action of vancomycin is through binding to precursor terminus of D-Ala-D-Ala and not in a calcium-related manner. Thus, possibly being bound to these precursors, vancomycin has displayed less effect on the transcriptional profile of *yloB* gene.

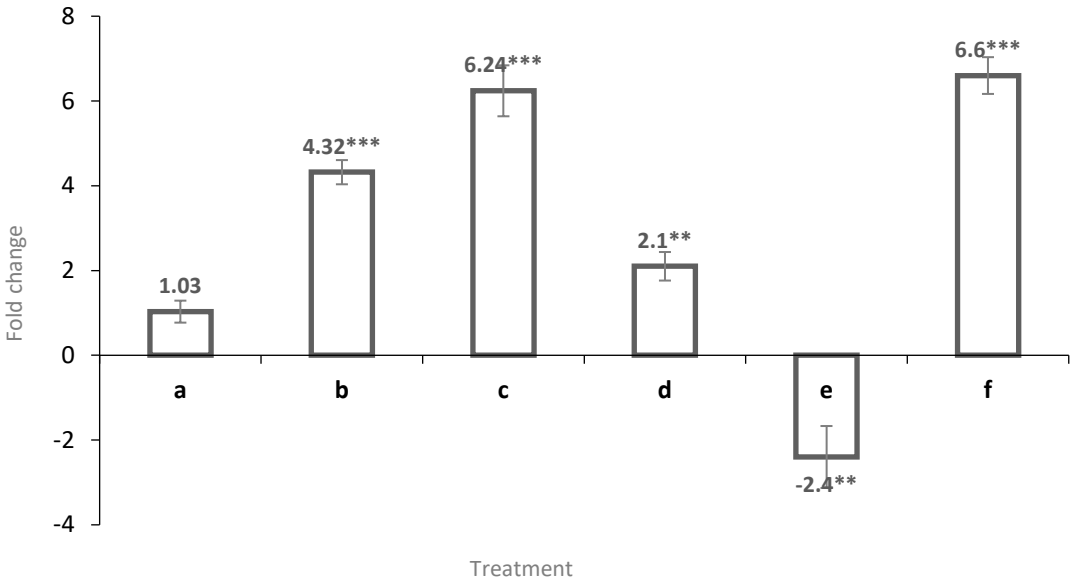


Figure 4.7. Changes in gene expression of *yloB* in response to all treatment conditions and in the presence of 1 mM Ca^{2+} at 60 minutes. The fold change of *yloB* gene was measured using qPCR at 60 minutes under the following conditions; **a)** control DMSO 1 %; **b)** 3.95 mM carvacrol; **c)** 537.65 mM cuminaldehyde; **d)** 0.49 mM carvacrol and 8.40 mM cuminaldehyde; **e)** 256 mg/l vancomycin; **f)** the formulation (1.98 mM carvacrol, 4.20 mM cuminaldehyde and 0.031 mg/l vancomycin) and all in the presence of 1 mM Ca^{2+}. Fold change was calculated as $2^{-\Delta\Delta Cq}$ against reference genes *rpoD* and *dnaB_2,* *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$ (n= 3, ±SEM).

The transcriptional response of the bacterium at 120 minutes in the presence of Ca^{2+} have elucidated recovery of the bacterial cell from the applied stresses by the EO components and in formulation. However, the effect of vancomycin continued to happen in the presence of Ca^{2+} with a 3.32-fold down-regulation of the expression level of *yloB* gene. And when in the presence of calcium, the vancomycin effect was reflected on the overall formulation effect that was less than its components. The expression level of *yloB* gene was up-regulated by 13.84-fold with carvacrol, 12.89-fold with cuminaldehyde (Figure 4.8), by 6.02-fold with both EO components that is similar to the transcriptional behaviour with the formulation at 60 minutes (Figures 4.7 and 4.8), and

a 3.08-fold up-regulation with the formulation was observed (Figure 4.8). The response towards the applied stress of vancomycin alone and the formulation contrasted with the effects in the absence of calcium at 120 minutes. Where there was an up-regulation by 2.23-fold with vancomycin and no-significant alteration with the formulation (Figure 3.6).

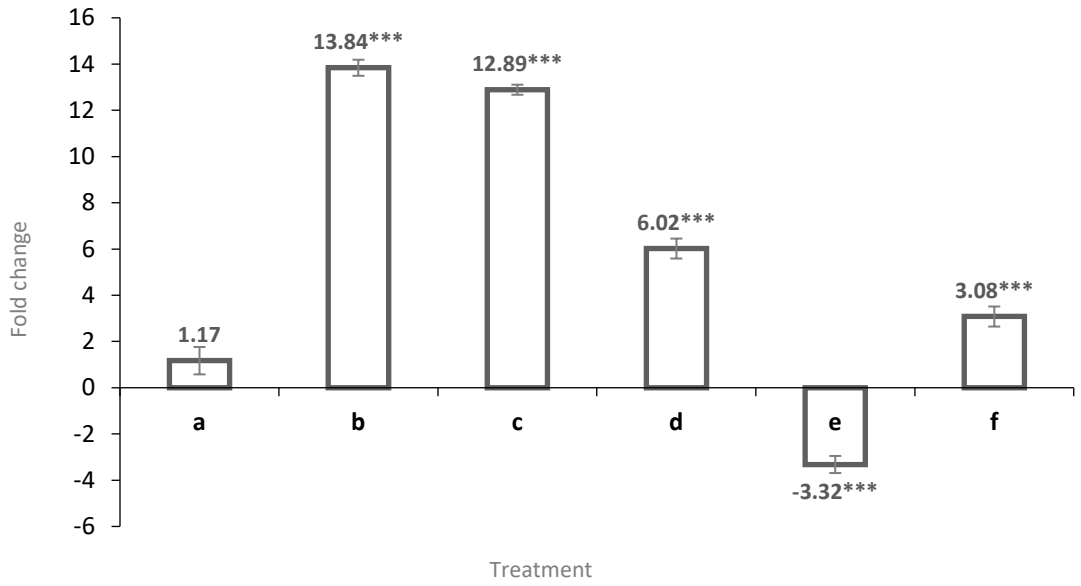


Figure 4.8. Changes in gene expression of *yloB* in response to all treatment conditions and in the presence of 1 mM Ca^{2+} at 120 minutes. The fold change of *yloB* gene was measured using qPCR at 120 minutes under the following conditions; **a)** control DMSO 1 %; **b)** 3.95 mM carvacrol; **c)** 537.65 mM cuminaldehyde; **d)** 0.49 mM carvacrol and 8.40 mM cuminaldehyde; **e)** 256 mg/l vancomycin; **f)** the formulation (1.98 mM carvacrol, 4.20 mM cuminaldehyde and 0.031 mg/l vancomycin) and all in the presence of 1 mM Ca^{2+}. Fold change was calculated as $2^{-\Delta\Delta Cq}$ against reference genes *rpoD* and *dnaB_2,* *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$ (n= 3, ±SEM).

4.4 Discussion

The investigations in this chapter were to ascertain the importance and involvement of a calcium transport system in the antimicrobial mechanism of action of the formulation. Moreover, to understand how *yloB* gene is differentially regulated when subjected to all treatment conditions, including EO components (carvacrol and cuminaldehyde), a combination of EO components, vancomycin and the formulation, over the time points of the time course study of 360 minutes as established in chapter 3.

Calcium is a divalent cation, and divalent cations are known to be essential bridging ions for bacterial polysaccharides and are assumed to take part in regulating gene expression (Sarkisova *et al.*, 2005). In addition, calcium plays a regulatory role for transporters and channels (Norris *et al.*, 1996).

The presence of extracellular Ca^{2+} in addition to all treatment conditions did not result in cell death or have an effect on the viability of the bacterial cells. However, carvacrol, EOs in combination, and vancomycin have significantly reduced the bacterial viability in the presence of 1 mM Ca^{2+} as shown in growth curves, and an increase, although not significant, was noted in the effect of cuminaldehyde and the formulation (Figure 4.2).

Introducing calcium alone and in combination with all treatment conditions of the study; including EO components, a combination of EO components, vancomycin and the formulation, has imposed a stress on the different functions of the bacterial cell from 10 to 30 minutes of being exposed to all conditions.

Subjecting the bacterial cells to capsaicin in the presence of 1 mM Ca^{2+} did not increase the inhibitory effect of capsaicin on *E. faecium* viability (Figure 4.3). Nevertheless,

evaluating the changes in [Ca^{2+}] showed an increased fluorescent signal when in the presence of calcium with capsaicin in comparison to calcium alone as control (data not shown). The increase in [Ca^{2+}] could be due to that capsaicin having increased the transportation of calcium across the membrane resulting in an accumulation of intracellular calcium. Similar results have been observed in *Streptococcus pneumoniae* loaded with Fura-2/AM. When exposed to the calcium ionophore, ionomycin, there was an increase in fluorescent signal reflecting an increased [Ca^{2+}] in comparison to that of the untreated bacterial cells (Clementi *et al.*, 2014). Even though, capsaicin has induced an elevation in [Ca^{2+}], this did not result in toxicity leading to cell death. Where at a genetic level, capsaicin in the presence of calcium induced up-regulation of *yloB* gene which in turn could be efflux pumping the excessive intracellular concentration of calcium and thereby the bacterial cells were not lysed as a result (Figure 4.4). Similarly, a study by Ulasli *et al*. (2014) has shown that the extracts of *Nigella sativa*, *Anthemis hyaline* and *Citrus sinensis* have increased the [Ca^{2+}] levels in coronavirus infected HeLa cells through downregulating the expression levels of TRP genes, where one of the cellular functions of TRPs is to modulate intracellular ion concentrations including calcium as reviewed by Gkika and Prevarskaya (2009). Despite the study being done in mammalian cells, the generic concept displaying effects of some EOs at the molecular level and on the physiological level through increasing [Ca^{2+}].

In addition, the effect of adding calcium in the presence of either to the EO components, a combination of EOs, vancomycin or to the formulation was assessed on the transcriptomic level of *yloB* gene over 120 minutes. The expression level of *yloB* was significantly down regulated at 10 and 30 minutes when being exposed to the effect of

the formulation in the presence of 1 mM Ca^{2+}, where it may have entered the bacterial cell through the Ca^{2+} channels in addition to calcium which was at an extracellular concentration. Consequently, the intracellular concentration of Ca^{2+} has increased, and when the formulation has entered the bacterial cell it prevented *E. faecium* from overcoming the formulation antibacterial action by down-regulating the expression of one of the genes responsible for calcium transportation (Figures 4.5 and 4.6).

Combining vancomycin with the EOs led to a 6-fold down-regulation in the expression level of *yloB* gene after 10 minutes of treatment, demonstrated in Figure 3.7. However, exposing *E. faecium* to similar treatment condition in the presence of 1 mM Ca^{2+} has induced a significant further 50-fold down-regulation in the expression level of *yloB* gene (Figure 4.5). One possible explanation for this is that under normal conditions the bacterial cell controls its [Ca^{2+}] in the presence of an extracellular concentration of Ca^{2+}, which when also presented with the formulation (carvacrol, cuminaldehyde and vancomycin) the regulation of the relative gene/genes, has enhanced the inhibitory effect of the formulation. This may have occurred through mediating the formulation transporting into the cell. This inhibitory effect of the formulation that has significantly increased in the presence of 1 mM Ca^{2+}, could lead to assumption that the formulation has exploited the calcium channels to enter the cell.

The transcriptional profile of *yloB* gene when exposing *E. faecium* to the effect of the formulation in the presence of an extracellular concentration Ca^{2+} for 30 minutes, could be explained by the fact that in order to overcome the effect of the formulation, the bacterial cell was trying to reverse its action; however, it could be that in the presence

of calcium the formulation was able to carry on displaying its effect even though not to same level, where it was 50-fold and 4.4-fold down-regulation in the expression level of *yloB* were noted under the effect of the formulation at 10 and 30 minutes, respectively (Figures 4.5 and 4.6).

E. faecium has shown recovery from the applied stresses after 60 and 120 minutes, this might indicate that in the presence of Ca^{2+}; even though, the formulation has partially restored its action and vancomycin continued to act on the bacterial cell in the presence of extracellular Ca^{2+} (Figure 4.8), similar actions were not observed in the absence of Ca^{2+}. Nevertheless, considering the concept that the formulation or the other treatment conditions could have used calcium transport channels to enter the bacterial cell, and the accumulation of calcium in the bacterial cell resulted in an up-regulation of *yloB*, which could have resulted in the efflux pumping of the calcium and potentially the capsaicin/EOs out of the cell; and thus there was limited effect on the inhibitory action of the formulation at 60 and 120 minutes (Figures 4.7 and 4.8).

Different studies have highlighted the importance of calcium during the life cycle of the bacterial cell. Maintaining the concentration gradient of calcium across the plasma membrane is crucial and involves highly dynamic fluxes through closely regulated channels (Bruni *et al.*, 2017). As outlined in the introduction of this chapter, calcium is known to have important roles in diverse processes, such as the bacterial differentiation, pathogenicity, chemotaxis and sporulation. In addition, it has been observed that exposure to external calcium in bacterial species, results in significant transcriptomic alterations (Domínguez, 2018; Gode-Potratz *et al*., 2010; Oomes *et al*., 2009). The

evidence of involving the Ca^{2+} signalling in cell structure maintenance, gene expression, the processes of cell differentiation and cell cycle has increased in recent years (Hu *et al.*, 2011).

Current published data has shown the effects of either carvacrol alone, EOs in combination, or vancomycin on *E. faecium* viability, were enhanced by the existence of a concentration of Ca^{2+} in the surrounding environment (Figure 4.2). A number of previous studies have reported increased effects of daptomycin, teicoplanin and vancomycin on methicillin-resistant *Staphylococci*, vancomycin-resistant *Enterococci* when extracellular concentrations of Ca^{2+} were increased (Eliopoulos *et al.*, 1986; Fuchs *et al.*, 2000; Greenwood and Palfreyman, 1987; Louie *et al.*, 1992). In addition, at the transcriptomic levels the effect of vancomycin in the current study was changed by the addition of extracellular calcium (Figures 4.7 and 4.8); furthermore, adding calcium in the surrounding environment has enhanced the activity of the formulation (Figures 4.5-4.8).

A study on *S. aureus* has evaluated the effects of cationic ions, such as Ca^{2+} and magnesium (Mg^{2+}). Ca^{2+} and Mg^{2+} are essential alkaline-earth-metal ions (M^{2+}) for almost all living organisms. When these cationic ions bind to cardiolipin (CL), which is a main lipid component in the membrane of *S. aureus*, they create cation-CL complexes of negative curvature, which is an important parameter for different processes of membrane-destabilisation. Moreover, the study findings showed that the viability of *S. aureus* cells decreased in a dose-dependent manner, where the highest tested dose of 40 mM, resulted in ~ 60 % bacterial cell viability loss which equates to an absolute

number density of ~ 3×10^5 CFU/ml. Furthermore, the study concluded that Ca^{2+} and Mg^{2+} could be species-specific bactericidal agents towards *S. aureus* as membrane-active factors (Xie and Yang, 2016). The current study is in line with Xie and Yang study (2016), in the mean of that Ca^{2+} affected the transportation across the cell wall of a Gram-positive bacterium in accordance to the existence concentration. Thus, the effect of externally added calcium on the antimicrobial action of the formulation (EOs-vancomycin) against *E. faecium* VRE could be through catalysing and/or mediating the influx of the formulation into the bacterial cell; though there is a difference between the extracellular concentrations of Ca^{2+} in the above study (40 mM) and the current study (1 mM).

Another study has reported that *B. cereus* exposed to carvacrol demonstrated reduced intracellular potassium ions levels. However, the potassium ion level increased proportionately in the extracellular, maintaining the total amount constant. This action of affecting the intracellular potassium concentration leads to the conclusion that carvacrol is allowing potassium ions to leave the cytoplasm by creating channels in the membrane by pushing apart the fatty acid chains of the phospholipids (Ultee *et al.*, 1999). The current study is in line with Ultee *et al.* (1999) study, where carvacrol increased the expression level of *yloB* gene after 60 minutes of incubation and the effect was carried on until 120 minutes with significant increase in the expression level by ~ 14-fold. Consequently, carvacrol could have increased calcium pumping outside the bacterial cells to reduce [Ca^{2+}]; however, this conclusion needs to be ascertained through measuring the [Ca^{2+}]. Nevertheless, when exposing *E. faecium* VRE to the effect of carvacrol in the presence of calcium, it significantly affected the bacterial viability

which could be due to the antimicrobial action of carvacrol that was reported by Magi *et al.* (2015) to disturb the bacterial cell wall and increase the membrane fluidity and permeability; in addition to that carvacrol reduces ATP synthesis, which is needed for Ca^{2+} transportation. Thus, more carvacrol entering the bacterial cell mediated by calcium, may have led to a decreased calcium efflux; the resultant increase in [Ca^{2+}] could then lead to cell lysis and death.

4.5 Conclusion

The findings of the current study suggest that the formulation has inhibited the growth of *E. faecium* through exploiting the calcium transport channels. Adding calcium to the external environment of *E. faecium* has increased the effect of the formulation and/or its components at the transcriptomic level through down-regulating the expression level of *yloB* upon exposure to all treatment conditions (carvacrol, cuminaldehyde, a combination of EOs, vancomycin, and a formulation of EOs-vancomycin), that would have reduced the efflux of these components and thereby had enough time to display their inhibitory actions. However, the role of calcium channels would need to be investigated more fully.

Chapter 5

5 The effect of an efflux pump inhibitor, EDTA, on the expression level of a VRE efflux pump transporter, *bcr,* in response to the formulation

5.1 Introduction

Due to the interesting transcriptomic profile of *bcr* under the effect of the formulation investigated (1.98 mM carvacrol, 4.20 mM cuminaldehyde and 0.031 mg/l vancomycin) (Figure 3.3), further analysis of this gene was carried out. Previously (Section 3.1.2.1), it was detailed that *bcr* is a member of the major facilitator superfamily that encodes efflux transporters. It therefore was hypothesised that by adding an efflux pump inhibitor, EDTA, alongside the formulation could have made it more effective and this may have an effect on the expression level of the gene of interest or other genes that encode efflux systems; thus, it was thought that EDTA might make the formulation more effective as the formulation will remain in the cell at a higher concentration due to the efflux being inhibited. It was found that the use of efflux pump inhibitors that have one or more of the mechanisms of inhibiting efflux pumps in combinations with antibiotics as adjuncts has been under investigation since the earliest of the 20th century (Lomovskaya *et al.*, 2001). Potentially, the results could provide further understanding of the mechanisms of action of the formulation on VRE at the genetic level.

In response to different environmental conditions and chemical agents, bacteria have been found to tightly regulate the expression of efflux pumps (Sun *et al.*, 2014), that are the main focus of the studies in this chapter.

5.1.1 Efflux pumps

Efflux pumps are widely described as one of the main bacterial constituents that are involved in antimicrobial resistance mechanisms towards a wide range of structurally unrelated conventional antibiotics (Jamshidi *et al.*, 2016). Extensive research has been conducted on efflux pumps to restore the efficacy of conventional antimicrobials towards multi-drug resistant microorganisms (Tegos *et al.*, 2012). Efflux pumps are transmembrane proteins found in bacterial cells, with the ability to exclude toxic compounds and antibiotics from inside the cell to outside mediating bacterial survival (Espinoza *et al.*, 2019). The importance of efflux pumps in bacteria is not only due to their ability to expel varied agents but also to them mediating other resistance's mechanisms through reducing the intracellular concentration of the antibiotic and enhancing the incidence of mutations (Sun *et al.*, 2014).

Efflux pumps are found in both Gram-positive bacteria, such as MRSA, *S. pneumoniae* and *Enterococcus spp.*, and Gram-negative bacteria including; *K. pneumoniae*, *Stenotrophomonas maltophilia* and *P. aeruginosa* (Li *et al.*, 2015; Schindler and Kaatz, 2016). Efflux pump transporters require energy to expel substrates against a concentration gradient and are categorised based on the mechanism they use to derive the energy needed to transport the substrates as explained in (3.1.2) (Kumar *et al*., 2020).

Genes that encode for efflux pumps are found in both antibiotic-susceptible and antibiotic-resistant bacteria, and mostly form part of an operon with a regulatory gene that controls its expression (Webber and Piddok, 2003). Overexpression of efflux pumps

is the result of mutations in the regulatory proteins or at the promoters, leading to drug or multidrug resistance (Webber and Piddok, 2003). Drug resistance due to efflux pumps could be intrinsic or acquired, the intrinsic resistance is encoded by genes found on the chromosome and is most dominant, while the acquired mode of resistance is encoded by genes exist on plasmids or transposons and it is transferable between bacterial cells (Costa *et al.*, 2010; Sharma *et al.*, 2019).

Multidrug efflux transporters have been well described in enterococci, in particular those that belong to MFS and ABC (Lerma *et al.*, 2014). A study by Pazoles *et al.* (2001) established 34 potential genes encoding multidrug resistance transporters in *E. faecalis* using bioinformatics studies, and out of these, 23 were transporters belonging to ABC multidrug transporters (Pazoles *et al.*, 2001).

The first transporters in enterococci to be reported with efflux activity were EmeA, a member of the MFS family and was first identified in *E. faecalis* (Lee *et al.*, 2003), and EfrAB, which is an ABC transporter and was established in *E. faecalis* (Lee *et al.*, 2003).

Efflux pump inhibitors

Inhibiting efflux pumps could be achieved through either; 1) dissipating the energy that is essential for the efflux action (Mahamoud *et al.*, 2007). 2) interfering in genetic regulation leading to down-regulation of the genes encoding efflux pumps. 3) modulating the antibiotic structures and thus they become non-recognisable as substrates. 4) disturbing the construction of functional efflux pumps leading to less ability to interact with the substrates or preventing the substrates from binding to the

site by blocking the pump. Or 5) misfunctioning the energising mechanism of the pump (Bhardwaj and Mohanty, 2012).

Different compounds have been established as efflux pump inhibitors, such as PaβN, polymyxin B nonapeptide (PMBN) and ethylenediaminetetraacetic acid (EDTA); however, due to their toxicity none of them has been utilised in potential medical applications (Lomovskaya and Bostian, 2006; Siriyong *et al.*, 2017).

EDTA is a polyamine carboxylic acid well described as a metal-chelating agent, which was in use for treating poisoning with heavy metal ions such as lead and mercury (De Almeida *et al.*, 2016). EDTA is a permeating and sensitising agent and has also been used as a cure for biofilm-associated infections in dentistry, on medical devices and in veterinary medicine (De Almeida *et al.*, 2016). As reviewed by Finnegan and Percival (2015), combining EDTA with either alcohol, antibiotics, citric acid, silver, surfactants, iodine or other antiseptics, has displayed a controlled effect on microorganisms and biofilms; growing evidence as well is demonstrating that EDTA is an antimicrobial and antibiofilm agent (Finnegan and Percival, 2015). At low concentrations, EDTA is used as a food preservative and in combination with commercial antibiotics (Lerma *et al*., 2014), where the activity of antimicrobial agents, such as piperacillin/tazobactam was considered to be potentiated by the presence of 500 mg/l EDTA (Lambert *et al.*, 2004).

EDTA was found to enhance the action of antibiotics through two mechanisms; it either facilitates the entrance of antibiotics into the cells by binding to metal ions that might be competing with antibiotics on the same receptors in the cell, or it renders the outer membrane more permeable through disrupting the lipopolysaccharides structure

(Lambert *et al.*, 2003; Kumar *et al.*, 2013). In addition to the effect of EDTA in preventing biofilm formation through chelating the divalent ions in the lipopolysaccharide layer of biofilm (Kumar *et al.*, 2013). EDTA as well has been reported to inhibit the action of efflux pumps through calcium ions chelating that are essential for the activity of ATPases (Chaudhary and Payasi, 2013).

The expression of efflux pump genes was found to be inhibited by the effect of different concentrations of EDTA in *E. coli* and *P. aeruginosa* (Chaudhary and Payasi, 2012; Chaudhary, 2012). It was reported that 10 mM EDTA enhanced *P. aeruginosa* susceptibility to a wide range of antibiotics, including piperacillin with tazobactam, amoxicillin with clavulanic acid, imipenem with cilastatin, meropenem and ceftriaxone with sulbactam. Efflux pumps have been well described in *P. aeruginosa*, such as MexA-MexB-OprM, MexC-MexD-OprJ, MexE-MexFOprN, and MexX-MexY-OprM (Chaudhary and Payasi, 2012). The study revealed that EDTA (10 mM) significantly reduced the MICs of meropenem and ceftriaxone with sulbactam by 8 and 32-fold respectively, in addition to the down-regulation of *mexA* (2.93-fold) and *mexB* (3.21-fold) gene expression in *P. aeruginosa* (Chaudhary and Payasi, 2012).

5.1.2 Essential oils and their effect on efflux pumps

To date, several efflux pumps inhibitors have been described (Martins *et al.*, 2008; Pagès and Amaral, 2009; Viveiros *et al.*, 2012), and these compounds are of either natural or synthetic sources (Sharma *et al.*, 2019).

EOs and their components have been shown to be effective against MDR microorganisms such as MRSA and VRE and the mechanism of action has been

suggested to be related to efflux pump inhibition (Espinoza *et al.*, 2019). Recently, studies have investigated the activity of natural products in reversing multidrug resistance mediated by efflux pumps in *S. aureus* strains, such as thymol and carvacrol (Sousa Silveira *et al.*, 2020; Espinoza *et al.*, 2019), 5´-methoxy-hydnocarpin from *Berberis fremontii* (Stermitz *et al.*, 2000), baicalein from *Scutellaria baicalensis* (Chan *et al.*, 2011), boeravinone B from *Boerhavia diffusa* (Singh *et al.*, 2017), and biochanin A and crysoplenetin from *Artemisia annua* (Tegos *et al.*, 2012).

Increased evidence is showing that the activity of natural products being either efflux system regulators or efflux pump inhibitors mediating the reintroduction of ineffective antibiotics back into usage (Pagès and Amaral, 2009; Abreu *et al.*, 2012). The efflux inhibition action of *Rosmairnus officinalis* EOs has been well described in potentiating the antibacterial action of erythromycin and tetracycline against *S. aureus* strains that express efflux pumps of TetK and MsrA (Piddock, 2006; Roberts, 2005).

Similarly, a steroidal alkaloid compound of *Holarrhena antidysenterica*, conessine, was found to restore the antibacterial susceptibility of *P. aeruginosa* towards novobiocin, cefotaxime, levofloxacin, tetracycline and erythromycin, which resistance is mediated by the overexpression of MexAB-OprM efflux pump. Conessine reduces the MICs of all tested antibiotics by at least 8-fold in comparison to those of wild-type strain (Siriyong *et al.*, 2017).

Previously in Chapter 4, the investigations of the effect of the formulation on the expression level of *yloB* gene showed that the action of the formulation and its individual components was enhanced by the presence of externally added 1 mM Ca^{2+} over time.

The *bcr* gene, as discussed in chapter 3, encodes an efflux pump system and was differentially expressed in response to the formulation and its individual components over time as shown in Figure 3.3. Carvacrol, one of the formulation components, has been reported to act as an efflux pump inhibitor (Sousa Silveira *et al*., 2020), efflux pumps require Ca^{2+} ions to derive energy for their action (Chaudhary and Payasi, 2013), and EDTA is a Ca^{2+} chelator (De Almeida *et al*., 2016). Thus, the investigations in this chapter was to assess how externally added EDTA might also alter the effect of the formulation on the expression level of *bcr* gene.

5.1.3 Aim and objectives

The aim of the investigation in this chapter was to determine if adding EDTA at non-inhibitory concentration to the formulation will alter the transcriptional profile of *bcr* gene upon exposing *E. faecium* (VRE) to the effect of the formulation over time.

Objectives

- To determine the non-inhibitory concentration of EDTA against *E. faecium* (VRE).
- To assess the effect of the formulation on VRE growth in the presence of EDTA.
- To establish the expression levels of *bcr* gene under the effect of the formulation and in the presence of EDTA.

5.2 Material and methods

5.2.1 Growth curves

Bacterial cultures and treatment conditions were prepared as described in sections 2.2.3 and 3.2.1. EDTA (10 mM) stock solution (E9884, Sigma Aldrich, UK) was prepared by dissolving 292.2 mg of EDTA in 100 ml dH2O and the pH adjusted to 8.0 with NaOH. The resulting solution was dispensed into aliquots and sterilised by autoclaving at 121˚C, 15 psi for 15 minutes, and were kept at room temperature.

Growth curves were conducted in the presence of a range of EDTA concentrations (0.01 to 5 mM), using the broth suspension method and following guidelines on EUCAST (EUCAST, 2021; Schön *et al.*, 2020).

Aliquots of 100 µl of the bacterial suspension (~1 x 10^7 CFU/ml) were added to each well of a 96-well plate, in addition to 100 µl of two-fold serial dilutions of EDTA at the intended concentration, resulting in a final inoculum of ~1 x 10^6 CFU/ml and the intended concentration of EDTA. The inoculated plates were sealed with permeable adhesive film and placed in the Spectramax plus spectrophotometer plate reader that was set to read the absorbance on hourly intervals and 600 nm wavelength for 18-24 hours. Controls were either bacterial suspension, EDTA at the intended concentration or BHI broth alone.

5.2.2 Viable counts and determination of sub-inhibitory concentration of EDTA

EDTA was assessed to be used at sub-inhibitory concentration for it to display a slight effect, yet non-significant, on the cells viability of *E. faecium* (VRE) when exposed to the formulation over time.

Viable counts were conducted in the presence of 0.025, 0.05 and 0.1 mM EDTA using neutralising method as explained in section 2.2.7. Aliquots of 100 µl of overnight bacterial suspensions were inoculated in 9.9 ml BHI broth and were grown at 37˚C with shaking (100 rpm) to the mid-exponential phase (5-5.30 hours). EDTA was added at the intended concentrations to the bacterial culture and incubated at 37˚C with shaking (100 rpm) and samples reserved for analysis at 10, 30, 60, 120 and 360 minutes, diluted in PBS as needed, and spiral plated on BHI agar plates. The plates were incubated overnight at 37˚C and then enumerated.

5.2.3 Gene expression analysis

All qPCR methods were described previously (Sections 2.2.6-2.2.8 and 3.2.2) unless stated below.

5.2.4 Statistical analysis

Methods were described previously (Sections 2.2.9 and 3.2.3) unless stated below.

5.3 Results

An EDTA concentration (0.1 mM) was established that would not modify the effect of the formulation on the bacterial viability at any of the time points tested. EDTA at 0.1 mM has altered the action of the formulation on the expression level of *bcr* at 60, 120 and 360 minutes; however, this effect of EDTA did not reflect on the bacterial viability when added to the formulation over time. EOs and EDTA were previously assessed by different studies for their synergistic effects with conventional antibiotics (Section 5.1.2). The current investigations were conducted to establish the effect of non-inhibitory concentration of EDTA on the action of the formulation (EOs-vancomycin) at the transcriptomic response of a gene involved in efflux pump system, *bcr*, over time.

5.3.1 Viable counts

The conducted growth curves did not provide sufficient information to determine which concentration of EDTA to carry out for further investigations (data not shown). Therefore, viable counts were established with different concentrations of 0.025, 0.05 and 0.1 mM EDTA. Viable counts with 0.025 and 0.05 mM EDTA did not show any difference between the control growth and the treated samples (data not shown) over the time points investigated. Wherein, viable counts with 0.1 mM EDTA demonstrated small, yet non-significant, inhibitory effect by 0.61 Log_{10} CFU/ml growth reduction of *E. faecium* (VRE) (Figure 5.1).

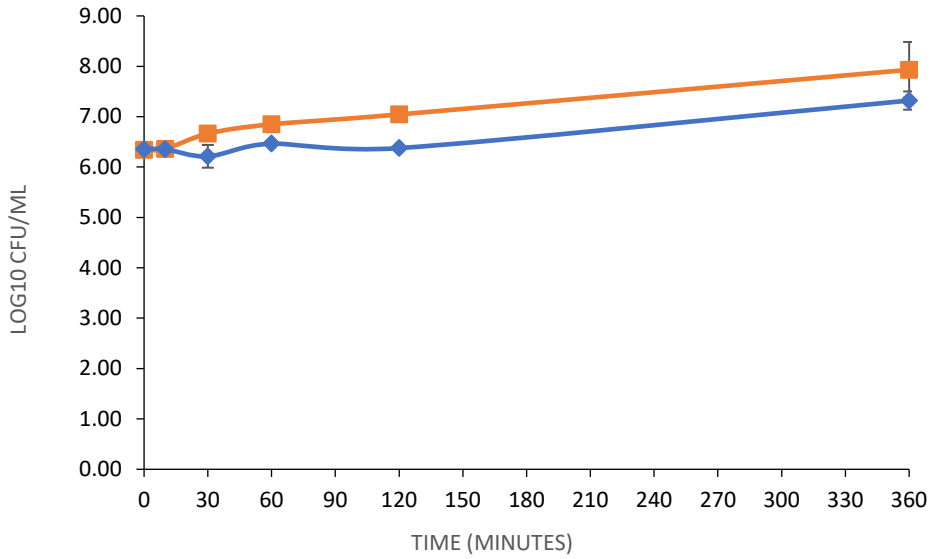


Figure 5.1. *E. faecium* (VRE) viable counts with 0.1 mM EDTA. ■ *E. faecium* (VRE) control viable count. ◆ *E. faecium* (VRE) viable count in the presence of 0.1 mM EDTA (n= 3, ±SEM).

EDTA (0.1 mM) was used to conduct another group of viable counts to establish the effect of EDTA on the action of the formulation, in order to determine that this concentration of EDTA does not alter the antibacterial action of the formulation on *E. faecium* (VRE). Viable counts demonstrated that EDTA (0.1 mM) did not significantly change the effect of the formulation on the bacterial cell viability at all times ($P > 0.05$) (Figure 5.2); thus, 0.1 mM EDTA was carried out for the transcriptomic investigations.

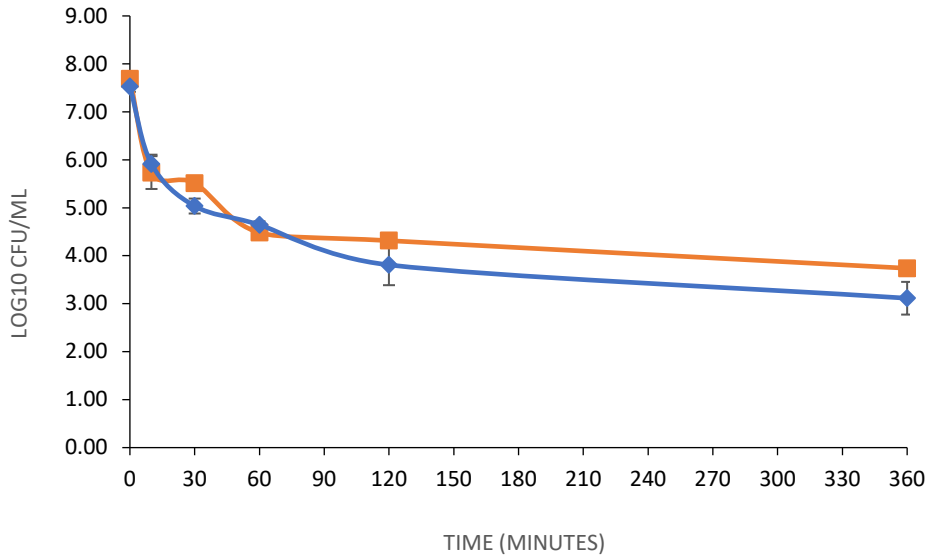


Figure 5.2. *E. faecium* (VRE) viable counts with the formulation and in the presence of 0.1 mM EDTA. ■ *E. faecium* (VRE) viable count with the formulation (carvacrol, cuminaldehyde and vancomycin). ◆ *E. faecium* (VRE) viable count with the formulation in the presence of 0.1 mM EDTA (n= 3, ±SEM).

5.3.2 Expression analysis of *bcr* in the presence of EDTA

The findings of the time course study in chapter 3 showed that *bcr* gene was differentially regulated when exposed to the formulation and its individual components over time (Figure 3.3). When in the presence of the formulation, *bcr* gene was significantly downregulated at 10 minutes and upregulated at 120 and 360 minutes. Thus, these time points were carried out for further investigations in the presence of an concentration of EDTA. No significant alteration in the expression level of *bcr* gene at 30 and 60 minutes was observed in the time course study in Chapter 3; however, these time points were still investigated here to find if the presence of EDTA might induce any change in the action of the formulation on the genetic level at these two particular time points.

Thus, the transcriptional response of *bcr* gene was investigated under the effect of 1 % DMSO (control), 0.1 mM EDTA and the formulation (carvacrol, cuminaldehyde and vancomycin) in the presence of 0.1 mM EDTA, over time points of 10, 30, 60, 120 and 360 minutes as shown in Figure 5.3.

In response to 0.1 mM EDTA at 10 and 30 minutes, *bcr* gene was non-significantly upregulated by 1.37 and 1.6-fold, respectively, *P > 0.05* (Figure 5.3a, b). Similarly, the expression level of *bcr* was not significantly altered when exposed to the formulation in the presence of 0.1 mM EDTA at 10 and 30 minutes, with 1.11-fold down-regulation and 1.63-fold up-regulation, respectively, $P > 0.05$ (Figure 5.3a, b). However, *bcr* was significantly upregulated by 2.52 and 8.9-fold upon exposure to 0.1 mM EDTA and the formulation in the presence of 0.1 mM EDTA for 60 minutes, $P \leq 0.05$ (Figure 5.3c). At a later time point of 120 minutes, it was noted that *bcr* was significantly expressed by 2.79-fold up-regulation when with 0.1 mM EDTA alone, $P \leq 0.05$, and non-significantly downregulated by 1.06-fold when exposed to the formulation in the presence of 0.1 mM EDTA, $P > 0.05$ (Figure 5.3d). Moreover, at 360 minutes' time point the expression level of *bcr* was significantly downregulated by 16.67 and 10-fold when exposed to 0.1 mM EDTA and the formulation in the presence of EDTA (0.1 mM), respectively, $P \leq 0.05$ (Figure 5.3e).

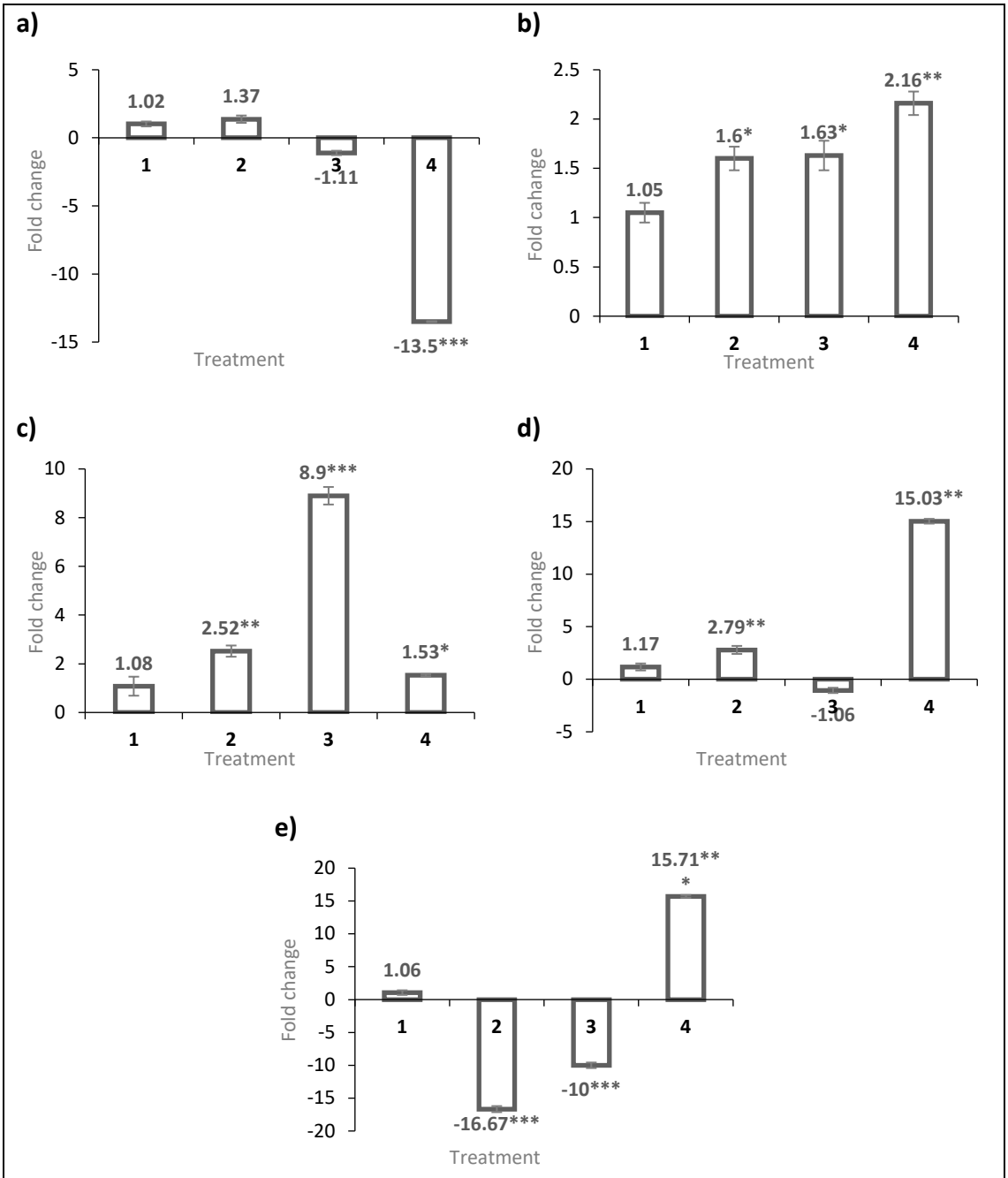


Figure 5.3. Changes in gene expression of *bcr* in response to EOs in formulation and in the presence of 0.1 mM EDTA over time. The fold change of *bcr* gene was measured using qPCR under the following conditions; **1)** 1% DMSO (control), **2)** 0.1 mM EDTA or **3)** formulation (1.98 mM carvacrol, 4.20 mM cuminaldehyde and 0.031 mg/l vancomycin) in the presence of 0.1 mM EDTA; **4)** formulation alone, all over time points of: **a)** 10 minutes, **b)** 30 minutes, **c)** 60 minutes, **d)** 120 minutes and **e)** 360 minutes. Fold change was calculated as $2^{-\Delta\Delta Cq}$ against reference genes *rpoD* and *dnaB_2,* *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$ (n= 3, ±SEM).

5.4 Discussion

Efflux pump inhibitors are being investigated as it is thought that inhibiting efflux pumps could lead to the restoration of the efficacy of existing antibiotics and reduce the emergence of multidrug resistance to existing antibiotics (Webber and Piddok, 2003). Nevertheless, it is difficult to determine if the inhibition effect includes one or more efflux pumps or inhibition of specific efflux pump, in addition to the fact that bacteria can have many efflux pumps with several being uncharacterised or unidentified (Rao *et al.*, 2018).

The data of the current study is a continuous investigation of the time course study constructed in Chapter 3. A major facilitator superfamily transporter, *bcr*, was identified to be differentially regulated under the effect of the formulation (carvacrol, cuminaldehyde and vancomycin) and its individual components over time (Figure 3.3). The results of the qPCR conducted in this study showed that 0.1 mM EDTA eliminated the effect of the formulation on the expression level of *bcr* at 10 and 30 minutes (Figure 5.3a, b), *bcr* was non-significantly expressed at these two time points. In comparison to the findings of exposing *E. faecium* (VRE) to the formulation alone, *bcr* gene was downregulated by 13.5-fold and upregulated by 2.16-fold (Figure 5.3a, b). Correlating these findings with the viable counts conducted in this study (Figure 5.2), there was no difference between the effect of the formulation alone and in the presence of 0.1 mM EDTA on the viability of VRE at 10 minutes; however, at 30 minutes the presence of 0.1 mM EDTA induced a small yet not significant reduction in the viability by 0.47 Log_{10} CFU/ml (Figure 5.2). The exposure to 0.1 mM EDTA alone as well did not induce a

significant alteration in the expression level of *bcr* gene at these two time points. This could be due to that EDTA was used at sub-inhibitory concentration and the amount of EDTA that entered the bacterial cell at 10 and 30 minutes was not enough for it to display an efflux pump inhibitory action, with previously published studies showing that EDTA action is concentration dependent (Lambert *et al.*, 2004; Chaudhary and Payasi, 2013). Nevertheless, 0.1 mM EDTA might have been enough for it to chelate the Ca^{2+} that is exist in trace concentrations in the cells' surrounding environment; and thus so, EDTA was able to eliminate the formulation action as it was assumed in chapter 4 that the mechanism of the formulation action is mediated by calcium channels and maybe Ca^{2+}.

Previously, *bcr* was not significantly expressed when exposed to the formulation alone at 60 minutes (Figure 5.3c); wherein, at the same time point in the current study and upon exposure to 0.1 mM EDTA alone and to the formulation in the presence of 0.1 mM EDTA, *bcr* was significantly upregulated by 2.52 and 8.9-fold, respectively (Figure 5.3c). This could be explained by that the cell is trying to overcome the applied stresses by overexpressing the gene involved in efflux pumps regulation.

However, the transcriptomic response at a later time point of 120 minutes showed that *bcr* was significantly expressed in response to 0.1 mM EDTA alone by 2.79-fold, and non-significantly downregulated when exposed to the formulation in the presence of 0.1 mM EDTA (Figure 5.3d). Comparing these results with the findings of the same time point in the absence of EDTA, where the bacterial cell could have recovered from the effect of the formulation by overexpressing *bcr* gene by 15-fold (Figure 5.3d). While with the formulation in the presence of 0.1 mM EDTA the cell could not display that recovery,

elucidating that the presence of 0.1 mM EDTA might have increased the effect of the formulation at the molecular level; despite that these changes were not reflected on the bacterial viability (Figure 5.2). Nevertheless, at 120 minutes' time point the bacterial cell was able to overcome the stress applied by 0.1 mM EDTA alone by significantly expressing *bcr* (Figure 5.3d).

Although, the concentration used of EDTA displayed sub-inhibitory action on the bacterial viability; thus, it might by the time point of 360 minutes, EDTA was accumulated inside the bacterial cell to a concentration that is enough to display a significant action alone and when added to the formulation at the molecular level. Where it was noted that the expression level of *bcr* was significantly downregulated when exposed to 0.1 mM EDTA alone and to the formulation in the presence of 0.1 mM EDTA by 16.67 and 10-fold, respectively (Figure 5.3e). The response towards the formulation when with 0.1 mM EDTA was in contrast to that when being alone (Figure 5.3e), where the bacterial cells have shown recovery of the applied stresses by significantly overexpressing *bcr* in order to expel these components to the surrounding area. And when correlating the current findings with the viable counts conducted in this study (Figure 5.2), it was noted that 0.1 mM EDTA when with the formulation has induced a non-significant reduction in the bacterial viability by 0.63 Log_{10} CFU/ml to that of the formulation alone, this could suggest an increase in the effect, yet non-significant. Overall, the findings of the present chapter could suggest that EDTA altered the expression of the *bcr* gene but this was not translated to a growth inhibition. Therefore, it can only be assumed that the cell is compensating for the efflux pump inhibition and potentially protecting the cell by making changes in gene expression.

Different studies have investigated chemical and natural efflux pump inhibitors. For example, a study evaluated the effect of EDTA as efflux pump inhibitor of EfrAB efflux pump in *E. faecium* and *E. faecalis*, found that 3 mM EDTA reduced the MICs of gentamicin, streptomycin, chlorhexidine and triclosan; all by > 8-fold, and significantly downregulated the expression of *efrA* and *efrB* genes by 10 to 140-fold regardless the enterococci strain used (Lerma *et al.*, 2014).

In contrast, a genomic analysis study by Cattoir *et al*. (2015) has established the mechanism of tigecycline resistance in *E. faecium* through fully sequencing *E. faecium* AUS0004, and using serial passages with gradually increased concentrations of tigecycline. Tigecycline MICs ranged from 0.5 mg/l to a final of 0.25 mg/l (according to EUCAST, resistance to tigecycline is with MIC > 0.5 mg/l) (EUCAST, 2021). The Cattoir study showed that no effects were observed on the MICs of tigecycline in the presence of efflux pump inhibitors, reserpine or pantoprazole, suggesting that an efflux mechanism was not involved in increasing the susceptibility to tigecycline. The involvement of the efflux pump inhibitors was assessed on the genetic level as well through detecting the transcriptional profile of *tet* genes that mediate either active efflux or ribosomal protection and lead to the acquisition of tetracycline resistance (Cattoir *et al.*, 2015). The findings of the current study suggest the involvement of an efflux system in the mechanism of action of the formulation of interest, and are in different with the results of Cattoir *et al*. (2015) study, that showed an efflux system inhibitor did not seem to be responsible for increasing susceptibility towards conventional antibiotic in the presence of natural product with efflux pump inhibitory action.

The activities of efflux pumps in MRSA was modulated by the diterpenes extracted from the cones of *Chamaecyparis lawsoniana*, such as ferruginol; whereby, the antibacterial activity of oxacillin was potentiated (Smith *et al.*, 2007). Similar inhibitory actions were noted against *Acinetobacter baumannii* by plant alkaloids that not only restore the efficacy of novobiocin, coumermycin, chlorobiocin, rifampicin and fusidic acid, they inhibited the efflux activities as well (Chusri *et al.*, 2009). The efflux pump in *Salmonella enteritidis* was reported to be inhibited by thymol and carvacrol, with noted effect of thymol in reducing the MICs of benzalkonium chloride and tetracycline (Miladi *et al.*, 2016).

5.5 Conclusion

Novel antimicrobial compounds with potential mechanism of actions of inhibiting efflux pumps could be promising in treating AMR microorganisms. The addition of chemical components, such as EDTA known to inhibit efflux systems in the bacterial cell, to the formulation has led to alteration in the formulation's effect at the molecular level, specifically changes in the expression of *bcr*, involved in the VRE efflux system. Suggesting that the mechanism of antimicrobial action of the formulation could be mediated by efflux pumps in addition to that of calcium channels as described in chapter 4. Future investigations of combining EOs with a variety of agents known to display efflux pump inhibition activity could be potential area of research.

Chapter 6

6 Attempted construction of *E. faecium* (VRE) *bcr* and *yloB* knockout strains

6.1 Introduction

The time course data in Chapter 3 suggests that the products of *bcr* and *yloB* may be involved in the antimicrobial mechanism of action of the formulation (carvacrol, cuminaldehyde and vancomycin) and this involvement may be as early as 10 and 30 minutes post-exposure. Therefore, it was thought to investigate if constructing gene knockouts of *bcr* and *yloB* in VRE could affect the bacterial strain, causing it to become more or less susceptible to the formulation. Also, gene knockout construction will provide greater insight about the importance of these transporters in the mechanism of action of the formulation towards *E. faecium* (VRE), and whether the bacterial cells are still able to grow and survive without either of these two genes. Furthermore, to find out if removing one gene could increase the expression of the other transporters in order to compensate the action of the removed transporter.

Creating strains of bacteria, whereby one gene has been knocked out is a useful and informative technique in studying the function and the products of a gene (Chen *et al.*, 2018). The impact of constructing gene knockout on restoring sensitivity to antibiotics has been demonstrated by Ferain *et al*. (1996) in *Lactobacillus plantarum,* which is naturally resistant to vancomycin through synthesising the D-lactate-ending peptidoglycan precursors using L- and D-lactate dehydrogenases that are encoded by *ldhD* genes. Gene knockout of the *ldhD* gene has been performed using a protocol

similar to the one used in the current study and concluded that constructing Δ*ldhD* restored vancomycin sensitivity in *L. plantarum* (Ferain *et al*., 1996).

The involvement of ABC efflux pump transporters in expressing multidrug resistance towards a varied group of antimicrobial agents, including ciprofloxacin, norfloxacin, doxycycline, tetraphenylphosphonium chloride, acriflavine and doxorubicin, has been investigated in *E. faecalis* ATCC29212 harbouring EfrAB transporters. A recombinant plasmid, pAEF82 containing the cloned DNA fragment from the *E. faecalis* chromosomal DNA, was introduced in mutant *E. coli* KAM32, which is drug-hypersensitive due to lack the major multidrug efflux pumps, AcrAB and YdhE. The transformed *E. coli* KAM32 restored antibiotic resistance towards acriflavine by expression of *efrAB* from *E. faecalis*. The efflux activity in transformed *E. coli* KAM32 was eliminated by using reserpine and verapamil, known efflux pump inhibitors (Lee *et al*., 2003).

There is limited published data on the production of strains of *E. faecium* harbouring gene knockouts and thus this chapter will focus on producing a novel protocol to produce these in VRE by adapting a protocol used in the similar species, *E. faecalis* (Mesnage *et al*., 2008). Upon the successful implementation of this protocol and production of these strains, it is hoped that the analysis of resultant strains will provide an insight on the role of *yloB* and *bcr* in the response to the novel formulation and its individual components.

6.1.1 Aim and objectives

The work in this chapter aims to create mutant strains of *E. faecium* (VRE) through constructing gene knockouts of *bcr* and *yloB* genes separately, and to establish the effect of the formulation on these strains.

Objectives

- To perform knockouts of *yloB* and *bcr* to produce mutant strains of VRE using a novel protocol.
- To study the viability of the resulting mutant strains in comparison to wild-type (WT) VRE.
- To determine the effects of the formulation and individual components on the Δ*bcr* and Δ*yloB* strains in comparison to WT.
- To determine whether the expression levels of the five genes discussed in chapter 3 are differentiated in these strains.
- To compare the results of conducted qPCR runs of wild and mutant *E. faecium* VRE in order to further elaborate the mechanism of action of the formulation.

6.2 Material and methods

6.2.1 Bacterial strain and chemicals

Methods were as described previously in Section 3.2.1 unless stated below. Luria-Bertani agar (LB) (L2897-250G) was purchased from Sigma Aldrich Ltd, UK.

6.2.2 Molecular biology techniques

PCRs were performed with appropriate template DNA in a volume of 50 µl using Q5® Hot Start High-Fidelity 2X Master Mix according to the manufacturer's guidelines in a Swift™ MiniPro® Thermal Cycler (ESCO LIFESCIENCES). Primers were purchased from integrated DNA Technologies (https://eu.idtdna.com) and annealing temperatures were calculated using the NEB Tm Calculator version 1.13.0 (https://tmcalculator.neb.com) for Q5® Hot Start High-Fidelity 2X Master Mix and a primer concentration 200 nM. Details of all primers used in this chapter are provided in Table 6.1. PCR products were analysed on 0.7-1 % (w/v) agarose gels set and electrophoresed (90 V for 45-60 minutes) in 1X Tris-acetate-EDTA (TAE) buffer (diluted from 50X stock, Thermo Fisher Scientific) stained with ethidium bromide (~ 0.5 µg/ml) and visualised on a UV light box. PCR products were purified from agarose gels using the Nippon Genetics FastGene Gel/PCR Extraction Kit.

Restriction digestion of PCR products and plasmid DNA (all plasmids described in this chapter are detailed in Table 6.2,) was performed using Thermo Scientific Fast Digest Restriction Enzymes according to the manufacturer's instructions. Ligation reactions were set up using T4 DNA ligase (NEB) according to the manufacturer's instructions and were introduced into chemically competent *Escherichia coli* TG1 (*repA*$^{+}$) cells (provided

by Dr Andrew Hitchcock, University of Sheffield) as follows: 3 µl of ligation reaction was transferred to a 25 µl aliquot of competent cells in a 1.5 ml Eppendorf tube on ice and incubated for 30 minutes prior to heat shock (42°C for 50 seconds and return to ice for 2 minutes). LB broth (1 ml) was added and the tube was transferred to a 37°C incubator with shaking (250 rpm) for 1 hour. The cells were pelleted in a bench top microcentrifuge (10,000 rpm, 2 minutes, room temperature), resuspended in 100 µl of LB and plated on LB agar containing the appropriate selective antibiotic. Plates were incubated at 37°C for ~16 hours. Plasmid DNA was purified from 5 ml overnight cultures using the Nippon Genetics FastGene Plasmid Mini Kit. Automated Sanger sequencing verification of generated plasmids was performed by Eurofins.

Table 6.1. Oligonucleotide primers and restriction enzymes with their digest sites highlighted in uppercase used in this study

Primer name	Sequence 5'-3'	Details
pG9_F	acgttAGATCTgtaatcactccttcttaattacaaatttttagc	*Bgl*II site uppercase
pG9_R	tgcatGAGCTCttctatgagtcgcttttgtaaatttgg	*Sac*I site uppercase
pG9-AbR_F	agctgtcagtagtatacc	
pG9-AbR_R	gaattgaagttaaattagatgc	
yloB_seq_check_F	aagaatcgattttgacgggag	
yloB_seq_check_R	tctgaacagatcactgtcatag	
yloB_PCR1_F	aagctGGTACCcagcaattgtcaatctaatgattttgg	*Kpn*I site uppercase
yloB_PCR1_R	taactgttgtgtcatccttgcttttatttcattatatccttgatc	
yloB_PCR2_F	gaaataaaagcaaggatgacacaacagttattgattggag	
yloB_PCR2_R	tgcaaGCGGCCGCtgcatcattctccatttttagaggt	*Not*I site uppercase
yloB_OLE-PCR_F	aagctGGTACCcagcaattgtcaatc	*Kpn*I site uppercase
yloB_OLE-PCR_R	tgcaaGCGGCCGCtgcatcattc	*Not*I site uppercase
yloB_screen_F	tctatataggaacgattttgtcg	
yloB_screen_R	tccctgtatgactctgcattg	
bcr_PCR1_F	aagctGGTACCtcgaatattatacggcgaatcg	*Kpn*I site uppercase

bcr_PCR1_R	caatgattcaatcaacatgtatgttatacattccccttaagct	
bcr_PCR2_F	tgtataacatacatgttgattgaatcattgaaaaatgaaatttgatc	
bcr_PCR2_R	tgcaaGCGGCCGCagaactgacaaaagaacagtatgc	*Not*I site uppercase
bcr_OLE-PCR_F	aagctGGTACCtcgaatattatacg	*Kpn*I site uppercase
bcr_OLE-PCR_R	tgcaaGCGGCCGCagaactgac	*Not*I site uppercase
bcr_screen_F	agctttccgtttaatagcatttgc	
bcr_screen_R	agaggcaatggtcaaaaaagc	
pG9_MCS_seq_F	aagttgggtaacgccagg	
pG9_MCS_seq_R	aaggagctaaagaggtccctag	
bla_F	agttcCTCGAGcgcggaacccctatttg	*Xho*I site uppercase
bla_R	tcctgACTAGTttaccaatgcttaatcagtgagg	*Spe*I site uppercase
pG9_F2	acgttCTCGAGgtaatcactccttcttaattacaaatttttagc	*Xho*I site uppercase
pG9_R2	tgcatACTAGTttctatgagtcgcttttgtaaatttgg	*Spe*I site uppercase

Table 6.2. Plasmids used and generated in this study

Plasmid name	**Details**	**Source/Reference**
pGhost9	Thermosensitive plasmid used for gene replacement in *E. faecalis*. Erythromycin resistant (Ery^R). See Figure 6.2A for plasmid map.	Dr Stéphane Mesnage, University of Sheffield.
pGhost9-*cat*	pGhost9 backbone with ery^R cassette replaced by the chloramphenicol acetyl transferase (*cat*) gene under control of the constitutive P2 promoter from a kanamycin resistance transposon (P2-*cat*). Chloramphenicol resistance (Cm^R). See Figure 6.2C for plasmid map.	This study
pGhost9-*cat*::*yloB*_KO	Fragment for deletion of *yloB* inserted into the *Kpn*I/*Not*I sites of pGhost9-*cat*. Cm^R. See Figure 6.4D for plasmid map.	This study

pGhost9-*cat*::*bcr*_KO	Fragment for deletion of *bcr* inserted into the *Kpn*I/*Not*I sites of pGhost9-*cat*. Cm^R. See Figure 6.4E for plasmid map.	This study
pGhost9-*bla*::*yloB*_KO	P2-*cat* fragment of pGhost9-*cat*::*yloB*_KO replaced with *bla* gene and promoter from pET21a(+). Ampicillin resistant (Amp^R). See Figure 6.7B for plasmid map.	This study
pGhost9-*bla*::*bcr*_KO	P2-*cat* fragment of pGhost9-*cat*::*bcr*_KO replaced with *bla* gene and promoter from pET21a(+). Amp^R. See Figure 6.7C for plasmid map.	This study

6.2.3 Preparation of BHI agar inoculated with chloramphenicol/ampicillin

In order to prepare a BHI-34 µg/ml chloramphenicol and BHI- 100 µg/ml ampicillin, a stock solution of chloramphenicol (C0378, Crystalline) (Sigma Aldrich Ltd, UK) was prepared in ethanol at a concentration of 34 and 50 mg/ml, and 100 µl was added to 100 ml molten agar cooled to ~ 40°C using a PTFE syringe filter to give a final concentration of 34 µg/ml. Similarly, a stock solution of 100 mg/ml ampicillin (A0166, Powder) (Sigma Aldrich Ltd, UK) was prepared, and 100 µl was added to 100 ml molten agar using a PTFE syringe filter. The antibiotic-BHI agar was mixed thoroughly and poured in 90 mm petri dishes. The plates were left to settle at the room temperature and were stored at 4˚C.

6.2.4 Preparation of VRE electro-competent cells

A 100 ml culture of pre-warmed (37˚C) BHI broth in a 250 ml conical flask was inoculated with 1 ml out of a 5 ml of bacterial suspension in BHI broth, was overnight incubated and grown at 37˚C without agitation to an optical density at 600 nm (OD_{600}) of ~ 0.5. The

culture was transferred to two pre-chilled 50 ml centrifuge tubes and the cells were pelleted at 5,000 rpm at 4˚C for 10 minutes. The supernatant was discarded and the pellet was gently re-suspended in 15 ml of chilled (4˚C) Sac-Gly solution (500 mM sucrose, 10% (w/v) glycerol, pH: 7). The re-suspended pellets were pooled and centrifuged at 5,000 rpm at 4˚C for 10 minutes. The Sac-Gly wash step was repeated and the cells were pelleted as above, re-suspended in 1 ml of ice-cold Sac-Gly solution and kept on ice. Cells were aliquoted (50 µl) into 1.5 ml Eppendorf tubes, flash frozen in liquid nitrogen and stored at -80˚C.

6.2.5 Electroporation protocol

A 1 mm electroporation cuvette (165-2089, Bio-Rad Labs, UK) was chilled on ice for ~ 15 minutes. *E. faecium* (VRE) competent cells (50 µl) were thawed on ice and mixed with 4 µl (100 ng to 1 µg) of the plasmid DNA by gentle pipetting. The cells-DNA mixture was transferred to the electroporation cuvette, and subjected to an electric shocked (25 µF, 200 Ohms and 2.0 kV) using an electroporator (MicroPulser, 411BR 1138) (Bio-Rad Labs, UK). Following the electric shock, 1 ml of ice cold SM17MC (M17 + glucose, 0.5 M saccharose, 10 mM $MgCl_2$, 10 mM $CaCl_2$, pH 6.8-7.0) or BHI broth was added, and the cuvettes were quickly returned to ice. After 5 minutes on ice, the cuvettes were transferred to 28°C for 3 hours without agitation, after which the cells were spread on BHI agar containing the appropriate selective antibiotic (34 and 50 µg/ml chloramphenicol or 100 µg/ml ampicillin). The plates were incubated for 48-27 hours at 28°C.

6.3 Results

The results of the time course study in Chapter 3 showed significant expression levels of *yloB* and *bcr* genes upon exposure to the formulation and its individual components at the early stages of treatment. Thus, it was decided to construct knockouts of these two genes and to assess the response of the mutant *E. faecium* VRE (Δ*yloB*, Δ*bcr*) to the effect of the formulation over the time points that were with the distinguished regulation of these two genes, which in turn could elaborate more the mechanism of action of the formulation.

6.3.1 Construction of chloramphenicol resistant derivative of pGhost9

Interestingly, chloramphenicol has maintained its activity towards most VRE isolates *in vitro* as has been shown in the United States (Eliopoulos *et al*., 1998), and this was exploited in the current study as a marker to select the mutated strains. Through establishing the sensitivity profile of the bacterial strain VRE NCTC12202, it was found that the VRE in addition to being resistant to vancomycin, is resistant to other antibiotics at the growth inhibitory concentrations of other enteric bacteria, such as *Escherichia coli* K12; where VRE grew on BHI agar in the presence of erythromycin at concentration of 200-250 µg/ml, kanamycin at 50 µg/ml or streptomycin at 50 µg/ml; however, was sensitive to chloramphenicol at 50 µg/ml and ampicillin at 100 µg/ml (Figure 6.1). The use of the replication-thermosensitive suicide plasmid (pGhost9) that was used to construct in-frame deletions by allelic exchange in *E. faecalis* (Mesnage *et al.*, 2008), and other Gram-positive bacteria (Tan *et al.*, 2008), was precluded due to the resistance to

erythromycin (Maguin *et al.*, 1992). In comparison, erythromycin 30 µg/ml is used for selection of pGhost9 genomic integration in *E. faecalis* OG1RF (Smith *et al.*, 2019).

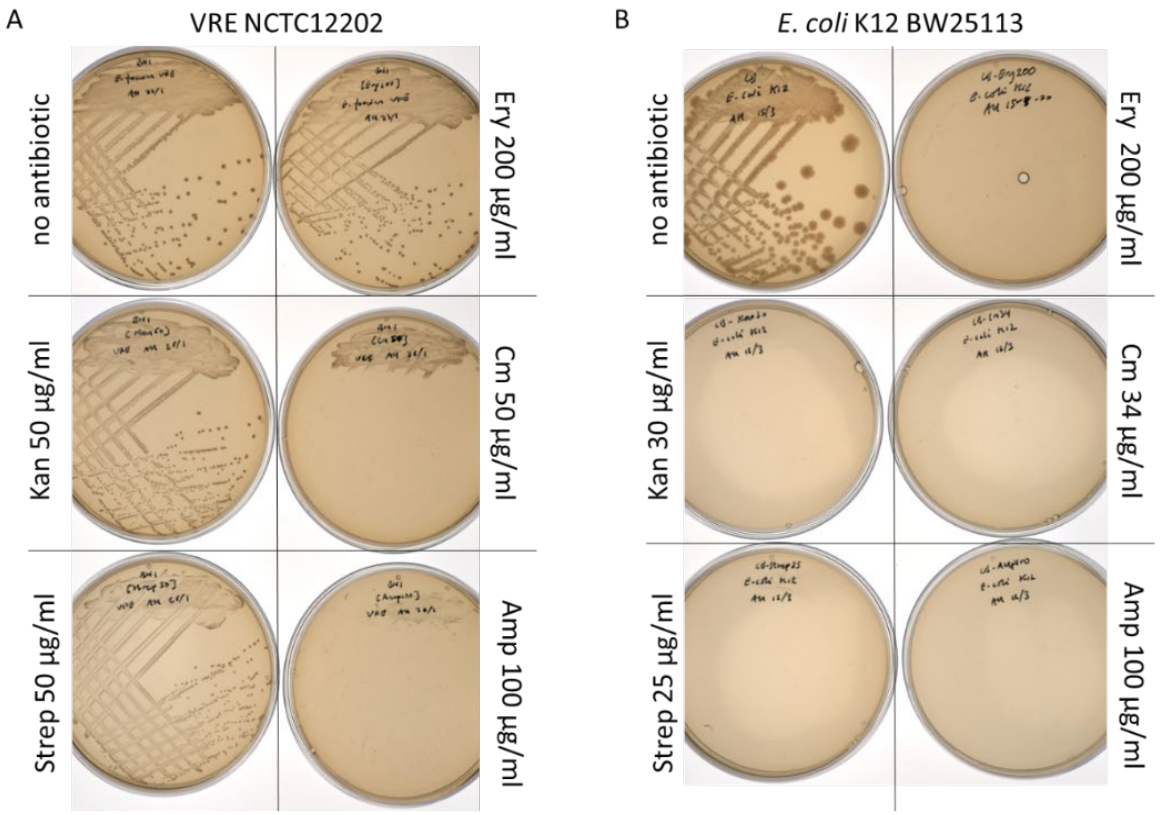


Figure 6.1. Comparison of the antibiotic sensitivity/resistance of VRE NCTC12202 and *E. coli* K12 BW25113. (A) VRE NCTC12202 was streaked onto BHI agar containing the indicated concentrations of kanamycin (Kan), streptomycin (Strep), erythromycin (Ery), chloramphenicol (Cm) and ampicillin (Amp). **(B)** *E. coli* K12 BW25113 was streaked onto LB agar with the indicated concentrations of the same antibiotics.

Therefore, the erythromycin resistance cassette (Ery^R) in pGhsot9 (Figure 6.2A) was switched for a fragment comprising the chloramphenicol acetyl transferase (*cat*) gene under control of the constitutive P2 promoter from a kanamycin resistance transposon, known to impart chloramphenicol resistance in *E. faecalis* (Dr Stéphane Mesnage, University of Sheffield, *pers*. *comm*.). This alteration process was achieved by PCR amplification of the 3012 bp pGhost9 backbone lacking Ery^R flanked by *Bgl*II/*Sac*I

restriction enzyme sites (PCR product size 3034 bp; Figure 6.2B) using primers pG9_F and pG9_R (All primers used in this study are detailed in Table 6.1) and ligating this PCR product with a synthetic P2-*cat* fragment flanked by the same sites (synthesised as a gBLOCK by integrated DNA Technologies, IDT). The ligation reactions were introduced to chemically competent *E. coli* TG1 *repA*$^{+}$ (genotype *supE Dthi (lac-proAB) hsdD5* (*F'*$^{+}$*traD36 proAB lacI*^{q}*ZDM15*) *glgB::repA*) with selection on LB agar containing chloramphenicol at 34 µg/ml. The resulting pGhost9-*cat* plasmid (Figure 6.2C) was screened by diagnostic restriction digestion (Figure 6.2D) and PCR (Figure 6.2E), and the sequence of the inserted P2-*cat* was verified by automated DNA sequencing with primers pG9-AbR_F and pG9-AbR_R (Eurofins; data not shown). As expected, pGhost9-*cat* conferred chloramphenicol but not erythromycin resistance to *E. coli* TG1 *repA*$^{+}$ (the opposite of pGhost9), and the temperature sensitivity of the plasmid-encoded RepA^{+} was confirmed by the lack of growth of *E. coli* JM109 transformed with the plasmid at 37°C (Figure 6.2F-G).

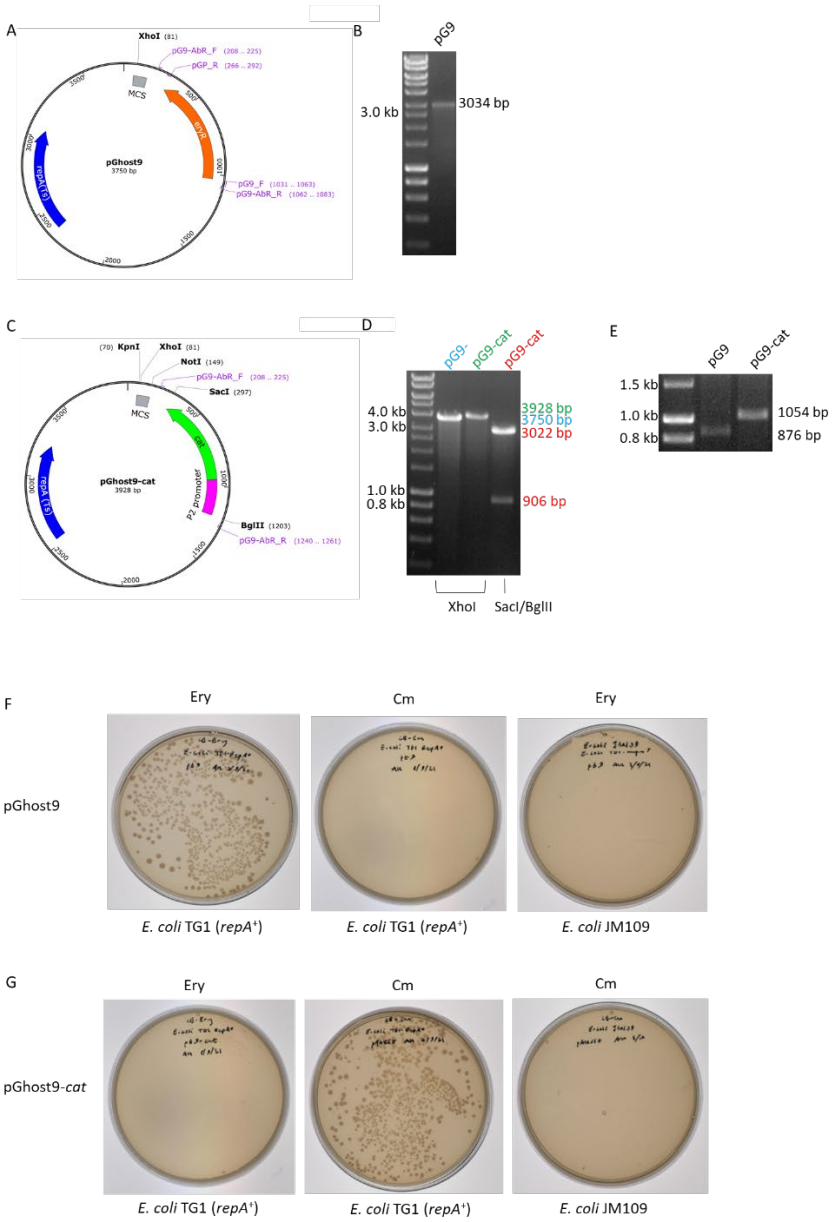


Figure 6.2. Verification of pGhost9-*cat*. (A) Plasmid map of pGhost9. The primers used for amplifying the plasmid backbone minus the ery^R cassette (pG9_F and pG9_R) and for plasmid screening (pG9-AbR_F and pG9-AbR_R) are shown, as is the position of the *Xho*I site. **(B)** PCR amplification of the pGhost9 backbone. **(C)** Plasmid map of pGhost9-*cat*. The primers used for PCR screening (pG9-AbR_F and pG9-AbR_R) are shown along with restriction sites used for diagnostic digests (*Xho*I, *Sac*I, *Bgl*II) and insertion of mutagenesis fragments (*Kpn*I and *Not*I). **(D)** Diagnostic restriction digestion of pGhost9-*cat*. **(E)** PCR screening of the antibiotic resistance cassette in pGhost9 and pGhost9-*cat* using primers pG9-AbR_F and pG9-AbR_R. **(F-G)** Verification of the antibiotic susceptibility/resistance of *E. coli* TG1 (*repA*$^+$) harbouring pGhost9 **(F)** and pGhost9-cat **(G)** and demonstration of temperature sensitivity of the plasmids in *E. coli* JM109.

6.3.2 Identification of an error in the VRE NCTC12202 genome sequence affecting the *yloB* locus

According to the genome sequence of VRE NCTC12202 strain (Accession number UFYT01000003.1; NCBI, 2018), the *yloB* (B02_00693; 1716 bp) and upstream B02_00692 (717 bp) open reading frames (ORFs) are separated by a 119 bp intergenic region (IGR). However, BLASTn searches (Altschul *et al.*, 1990) with the VRE NCTC12202 *yloB*, B020069 and the IGR sequences revealed that in other strains of *E. faecium* the two genes and the IGR are combined as a single 2553 bp *yloB* ORF encoding an 850 amino acid protein. Sequence alignments show that due to an extra adenosine (A) nucleotide at position 700 in these longer *yloB* genes (Figure 6.3A), and which is absent in NCTC12202 resulting in an apparent frameshift mutation in B02_00692 that in turn introduces a stop codon in this ORF (Figure 6.3B). The residue in question is part of a run of 6 (in B02_00692 in NCTC12202) or 7 (in other *E. faecium yloB* genes) A bases; therefore, to find out if the frameshift mutation in NCTC12202 is real or is only an error in the genome sequence, PCR runs were conducted to amplify the ~ 500 bp region covering the end of B02_00692, the IGR and the beginning of *yloB* gene using primers yloB_seq_check_F and yloB_seq_check_R (Figure 6.3C). Sequencing this fragment confirmed a run of 7 A bases, illustrating that B020069, the IGR and *yloB* are altogether in frame with each other as such that they encode a single 2553 bp *yloB* gene homologous to those in other *Enterococcus* species.

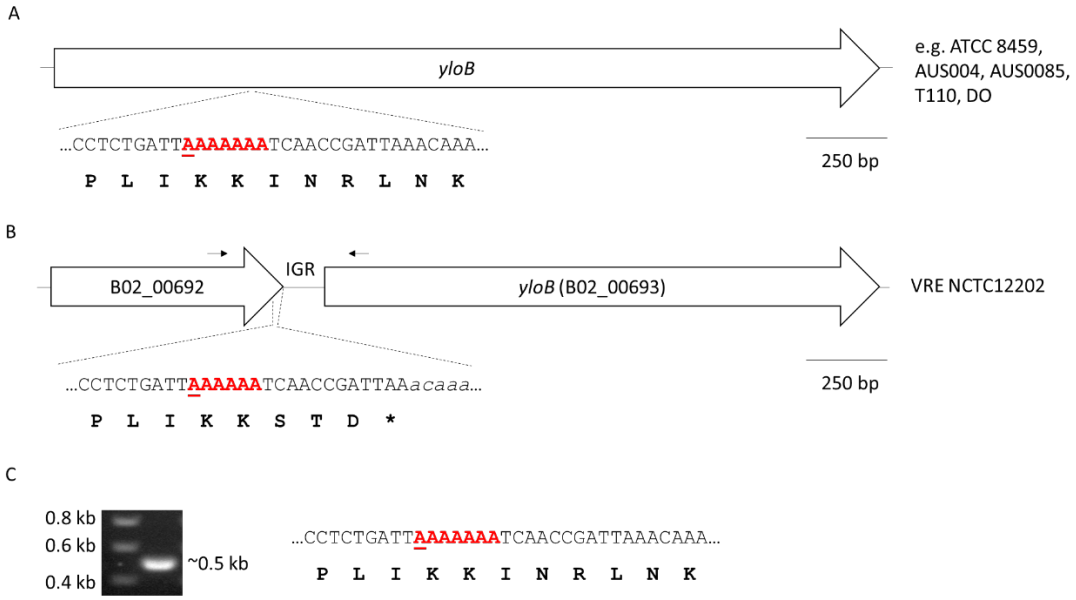


Figure 6.3. Analysis of the *yloB* locus in VRE NCTC12202. (A) The 2553 bp *yloB* gene in *E. faecium* genomes in the KEGG database. A run of 7 A bases starting at nucleotide 700 (underlined) is highlighted in red. **(B)** The *yloB* gene in VRE NCTC12202 is predicted to be split into two ORFS, B02_00692 (717 bp) and *yloB*/B02_00693 (1716 bp), separated by a 119 bp intergenic region (IGR). This is due to a run of only 6 A bases at the equivalent genomic position resulting in a frameshift that introduces the premature stop codon in B02_00692 (indicated with an asterisk). The position of primer annealing used for the PCR analysis in (C) is shown with small black arrows. **(C)** PCR amplification of the ~ 0.5 kb region indicated in (B); automated DNA sequencing revealed a run of 7 As, indicating B02_00692, the IGR and B02_00693 are in frame and together constitute a single 2553 bp *yloB* gene in VRE NCTC12202 as in other strains of *E. faecium*.

6.3.3 Construction of pGhost9-*cat* derivatives for generation of Δ*bcr* and Δ*yloB* deletion strains

Homology regions (700-750 bp) upstream and downstream of the portion of the gene to be deleted were amplified by PCR (Table 6.1 for primer details) and fused by overlap extension (OLE)-PCR (Ho *et al.*, 1989) (Figure 6.4A-C). The OLE-PCR products were flanked by *Kpn*I/*Not*I sites to allow digestion and cloning into similarly cut pGhost9-*cat*, generating the pGhost9-*cat::bcr*_KO and pGhost9-*cat::yloB*_KO constructs (KO:

knockout) (Figure 6.4D-E). The plasmids were screened by diagnostic restriction digests (Figure 6.4F) and the inserts were verified by automated DNA sequencing (Eurofins, data not shown) with primers pG9_MCS_seq_F and pG9_MSA_seq_R. All cloning steps were performed in *E. coli* TG1 *repA*$^{+}$.

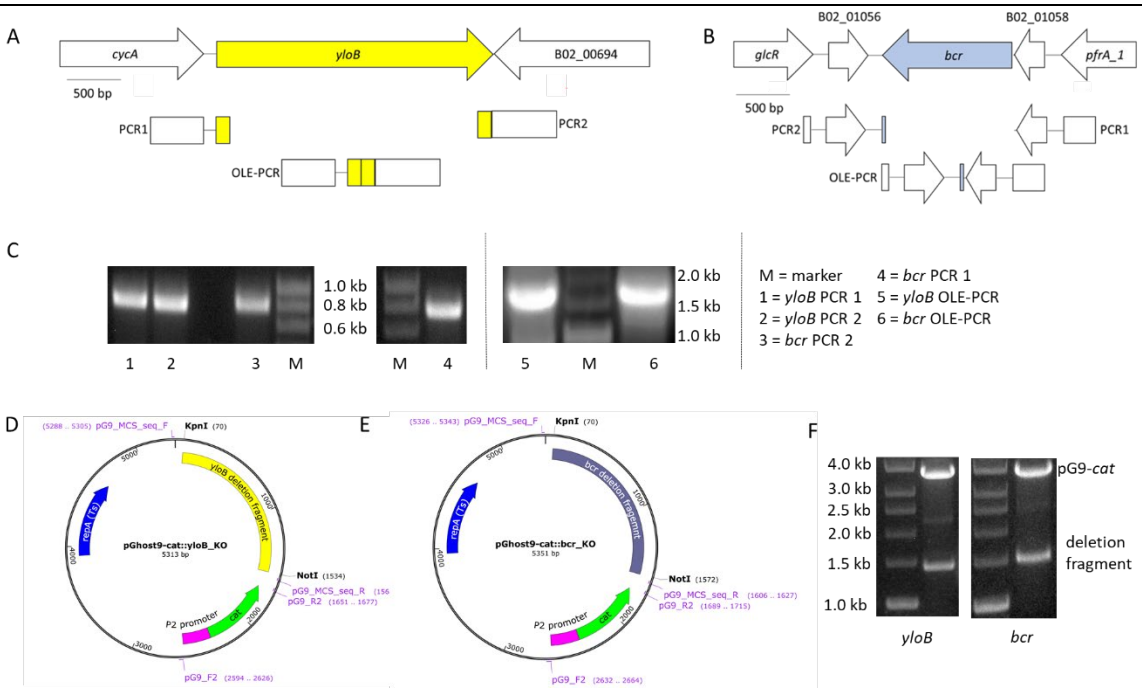


Figure 6.4. Generation of pGhost9-*cat* constructs for deletion of *yloB* and *bcr*. (A) Schematic of the *yloB* locus in WT VRE NCTC12202 showing the flanking homology regions amplified by PCR and joined by OLE-PCR. The flanking genes encode a putative amino acid permease (*cycA*) and a helix-turn-helix domain protein (B02_00694). **(B)** As in (A) but for the *bcr* locus. The flanking genes encode a DeoR family transcriptional regulator (*glcR*), a putative inner membrane protein (B02_01056), a hypothetical protein (B02_01058) and a CarD family transcriptional regulator (*prfA_1*). **(C)** Agarose gels of the PCR and OLE-PCR products depicted in panels (A) and (B). The OLE-PCR products were gel purified prior to digestion. Marker = HyperLadderTM 1 kb marker. **(D, E)** Plasmid maps of pGhost9-*cat::yloB*_KO and pGhost9-*cat::bcr*_KO. The positions of pertinent primer binding and restriction enzyme recognition sites are shown. **(F)** Agarose gels of *Kpn*I/*Not*I digestion of pGhost9-*cat::yloB*_KO and pGhost9-*cat::bcr*_KO showing successful insertion of the respective *yloB* or *bcr* fragments into the pGhost9-*cat* (pG9-cat) plasmid.

6.3.4 Attempted generation of Δ*bcr* and Δ*yloB* VRE mutants

pGhost9 has previously been used for allelic exchange in *E. faecalis* (Mesnage *et al.*, 2008); the protocol is outlined in Figure 6.5. Briefly, pGhost9 plasmids are introduced to the recipient strain by electroporation and erythromycin resistant colonies are isolated at the permissive temperature (28°C). Next, single crossover (SCO) recombinants are selected by streaking onto fresh BHI-erythromycin plates and incubating at the non-

permissive temperature (42°C) for 18 hours. To induce a second recombination event to excise the plasmid backbone, SCO recombinants are serially sub-cultured in liquid broth five times and are incubated at 42°C in the absence of erythromycin; the final overnight culture is plated on BHI (without erythromycin) and incubated for 18 hours at 42°C. Mutants that have undergone the desired double crossover are identified by PCR with primers binding outside the homology regions and verified by sequencing of the target locus.

An attempt was made to generate isogenic Δ*bcr* and Δ*yloB* strains of VRE using pGhost9-*cat* derivatives by following the protocol outlined above but with selection on BHI agar with 50 µg/ml chloramphenicol. However, despite repeated attempts with varying chloramphenicol concentration (34 and 50 µg/ml), it was not successful to isolate chloramphenicol resistant VRE colonies following electroporation; members of the Mesnage lab (University of Sheffield) have also failed to use pGhost9-*cat* for allelic exchange in *E. faecalis* (personal communication) suggesting the plasmid copy of *cat* gene may not be sufficient to impart chloramphenicol resistance to *Enterococcus app*.

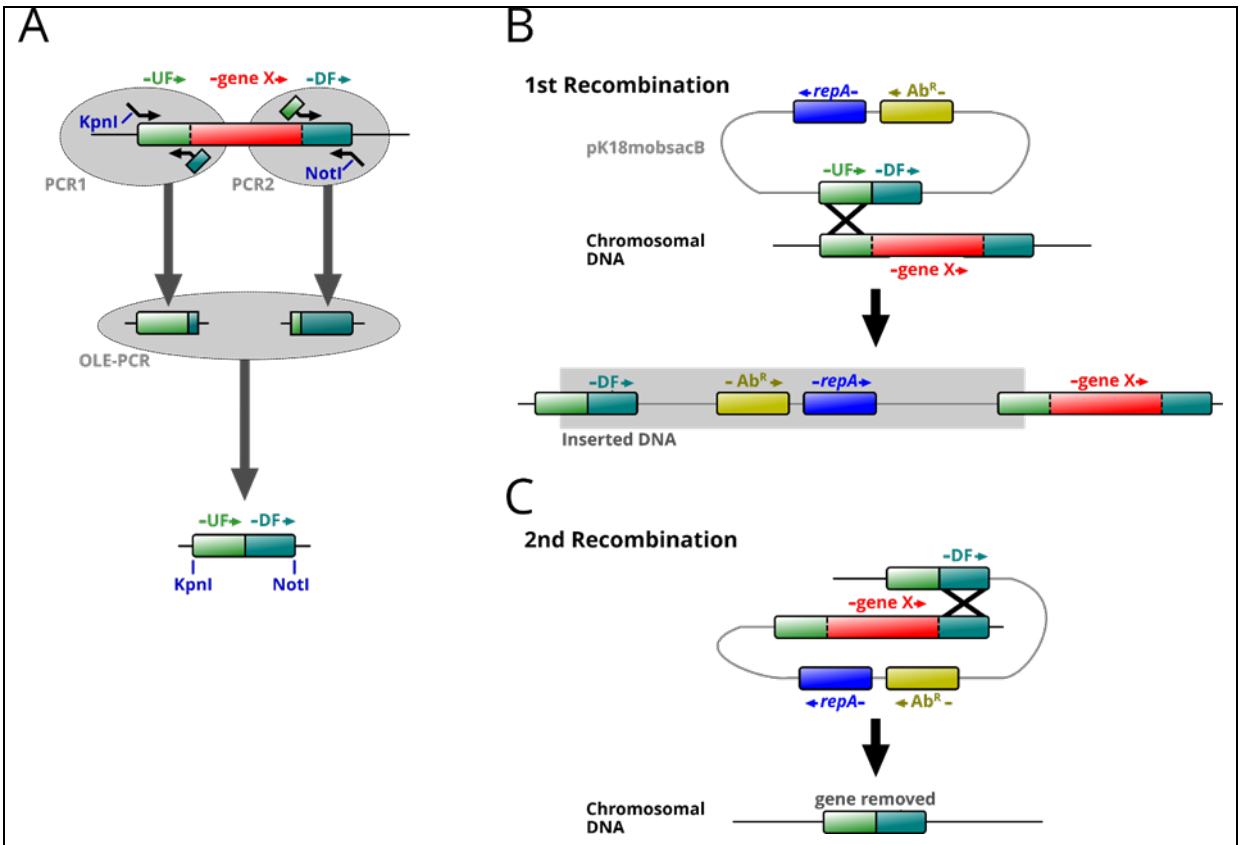


Figure 6.5. Strategy for markerless gene deletion using pGhost9. (A) Generation of a construct for the deletion of gene X (red). The primer extensions that add overlap sequences to the initial PCRs (PCR1 and PCR2) that amplify the upstream and downstream flanking regions (UF and DF, respectively) to permit their joining by OLE-PCR are indicated. The primers also add *Kpn*I and *Not*I restriction sites for cloning of the OLE-PCR product into the multiple cloning site (MCS) of pGhost9. **(B-C)** The recombination events that lead to the deletion of gene X. **(B)** A single crossover mediated by homologous recombination with the upstream flanking region (UF) is shown (note recombination at the DF could also have occurred). This results in integration of the entire plasmid into the genome (the inserted plasmid DNA is indicated by the grey box). The resulting first recombinants contain an antibiotic-resistance marker (Ab^R) for selection and the temperature-sensitive *repA*$^+$ gene, which permits growth only at the permissive temperature (28°C). **(C)** Growth at the non-permissive temperature (42°C) promotes a second recombination event resulting in excision of the plasmid from the genome by homologous recombination; only the desirable double crossover mediated by the downstream homology region (DF) is shown here; excision by recombination at the UF would result in restoration of the WT gene X locus. Figure adapted from Sutherland *et al.*, 2021 with permission from the authors.

6.3.5 Construction of an ampicillin resistant derivative of pGhost9

Due to the failure to generate chloramphenicol resistant strains using pGhost9-*cat*, it was decided to try another antibiotic resistance marker for selection of VRE transformants. Wild type VRE is also sensitive to ampicillin (Figure 6.6A-B) and 50 µg/ml gentamycin (Figure 6.6C-D); therefore, the *cat* gene and P2 promoter in the pG9*cat-bcr* and pG9*cat-yloB* plasmids were switched with the *bla* gene (988 bp) and its promoter from pET21a(+) (Novagen) which confers ampicillin resistance. The *bla* cassette flanked by *Xho*I and *Spe*I recognition sequences, both of which are absent in pG9*cat-bcr* and pG9*cat-yloB*, was PCR amplified from pET21a(+) using primers bla_F and bla_R (Figure 6.7A). The entire pG9*cat-bcr* and pG9*cat-yloB* plasmids lacking the P2-*cat* region were also PCR amplified with flanking *Xho*I and *Spe*I restriction sites using primers PG9-F2 and pG9-R2 (see Figure 6.7B, C for primer binding sites). Following digestion, the *bla* insert was inserted into each of the plasmid backbones (Figure 6.7B-C) resulting in ampicillin resistant *E. coli* TG1 *repA*$^{+}$ transformants (Figure 6.7D). The temperature sensitivity of pGhost9-*bla* was confirmed by the absence of growth of *E. coli* JM109 at 37°C (Figure 6.6D). The plasmids were analysed by diagnostic restriction digests (Figure 6.7E) and automated DNA sequencing (Eurofins) of the *bla* insert and *bcr*/*yloB* knockout regions confirmed they were present and correct (data not shown). However, as above with pGhost9-*cat* plasmids, it was not possible to select ampicillin resistant colonies following electroporation of VRE with the pGhost9-*bla* knockout constructs.

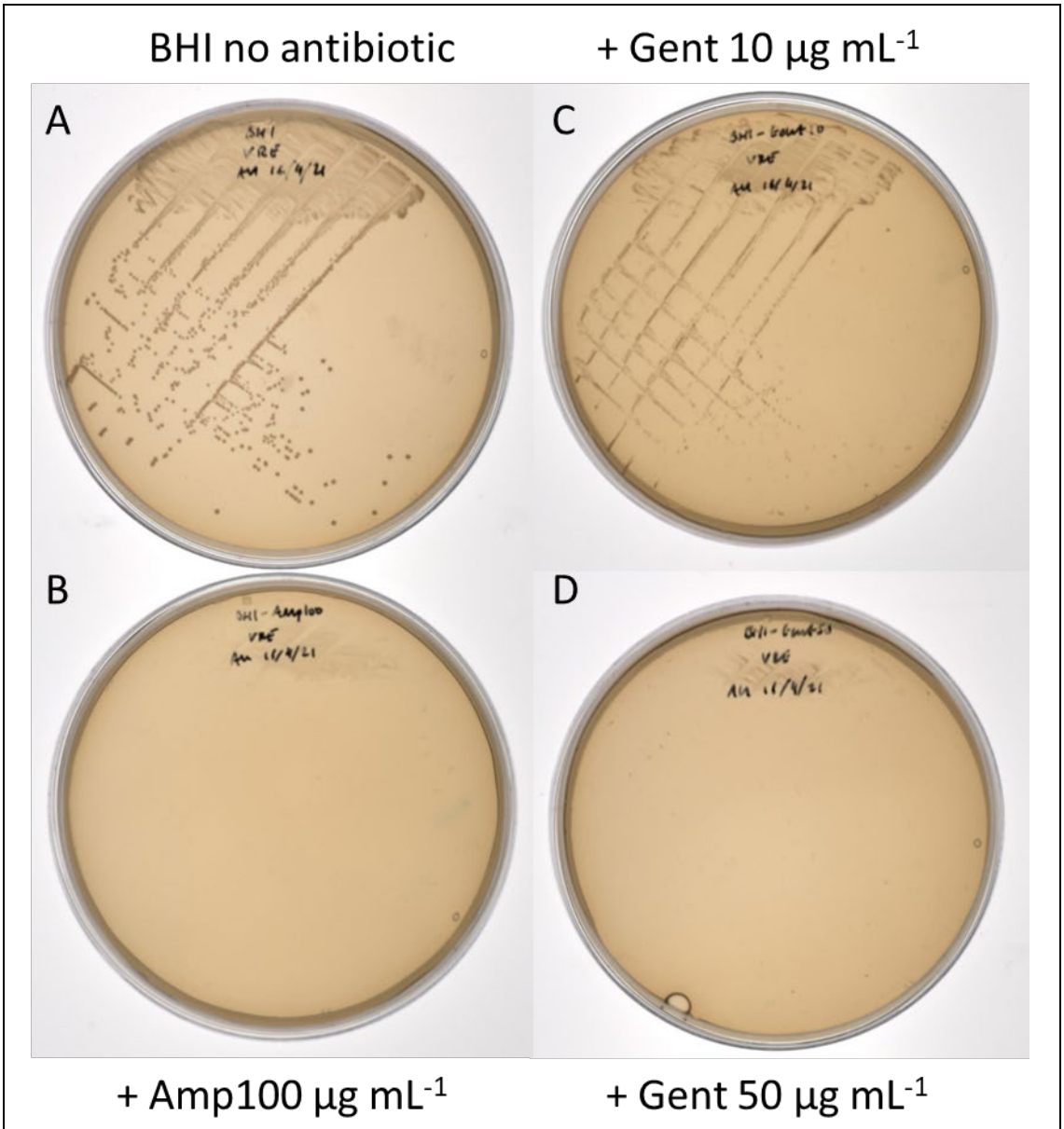


Figure 6.6. *E. faecium* VRE NCTC12202 is sensitive to ampicillin and gentamycin. (A) Growth of VRE NCTC12202 on BHI without any antibiotic. **(B)** VRE NCTC12202 is sensitive to 100 µg/ml of ampicillin. **(C-D)** VRE NCTC12202 was able to grow on BHI agar with 10 µg/ml (D) but not 50 µg/ml (C) gentamycin.

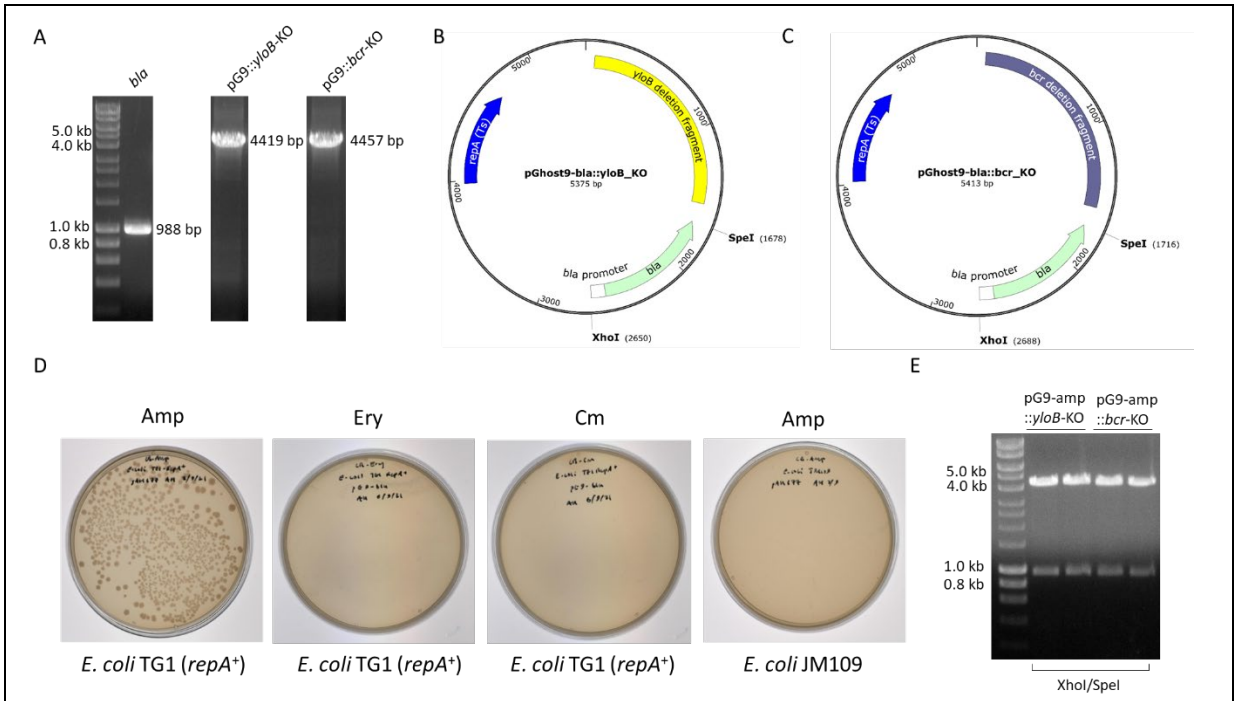


Figure 6.7 Generation of ampicillin resistant variants of the *yloB* and *bcr* deletion constructs. (A) PCR amplification of the *bla* promoter-gene fragment from pET21a(+) and the pGhost9-*cat*::*yloB*_KO (pG9::*yloB*_KO) and pGhost9-*cat*::*bcr*_KO (pG9::*bcr*_KO) backbones minus their P2-*cat* regions. **(B-C)** Plasmid maps of (B) pGhost-9-*bla*::*yloB*_KO and (C) pGhost-9-*bla*::*bcr*_KO, showing the restriction sites used for cloning and the diagnostic restriction digests in panel (E). **(D)** The pGhost9-*bla*::*yloB*_KO construct confers ampicillin resistance to *E. coli* TG1 (*repA*$^+$) but does not allow growth of *E. coli* JM109 due to the thermosensitive RepA. **(E)** *Xho*I/*Spe*I restriction digestion of pGhost-9-*bla*::*yloB*_KO and pGhost-9-*bla*::*bcr*_KO showing excision of the *bla* promoter/gene from the plasmid backbones.

6.3.6 Electroporated cells

The electro-competent VRE cells were electroporated following the protocol detailed in section 6.2.5 and were incubated for 48-72 hours at 28˚C. After incubation, no growth was noted on the inoculated BHI agar with 100 µg/ml ampicillin of either the mutated cells or the control cells, while both of these cells grew on BHI agar alone. That could be due to that the cells either did not uptake the plasmids that contain the mutated genes or the cells up took the plasmids but did not express the relative gene involved in resistance to ampicillin as shown in Figure 6.1.

6.4 Discussion

Production of bacterial strains containing gene knockouts is an informative tool to investigate the bacterial genes and their functions, the involvement of these genes in conferring antibiotic resistance, and the possibility of restoring sensitivity towards antibiotics (Sinel *et al*., 2017). Thus, if knocking out specific genes leads the mutated bacterial cells to become resistant or sensitive to antimicrobial agents, this transformant could be exploited in many important clinical applications, such as creating new formulas of antibiotics that might affect multidrug resistant microorganisms (Chen *et al.*, 2018).

The protocols in the current study are the first of their kind established for producing gene knockouts in *E. faecium* (VRE) NCTC12202. It was decided to construct knockouts of *yloB* and *bcr* genes due to their importance in transportation in *E. faecium* and their interesting transcriptional profiles in the conducted qPCR studies (Chapters 3, 4 and 5); with *bcr* being a member of the major facilitator superfamily involved in transporting antibiotics; and *yloB* belonging to the superfamily of P-type ATPases (Kumar *et al*., 2013; Lekshmi *et al*., 2018). From the studies in previous chapters, it is thought that the products of *bcr* and *yloB* are involved in the mechanism of action of the formulation; and thus, the subject of this chapter is to establish the effect of knocking out these two genes on the fitness of Δ*yloB* and Δ*bcr* VRE strains and on the subsequent antimicrobial action of the formulation on them.

The production of the knockout constructs and subsequent electroporation into *E. coli* TG1 ($RepA^+$) was successful; however, upon transformation into VRE, no colonies were

seen. This may have been due to a number of different reasons, for example they were successfully electroporated into the cell and integrated into the genome, but the chloramphenicol/ampicillin resistance cassette was not expressed. Alternatively, the plasmids may not have been taken up due to different reasons, such as the cells were not electrocompetent, or the temperature sensitivity aspect did not work; thus, further work is required to try and troubleshoot the process and produce a novel protocol for the production of mutant *E. faecium* strains using this technique.

Similarly, a study by Nallapareddy *et al*. (2006) found it difficult to construct mutations in the adhesin encoding gene, *acm*, in the emerging nosocomial *E. faecium* by allelic exchange. Attempts were unsuccessful in using suicide, temperature-sensitive, or mobilizable vector systems developed for *E. faecalis* mutagenesis to produce targeted mutations in clinical strains of *E. faecium* (Nallapareddy *et al*., 2006).

There is limited published research on the importance and applications of knocking out *yloB* and *bcr* in the literature; however, limited studies have established the effect of knocking out varied genes on the virulence of *E. faecium* strains and other bacteria (Zhang *et al*., 2017; Raeymaekers *et al.*, 2002).

The expression of P-type Ca^{2+}-transport ATPase in *Bacillus subtilis* has been investigated to establish its importance for the growth of vegetative cells (Raeymaekers *et al.*, 2002). It has been found that the mutant cells grew similarly to the wild-type strain. The product of *yloB* gene is only expressed following the induction of sporulation in *B. subtilis*; thus, the P-type Ca^{2+} transporter mediates the formation of spores but it is not essential for *B. subtilis* survival (Raeymaekers *et al.*, 2002). Similarly, it was thought in

the current study to investigate the importance of *yloB* product in the survival of VRE (WT) upon exposure to the formulation and individual components; however, this was not achievable due to unsuccessful transformation of the knockout constructs into the bacterial cell following electroporation.

Efflux systems are important for antibiotic resistance in bacteria; therefore, it was expected in the current study that knocking out *bcr* could increase the action of the formulation, due to the fact that *bcr* is involved in encoding an efflux system. Thus, when *bcr* is absent the existence of the formulation inside the cell will last longer leading to more effective antimicrobial action.

Similarly, gene knockouts have been used to analyse the mechanism of chloramphenicol resistance in *Myxococcus* xanthus, whereby knocking out genes of MFS the family of transporters, led to the mutant strain having restoring susceptibility to chloramphenicol (Yang *et al.*, 2019). It is hoped that successful production of Δ*bcr* would lead to an increase in action of the formulation in VRE.

6.5 Conclusion

In this study, attempts were made to construct gene knockouts in *E. faecium* NCTC12202. The current developed protocol of constructing gene knockouts was unfortunately not successful. Therefore, future work will focus on troubleshooting the methodology including preparing new electrocompetent cells, producing new constructs with smaller molecular sizes that might be easier to be taken up, changing the selection marker, and looking for a different vector to produce the constructs. Furthermore, different methods of transforming the constructs into the bacterial cell,

such as incubating the bacteria with the constructs at 4°C for 24 hours or following the conjugation protocol developed by Nallapareddy *et al*. (2006), will also be trialled. Analysis of the resulting strains can then be completed to determine the true role of YloB and Bcr in the mechanism of action of the novel formulation.

Chapter 7

7 Discussion

Antimicrobial resistant microorganisms are evolving in hospitals as well as in community arenas and are a serious global health concern with available antibiotics no longer effective. New regimes of treatment to replace the current ineffective antibiotics are of critical need (Vivas *et al*., 2019).

More interest is being paid to EOs as potential treatments to combat bacterial antimicrobial resistance, creating a promising new era of research (Yap *et al.*, 2014). The antimicrobial actions of EOs have been empirically proven, while the mechanisms of action of these compounds are yet to be fully established as potential antimicrobials. Elucidating the targets of EOs in the bacterial cells might help in understanding the mechanisms of action and developing new strategies in combating bacterial antimicrobial resistance (Božik *et al.*, 2018).

The antimicrobial properties of EOs are beginning to be elucidated on both the cellular and molecular levels. The findings in the published literature so far indicate that EOs act as antimicrobials (Mangalagiri *et al*., 2021; Rao *et al*., 2019; Owen and Laird, 2019; Christensen and Anderson, 2017).

The current study considers the role of transport channels in the mechanism of action of EOs individually, in combination and in formulation with vancomycin. The studies in this thesis were conducted in order to establish if the mechanisms of action of a formulation (carvacrol, cuminaldehyde and vancomycin) against *E. faecium* (VRE and VSE) may involve transport channels in the bacterial cell.

The data obtained showed that the channels, that are hypothesised to be involved in the mechanism of antimicrobial action of EOs against *E. faecium*, were not found to be similar to TRPV1 mammalian channels. Where neither the antibacterial effect of capsaicin, a TRPV1 channels agonist, nor the EOs' against *E. faecium*, were reversed by the TRPV1 channels antagonist, AMG517 (Figures 2.3-2.8) (Table 2.2). Different studies however have shown that TRPV1 mammalian channels are activated by EOs, these channels did not appear to be expressed in bacteria (Ghosh *et al*., 2020; Ohkawara *et al*., 2010). A study demonstrated that TRPV1 mammalian channels were activated by 4 out of 31 screened EOs, including rose, thyme, geraniol, palmarosa and tolu balsam, as potential targets for skin disorders (Ohkawara *et al*., 2010). Another study provided the cellular and molecular basis of the pharmacological effects of EOs from *Monarda fistulosa* L on TRP channels. The EOs, including; *p*-cymene, γ-terpinene, α-terpinene, α-thujene, carvacrol and thymol, were found to stimulate the transient increase in $[Ca^{2+}]$ by activating TRPA1 but not TRPV1 or TRPV4 channels, and at the molecular level, the study reported that the binding affinity of carvacrol, thymol and β-myrcene showed substantial similarity in the binding site of human TRPA1 (Ghosh *et al*., 2020). The findings of this study were further elaborated by bioinformatic analysis that elucidated that channels similar to TRPV1 mammalian channels are not found in *E. faecium*. Nevertheless, the microarray data (Owen *et al.*, 2020) suggests that bacterial cell channels are involved in the mechanism of action of the formulation. The microarray data serves as a preliminary investigation for further analysis of the mechanism of action of the formulation. Similarly, it has been elaborated by Evans (2015) that transcriptomics

have emerged as an important goal to be exploited in establishing the physiological responses of bacteria to the environmental stresses (Evans, 2015).

The microarray data described a wide range of genes of *E. faecium* (VRE) that were differentially regulated in response to EOs individually (carvacrol, cuminaldehyde) and in formulation with vancomycin. The effect of these compounds was determined on the expression levels of genes chosen from the array and are involved in transportation in *E. faecium* (VRE), that could be a preliminary elucidation of the mechanisms of action displayed by this EOs-vancomycin combination to re-sensitise *E. faecium* (VRE and VSE) to vancomycin. Conducting qPCR to validate the microarray data and to establish a time course study showed that the investigated genes including; *ecfA1*, *esfA-1*, *bcr*, *yloB* and *nhaC_2*, were differentially regulated when exposed to the formulation or its individual components over time (Figures 3.3-3.8).

Furthermore, the action of the formulation against the bacterial cell at the molecular level was found to happen at the early stages of treatment, 10 and 30 minutes, where it was noted that both the formulation and its individual EO components have significantly down-regulated the expression levels of *bcr* and *yloB*, that are involved in efflux systems and Ca^{2+} transportation in the bacterial cell, respectively (Figures 3.3 and 3.6). Whereby, through down-regulating the expression of efflux pumps related genes in the bacterial cell, the formulation was able to reach its targets inside the bacterial cell and function displaying an antimicrobial action. It was also noted that the bacteria displayed a recovery from the applied stresses by the formulation and its individual components at later time points, 120 and 360 minutes. The bacterial cell recovery could have happened

through upregulating the genes that are involved in encoding efflux systems in addition to the nutrients transportation related genes, *ecfA1*, *ecsA-1* and *nahC_2*, in order to bring the intracellular environment to a stable state (Figures 3.4, 3.5 and 3.7).

Similarly, microarray test has been used to establish the response of *E. faecalis* towards cell wall stress stimulus, the transcriptional profile of *E. faecalis* OG1RF has been assessed after being exposed to ampicillin, bacitracin, cephalothin and vancomycin, and the microarray data was validated conducting qPCR runs (Abranches *et al.*, 2013). And a study by Solheim *et al.* (2014) has assessed the results of a time course microarray of *E. faecalis* V583 in response to a high concentration of 6.5 % NaCl conducting qPCR runs. The findings of the current study there maybe similarities with the microarray data of Solheim *et al.* (2014) study that showed 515 genes were differentially regulated in response to 6.5 % NaCl at one or more time points of a time course study, and those genes are involved in transportation in *E. faecalis* V583, such as Na^+/H^+ antiporter and potassium transporting ATPase. Seven genes were differentially regulated over all time points studied and are responsible for encoding ABC transporters and transport and binding proteins. *mscL* gene (encodes mechanosensitive channels), was downregulated immediately upon exposure to 6.5 % NaCl, and genes responsible for potassium and glycine uptake from the extracellular area were up-regulated (Solheim *et al.*, 2014).

Previously, cuminaldehyde has been reported to act as an inhibitor of MFS LmrS multidrug efflux pump in a dose dependent manner in *S. aureus* (MRSA) (Kakarla *et al.*, 2017). It has been hypothesised that cuminaldehyde could be inhibiting LmrS drug transportation. LmrS efflux pump, described as a secondary active drug/H^+ antiporter,

confers resistance to structurally unrelated antibiotics including linezolid, trimethoprim, lincomycin, chloramphenicol, kanamycin, streptomycin and tetra-phenyl phosphonium chloride (Kakarla *et al.*, 2017). In line with Kakarla *et al.* (2017), it was noted in the present study that the main transcriptional response of all genes investigated in the current study towards the antibacterial action of cuminaldehyde happened after 120 minutes of treatment, that could be demonstrating a dose dependent manner, whereby cuminaldehyde could have accumulated inside the bacterial cell to a concentration that is enough to induce the transcriptional response as shown in Figures 3.3-3.8.

The addition of 1 mM Ca^{2+} to either capsaicin, the formulation or its individual components when applied to *E. faecium* (VRE) showed significant results at both of the viability and molecular levels. Where 1 mM Ca^{2+} increased the effect of capsaicin on *E. faecium* (VRE) inducing elevated [Ca^{2+}], suggesting that capsaicin could have promoted the transportation of Ca^{2+} into the bacterial cell through channels from the surrounding environment. These findings could be partly exploited in elucidating the mechanism of action of the formulation, where capsaicin may have used calcium channels to enter the bacterial cell prompting more Ca^{2+} to enter the cell; thus, the EOs included in the formulation (carvacrol, cuminaldehyde and vancomycin) could have use the very similar channels to display their antibacterial action towards *E. faecium* VRE.

Furthermore, the presence of externally added Ca^{2+} (1 mM) significantly increased the expression level of *yloB* gene*,* when exposed to either capsaicin or to all treatment conditions by at least double the fold change (at 10 minutes 11.18-fold up-regulation with capsaicin, and 50-fold down-regulation with the formulation) to what it was in the

absence of Ca^{2+} (6.12-fold upregulation with capsaicin and 5.67-fold down-regulation with the formulation), and most importantly this effect was not only at the early stages of the exposure; in contrast, it was carried over all time points investigated (Figures 4.4-4.8). That maybe elaborating more the involvement of calcium transporting channels in the mechanism of the antimicrobial action of the formulation.

EOs have been reported to alter the expression levels of genes involved in efflux systems as described by Sharma *et al*. (2019), Espinoza *et al*. (2019) and Singh *et al*. (2017). Similarly, the EOs included in the formulation investigated in the current study showed significant changes to the expression levels of genes involved in transportation in the bacterial cell over time; such as the major facilitator transporter, *bcr* (Figure 3.3). However, the addition of EDTA, a chemical known to be an efflux pump inhibitor, to the formulation significantly changed the effect of the formulation on the expression level of *bcr* over time (Figure 5.3). Albeit, the used concentration of EDTA did not alter the action of the formulation on the bacterial viability (Figure 5.2), but it disturbed the formula's action at the regulation level of an efflux system, suggesting the involvement of different mechanisms in the antimicrobial action of the formulation. Furthermore, these findings could be related to the findings in chapter 4 that suggested the involvement of calcium channels in the mechanism of action of the formulation (Figures 4.4-4.8). Thus, if calcium channels and Ca^{2+} are involved in the mechanism of action of the formulation, EDTA, through chelating Ca^{2+}, could have modulated the action of the formulation at the regulation level, leading to non-significant alteration to the expression level of *bcr* at the early stages, 10 and 30 minutes (Figure 5.3), which is indifferent to the effect of the formulation in the absence of EDTA at the very similar

time points (Figure 3.3). In contrast, the expression level of *bcr* at later time points, 60, 120 and 360 minutes was significantly downregulated (Figure 5.3), which could be due to the accumulation of EDTA inside the bacterial cell to a concentration that led to an efflux pump inhibitory action. The action of EDTA has been reported to be a concentration dependant (Lerma *et al*., 2014). Thus, the action of EDTA of eliminating the effect of the formulation at the transcriptional level of gene, yet not on the viability of the bacterial cells, could be due to that EO components have multiple targets in the bacterial cell (Rao *et al*., 2019; Burt, 2004).

Different studies have described the synergistic effect of EDTA and antimicrobial combinations against pathogens. Cinnamon oil and cinnamaldehyde alone and in combination with EDTA have shown synergistical antimicrobial effect against Gram-positive and Gram-negative pathogens including *Staphylococcus pseudintermedius*, *B-haemolytic, Streptococcus sp*., *P. aeruginosa* and *Proteus mirabilis* (Sim *et al*., 2019). The synergistic effect against *P. aeruginosa* has occurred with high concentrations of 672 µg/ml EDTA, 41 µg/ml cinnamon oil and 22 µg/ml cinnamaldehyde, due to overexpression of efflux pumps and outer membrane permeability barrier (Sim *et al*., 2019). The findings of Sim *et al*. (2019) may support the findings of the current study that EDTA displayed an efflux system inhibitory action when it existed at an effective concentration. The alterations in the formulation action at the genetic level of an efflux system due to the existence of EDTA could back up the involvement of transport channels in the mechanism of action of the formulation.

In accordance to the data obtained in the current study, it was thought to establish how constructing gene knockouts might affect the fitness of *E. faecium* (VRE) at the viability and molecular levels in response to the formulation and its components over time. These knockouts considered the genes that were regulated in response to the formulation at 10 and 30 minutes, *bcr* and *yloB* to determine the importance of these two particular genes in the antimicrobial mechanism of action of the formulation and on the survival of the resulting VRE strains. However, it was not successful to isolate chloramphenicol-resistant VRE strains through generating Δ*bcr* and Δ*yloB* using pGhost9-*cat* despite using varied chloramphenicol concentrations and successfully inducing chloramphenicol-resistance in *E. coli* TG1 *repA*$^{+}$ with keeping the temperature sensitivity; suggesting that the pGhost9-*cat* failed to impart chloramphenicol resistance to *E. faecium* (VRE). Similarly, using pGhost9-*cat* to induce chloramphenicol resistant in *E. faecalis* was not achievable by the Mesnage research group at the University of Sheffield (unpublished data, personal correspondence). Furthermore, isolating ampicillin-resistant VRE following electroporation of pGhost9-*bla* knockout constructs was not achievable; despite the successful transformation into *E. coli* TG1 *repA*$^{+}$.

7.1 Future work

The microarray data described different genes were differentially regulated in response to the formulation and its individual components. These genes are involved in varied cellular functions, in particular carbohydrate transport and metabolism. Therefore, investigating another group of genes in the array could be a fundamental thought for future study for further establishment of the mechanism of action of the formulation of interest.

The research conducted in this thesis shows that the action of the formulation of interest could have happened through transport channels with the main inhibitory action occurring at the early stages of treatment. Furthermore, one of the genes responsible for calcium transportation was differentially regulated under the effect of the EOs-vancomycin formulation and its components over time. Interestingly, the effects of either the formulation or its components were increased in the presence of externally added Ca^{2+}. Thus, calcium influx and efflux systems, such as 42912_B02_00594 calcium-translocating P-type ATPase and 42912_B02_01469 calcium-translocating P-type ATPase, could be targets to be investigated in a future work under the same treatment conditions used in the current study and in the presence of ion chelator in particular Ca^{2+}, such as EDTA.

Furthermore, RR and AMG517 did not reverse the inhibition effect of capsaicin *in vitro*; however, it could be informative to find out if they affect the expression level of *yloB* gene, that was significantly expressed when exposed to capsaicin in the absence and

presence of externally added Ca^{2+}, and similarly under the effect of all treatment conditions investigated in this study.

Further investigations to be conducted when the novel gene knockout protocol completes, to establish the effects of the formulation/components on the constructed ΔVRE strains at the viability and molecular levels. All to understand the role of the genes that were knocked out, *bcr* and *yloB*, in the antimicrobial resistance of VRE and how knocking *bcr* and *yloB* out has affected the bacterial ability to survive or not under the effect of the formulation and its components.

7.2 Conclusion

Natural products have been in use since the ancient times as traditional remedies with different effects including antimicrobial actions. Today, natural products and in particular EOs, are emerging as an interesting area of research due to them being found to act effectively alone and in combinations with commercial antibiotics as antimicrobials.

Bacterial cellular functions are happening through regulating the responsible genes, and when the regulation of these genes is being affected by a variety of factors, the bacterial functions in turn including motility, metabolism, virulence and eventually antibiotic resistance will be affected. This has created a new potential research to investigate the effects of antibiotic formulations at the molecular level. The transcriptional response of *E. faecium* (VRE) towards all tested treatment conditions in the current study, ranging from single treatment to combined agents, have resulted in alterations in the regulation of the channels-related genes in the mean of being expressed or repressed resulting in increasing or decreasing of transportation across the bacterial cell. These transcriptional changes could have participated to some extent in restoring the efficacy of vancomycin against *E. faecium*, and eventually might be an alternative method in order to combat the dilemma of AMR that is an increasing threat of human health.

Different possibilities of combining EOs with antibiotics could be new cures for bacterial infections. However, there are issues that still need to be addressed, despite the promising findings of the *in vitro* pharmacological studies, these issues include bioavailability, stability and selectivity of the EOs inside the human body, in addition to

possible adverse interaction between the EOs and the drug. Therefore, in order to deliver sufficient evidences to launch through the stages of clinical trials, the above-mentioned issues have to be addressed and fully assessed. Hoping that the discovery of combining EOs with conventional antibiotics will be in full use, the future will possibly be for EOs to move from being one of the traditional therapeutic agents to a widely used cure in the modern medical field.

Chapter 8

8 References

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Chapter 9

9 Appendix

Chapter 2

9.1 Evaluating the toxicity and efficacy of the neutralising method

Table 9.1. The survival of the tested microorganism in the neutraliser

Condition	Mean Log_{10} CFU/ml	SEM
E. faecium VSE	5.11	0.22
VSE+Neutraliser	4.91	0.05
VSE+AMG517+Neutraliser	4.93	0.04
VSE+capsaicin+Neutraliser	4.91	0.13
VSE+carvacrol+Neutraliser	4.98	0.16
VSE+cuminaldehyde+Neutraliser	4.88	0.06
VSE+EOs combination+Neutraliser	5.02	0.16

9.2 *E. faecium* growth and growth curves with all treatment conditions and in the presence of 10, 30 and 90 nM AMG517

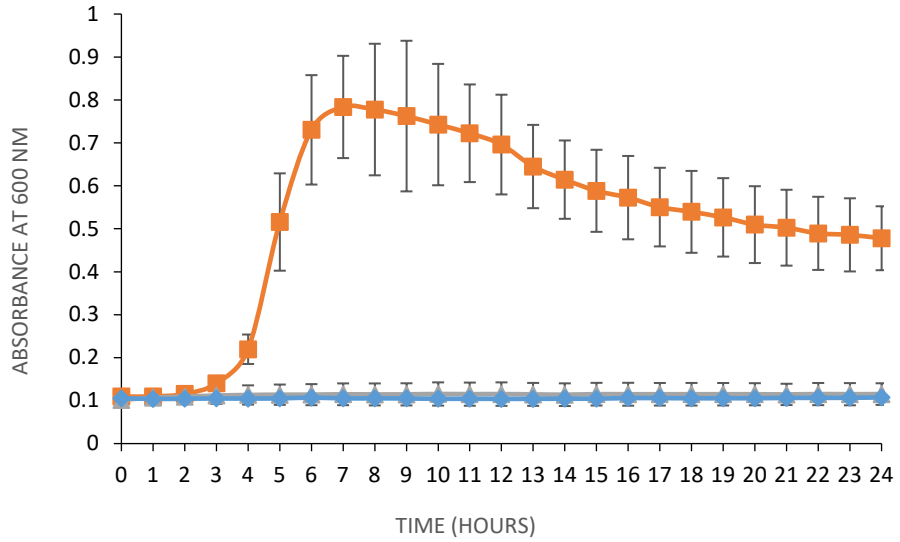

Figure 9.1. *E. faecium* (VSE) growth curves with carvacrol and AMG517 (10 nM). ■ *E. faecium* (VSE) control growth curve. ▲ *E. faecium* (VSE) with 3.95 mM carvacrol. ◆ *E. faecium* (VSE) with 3.95 mM carvacrol and 10 nM AMG517 (n=4, ±SEM).

Carvacrol alone caused significant inhibition of the bacterial growth over the 24 hours of incubation. The addition of 10 nM AMG517 did not reverse the inhibitory effect of carvacrol on *E. faecium* growth at any time point.

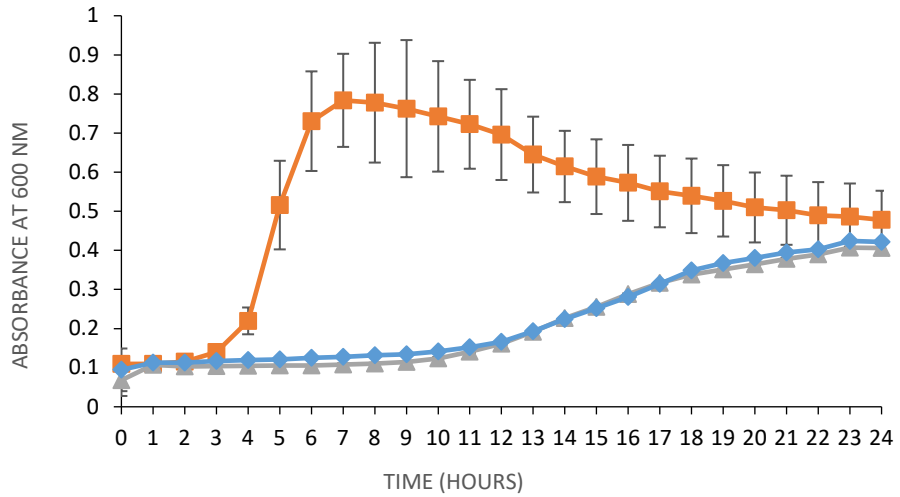

Figure 9.2. *E. faecium* (VSE) growth curves with cuminaldehyde and AMG517 (10 nM). ■ *E. faecium* (VSE) control growth curve. ▲*E. faecium* (VSE) with 134.41 mM cuminaldehyde. ◆ *E. faecium* (VSE) with 134.41 mM cuminaldehyde and 10 nM AMG517 (n= 4, ±SEM).

Cuminaldehyde significantly inhibited the growth of *E. faecium*, the effect was carried out up to 12 hours; however, after that the inhibitory action was eliminated gradually. Adding 10 nM AMG517 did not significantly alter the profile of the inhibitory action of cuminaldehyde at any time over the 24 hours.

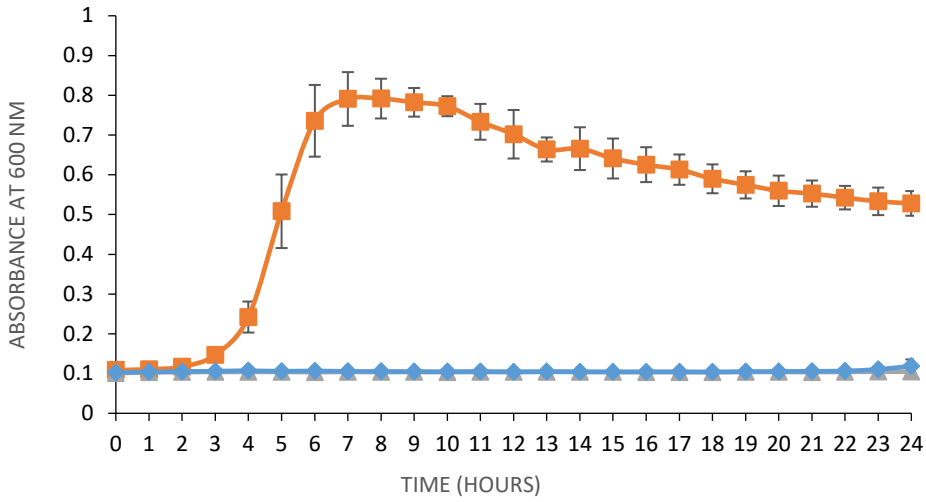

Figure 9.3. *E. faecium* (VSE) growth curves with carvacrol and AMG517 (30 nM). ■ *E. faecium* (VSE) control growth curve. ▲ *E. faecium* (VSE) with 3.95 mM carvacrol. ◆ *E. faecium* (VSE) with 3.95 mM carvacrol and 30 nM AMG517 (n= 4, ±SEM).

Carvacrol has significantly inhibited the growth of *E. faecium*; however, adding 30nM AMG517 in combination with carvacrol has not changed the inhibitory effect of carvacrol on *E. faecium* at any time.

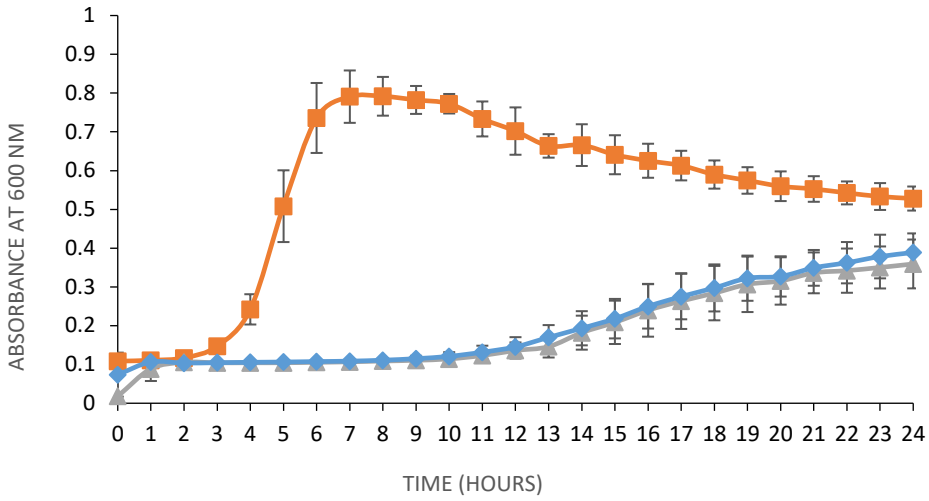

Figure 9.4. *E. faecium* (VSE) growth curves with cuminaldehyde and AMG517 (30 nM). ■ *E. faecium* (VSE) control growth curve. ▲ *E. faecium* (VSE) with 134.41 mM cuminaldehyde. ◆ *E. faecium* (VSE) with 134.41 mM cuminaldehyde and 30 nM AMG517 (n= 4, ±SEM).

Cuminaldehyde inhibited the growth of *E. faecium* for about 12 hours where after that the inhibitory action was reduced gradually (Appendix, Figure 9.4). Similar profile of the inhibitory action of cuminaldehyde was seen when with 10 nM AMG517 and similar incubation circumstances (Appendix, Figure 9.2).

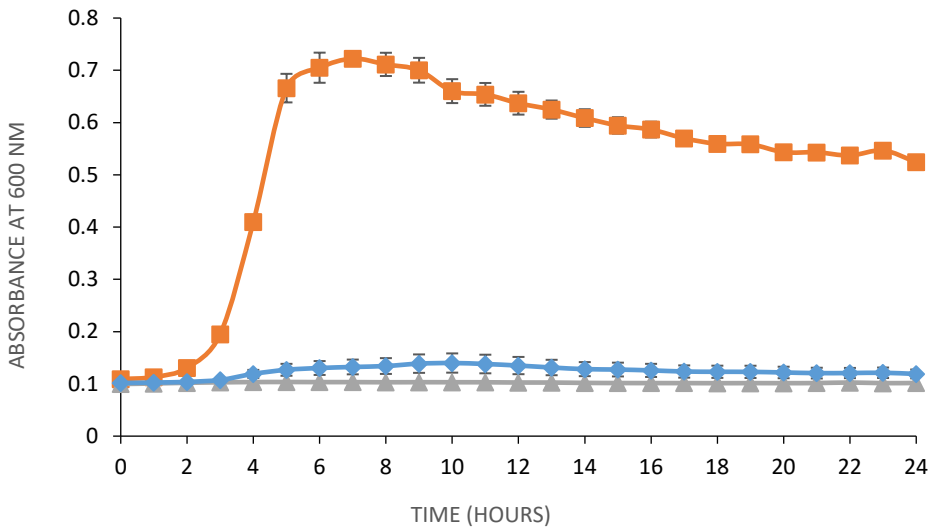

Figure 9.5. *E. faecium* (VSE) growth curves with carvacrol and AMG517 (90 nM). ■ *E. faecium* (VSE) control growth curve. ▲ *E. faecium* (VSE) with 3.95 mM carvacrol. ◆ *E. faecium* (VSE) with 3.95 mM carvacrol and 90 nM AMG517 (n= 4, ±SEM).

The growth of *E. faecium* was totally inhibited by carvacrol over 24 hours. The addition of 90 nM AMG517 to carvacrol did not lead to a reduction in the inhibitory action of carvacrol at any time point.

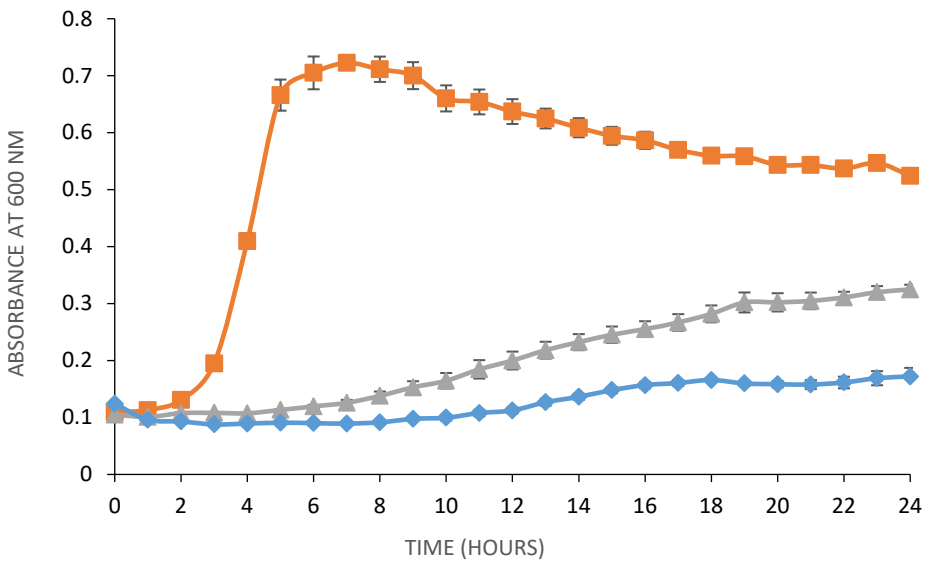

Figure 9.6. *E. faecium* (VSE) growth curves with cuminaldehyde and AMG517 (90 nM). ■ *E. faecium* (VSE) control growth curve. ▲ *E. faecium* (VSE) with 134.41 mM cuminaldehyde. ◆ *E. faecium* (VSE) with 134.41 mM cuminaldehyde and 90 nM AMG517 (n= 4, ±SEM).

It was noted that the inhibitory effect of cuminaldehyde was lessened gradually towards the end of the 24-hour incubation period. The addition of 90 nM AMG517 resulted in a significant increase of the inhibitory action of cuminaldehyde from 12 hours of incubation onwards.

9.3 Reference genes stability analysis

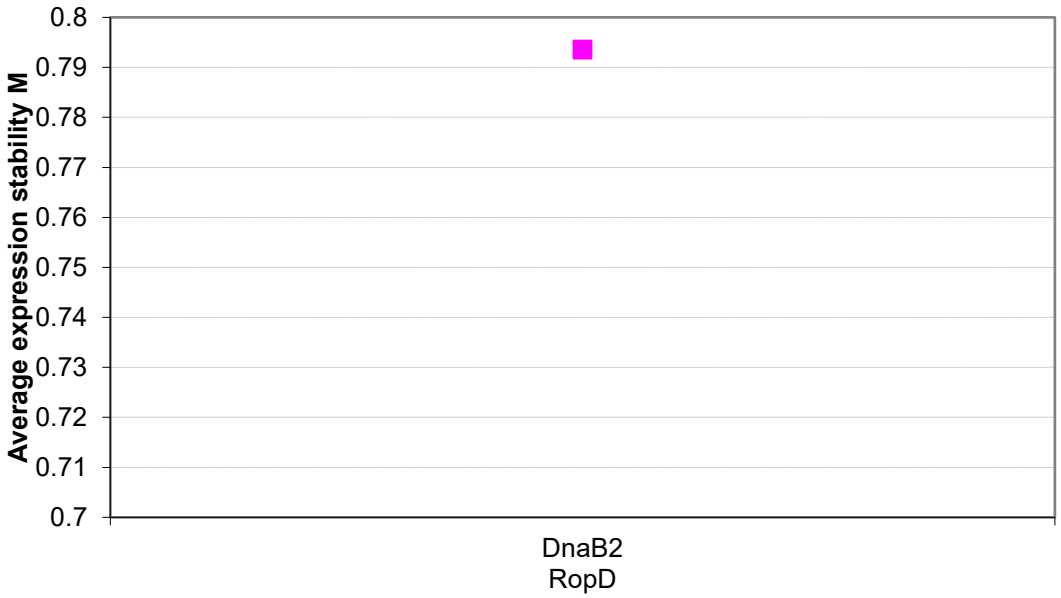

Figure 9.7. The average of the expression stability values of reference genes (*dnaB_2*, *ropD*). M < 1 for both reference genes.

9.4 Extracted RNA quantities of the biological triplicate of samples treated with all treatment conditions and at all time points investigated in the time course study

Table 9.2. The quantities of the extracted RNA (ng/µl) at 60 minutes treatment of the biological triplicate

Treatment	First Run	Second Run	Third Run
DMSO	696	815	710
Carvacrol	707.3	852.9	845.6
Cuminaldehyde	521.2	415.7	590.6
Carvacrol and Cuminaldehyde	519.8	631.6	471.9
Vancomycin	645.7	880.1	350.6
Carvacrol, Cuminaldehyde and Vancomycin	334.4	683	692

Table 9.3. The quantities of the extracted RNA (ng/µl) at 10 minutes treatment of the biological triplicate

Treatment	First Run	Second Run	Third Run
DMSO	162.3	63.7	308.8
Carvacrol	147.6	61.1	148.7
Cuminaldehyde	81.4	85.8	183.6
Carvacrol and Cuminaldehyde	91.2	65.8	160.9
Vancomycin	199.3	81.2	205.8
Carvacrol, Cuminaldehyde and Vancomycin	64.9	151	126.6

Table 9.4. The quantities of the extracted RNA (ng/µl) at 30 minutes treatment of the biological triplicate

Treatment	First Run	Second Run	Third Run
DMSO	315.1	558.6	77.4
Carvacrol	748.8	460.4	133.4
Cuminaldehyde	544.8	53.3	87.3
Carvacrol and Cuminaldehyde	559.6	50.6	84.2
Vancomycin	525.4	402.3	100.4
Carvacrol, Cuminaldehyde and Vancomycin	299.6	1001.4	154.0

Table 9.5. The quantities of the extracted RNA (ng/µl) at 120 minutes treatment of the biological triplicate

Treatment	First Run	Second Run	Third Run
DMSO	64.7	224.2	106.2
Carvacrol	52.2	110.9	75.4
Cuminaldehyde	55.1	110.1	62.8
Carvacrol and Cuminaldehyde	83.0	53.9	53.8
Vancomycin	47.3	64.7	62.0
Carvacrol, Cuminaldehyde and Vancomycin	88.2	71.3	59.6

Table 9.6. The quantities of the extracted RNA (ng/µl) at 360 minutes treatment of the biological triplicate

Treatment	First Run	Second Run	Third Run
DMSO	232.1	298.5	47.4
Carvacrol	126.8	285.7	998.3
Cuminaldehyde	214.5	115.4	65.9
Carvacrol and Cuminaldehyde	101.9	196.1	46.5
Vancomycin	689.9	89.7	537.9
Carvacrol, Cuminaldehyde and Vancomycin	318.7	123.6	381

9.5 Gene, primer efficiency and melting curves

Establishing amplification efficiency is very important step in constructing qPCR runs and accurate interpretation of the data. During each replication cycle the number of molecules of the target sequence should be doubled correspondingly to a 100 % amplification efficiency (Sreedharan *et al*., 2018).

In order to assess primer efficiencies, gDNA templates were quantified in nanodrop and 5 serial dilutions of 1:10 were prepared for constructing standard curves. Each template dilution was used in triplicate with each primer set for higher accuracy of the assessed efficiency. Amplification efficiency could be below 100 % due to reasons including; incorrect primer design, non-optimal reaction conditions or reagent concentrations. In addition to structures errors including dimers and hairpins or inappropriate melting temperatures that can influence the annealing of primer template. Ideally, amplification efficiencies range from 90 % to 110 %, with a desired efficiency of 100 % and correlation coefficient (R2) of 0.99 or preferably greater. Efficiency higher than 100 % is due to polymerase inhibition that is because of increased amounts of DNA/RNA or carry-over

material from the isolation step like ethanol. Primer efficiencies used in this study are listed in (Appendix, Table 9.7).

Melting curve analysis is used as a diagnostic tool to evaluate if the products of the intercalating dye PCR/qPCR runs are single and specific. Intercalating dye assays are of greater concern in the mean of amplification specificity due to that intercalating dyes are not sequence specific and bind to any double-stranded DNA available. While with probe-based assays, the probe adds higher specificity to amplicon specific primers, due to it being a third sequence that in order to produce a fluorescent signal has to bind to the target sequence.

Table 9.7. Efficiency of primers used in the current study

The primers set of each gene	**Efficiency %**	**R2 factor §**
dnaB_2 (reference)	104	0.98
rpoD (reference)	104	0.99
bcr	103	0.99
ecfA1	107	0.99
ecsA_1	98	0.98
nhaC_2	104	0.99
yloB	101	0.99

§: Pearson correlation coefficients ranging from 0.974-0.988

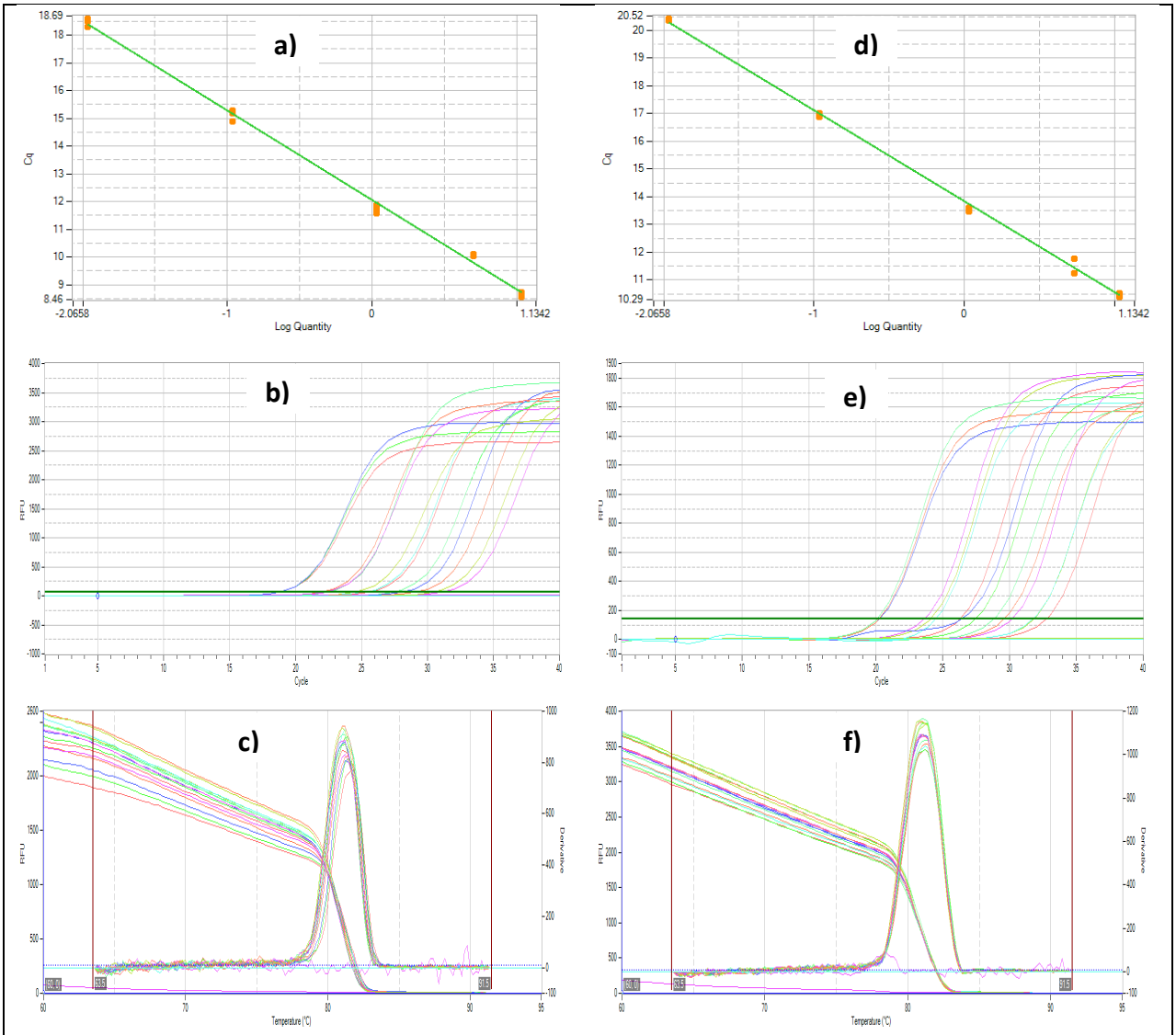

Figure 9.8. Examples of gene primer set efficiency and melting curve. Primer efficiencies were measured using genomic DNA. **a-c**: *bcr* primer efficiency 103 %, R2: 0.996 and no primer dimers; **d-f**: *yloB* primer efficiency 101 %, R2: 0.997 and no primer dimer.

9.6 qPCR results of the time course study

Table 9.8. The fold change in the expression levels of the studied genes at 60 minutes exposure to all treatment conditions

Gene Name	Fold change ($2^{-\Delta\Delta Cq}$)				
	Carvacrol	Cuminaldehyde	Carvacrol and Cuminaldehyde	Vancomycin	EOs-Vancomycin combination
bcr	-1.41	-3.52	-4.22	-1.30	1.53
ecfA1	1.23	2.10	1.60	1.58	2.13
ecsA_1	-1.64	2.16	-4.69	-1.21	-1.14
yloB	-2.41	-2.07	-4.93	-2.07	-1.8
nhaC_2	-2.36	-2.52	-4.53	-2.35	-3.09

Table 9.9. The fold change in the expression levels of the studied genes at 10 minutes exposure to all treatment components

Gene Name	Fold change ($2^{-\Delta\Delta Cq}$)				
	Carvacrol	Cuminaldehyde	Carvacrol and Cuminaldehyde	Vancomycin	EOs-Vancomycin combination
bcr	-2.95	1.19	1.66	-2.53	-13.50
ecfA1	-1.65	-1.82	1.98	-1.55	-1.41
ecsA_1	-1.48	1.30	2.04	-1.24	-3.95
yloB	-2.13	1.61	1.92	1.47	-5.67
nhaC_2	-2.16	1.09	1.17	-5.31	-6.31

Table 9.10. The fold change in the expression levels of the studied genes at 30 minutes exposure to all treatment components

Gene name	Fold change ($2^{-\Delta\Delta Cq}$)				
	Carvacrol	Cuminaldehyde	Carvacrol and Cuminaldehyde	Vancomycin	EOs-Vancomycin combination
bcr	1.12	-1.31	-1.24	-1.37	2.16
ecfA1	1.11	-3.89	-2.67	-6.53	-1.82
ecsA_1	1.28	-1.04	1.21	-1.04	1.39
yloB	1.08	-1.07	1.55	-1.04	3.68
nhaC_2	1.29	1.2	1.03	-1.05	2.84

Table 9.11. The fold change in the expression levels of the studied genes at 120 minutes exposure to all treatment components

Gene Name	Fold change ($2^{-\Delta\Delta Cq}$)				
	Carvacrol	Cuminaldehyde	Carvacrol and Cuminaldehyde	Vancomycin	EOs-Vancomycin combination
bcr	4.89	12.26	12.94	2.34	15.03
ecfA1	4.96	9.70	7.06	1.60	2.85
ecsA_1	1.26	6.36	5.09	2.11	-1.82
yloB	2.81	6.57	7.48	2.36	1.21
nhaC_2	3.96	15.5	4.79	1.83	4.7

Table 9.12. The fold change in the expression levels of the studied genes at 360 minutes exposure to all treatment components

Gene Name	Fold change ($2^{-\Delta\Delta Cq}$)				
	Carvacrol	Cuminaldehyde	Carvacrol and Cuminaldehyde	Vancomycin	EOs-Vancomycin combination
bcr	3.29	1.04	1.41	1.16	15.71
ecfA1	1.01	-1.79	-1.46	-3.99	-1.76
ecsA_1	1.60	-1.06	1.15	2.25	25.87
yloB	1.97	-1.46	-1.19	-4.10	1.32
nhaC_2	-1.73	-5.18	-2.1	-3.13	-1.02

Chapter 4

9.7 Extracted RNA quantities of samples treated with capsaicin and all treatment conditions at defined time points, all in the presence of 1 mM Ca^{2+}

RNA was extracted at defined time points and with defined treatment conditions in accordance to the transcriptional profiles of *yloB* gene under similar circumstances that have previously been established.

Table 9.13. RNAs of samples incubated with capsaicin 1.6 mM alone and in combination with 1 mM Ca^{2+} for 10 minutes

VRE with treatment condition	RNA (ng/µl)/Purity		
	First biological	Second biological	Third biological
Capsaicin 1.6 mM	112.1/2.01	362.0/2.06	93.3/ 1.93
Capsaicin and 1 mM Ca^{2+}	127.0/1.98	149.2/1.99	59.5/2.04

Table 9.14. RNAs of samples incubated with 1 mM Ca^{2+} alone and in combination with EO-vancomycin for 10 minutes

VRE with treatment condition	RNA (ng/µl)/ Purity		
	First biological	Second biological	Third biological
1 mM Ca^{2+}	319.7/ 1.94	436.6/ 1.92	651.7/ 2.00
1 mM Ca^{2+} and the formulation	347.1/ 1.90	250.3/ 1.98	129.3/ 1.95

Table 9.15. RNAs of samples incubated with 1 mM Ca^{2+} alone and in combination with EO-vancomycin for 30 minutes

VRE with treatment condition	RNA (ng/µl)/Purity		
	First biological	Second biological	Third biological
1 mM Ca^{2+}	136.3/1.94	289.2/1.93	267.1/1.96
1 mM Ca^{2+} and the formulation	404.8/2.01	277.8/2.08	109.4/1.99

Table 9.16. RNAs of samples incubated with 1 mM Ca^{2+} alone and in combination with different treatment conditions for 60 minutes

VRE with treatment condition	RNA (ng/µl)/Purity		
	First biological	Second biological	Third biological
1 mM Ca^{2+}	152.5/1.97	379.9/1.98	168.9/1.97
1 mM Ca^{2+} and carvacrol	224.7/1.97	418.2/1.92	271.3/1.98
1 mM Ca^{2+} and cuminaldehyde	160.9/1.98	256.1/1.99	298.8/2.00
1 mM Ca^{2+}, carvacrol and cuminaldehyde	120.4/2.06	286.5/1.93	371.6/2.02
1 mM Ca^{2+} and vancomycin	85.4/2.08	69.5/1.90	92.5/1.99
1 mM Ca^{2+} and the formulation	125.6/1.94	263.8/2.02	243.3/1.96

Table 9.17. RNAs of samples incubated with 1 mM Ca^{2+} alone and in combination with different treatment conditions for 120 minutes

VRE with treatment condition	RNA (ng/µl)/Purity		
	First biological	Second biological	Third biological
1 mM Ca^{2+}	624.5/2.04	478.8/2.01	221.0/2.04
1 mM Ca^{2+} and carvacrol	517.2/2.03	99.8/2.01	171.0/2.03
1 mM Ca^{2+} and cuminaldehyde	541.8/2.01	244.8/2.01	49.5/2.02
1 mM Ca^{2+}, carvacrol and cuminaldehyde	451.0/2.02	319.4/2.00	89.0/1.96
1 mM Ca^{2+} and vancomycin	270.5/1.98	162.5/2.00	102.8/2.03
1 mM Ca^{2+} and the formulation	367.1/1.98	397.9/1.99	113.6/1.98

Chapter 5

9.8 Extracted RNAs of samples treated with the formulation and in the presence of 0.1 mM EDTA at all time points

Table 9.18. Extracted RNAs of samples treated with the formulation and 0.1 mM EDTA at 10 minutes

Treatment condition	First biological		Second biological		Third biological	
	RNA	Purity	RNA	Purity	RNA	Purity
DMSO 1%	42.1	1.97	146.1	2.04	123.1	2.04
EDTA 0.1 mM	116.3	2.08	154.7	2.07	143.4	2.00
Formulation and 0.1 mM EDTA	107.5	1.97	63.7	1.98	43.0	1.98

Table 9.19. Extracted RNAs of samples treated with the formulation and 0.1 mM EDTA at 30 minutes

Treatment condition	First biological		Second biological		Third biological	
	RNA	Purity	RNA	Purity	RNA	Purity
DMSO 1%	107.8	2.02	173.8	2.05	137.0	1.99
EDTA 0.1 mM	1.08.8	2.04	42.3	2.02	124.4	2.04
Formulation and 0.1 mM EDTA	88.4	1.99	107.3	2.04	47.1	1.99

Table 9.20. Extracted RNAs of samples treated with the formulation and 0.1 mM EDTA at 60 minutes

Treatment condition	First biological		Second biological		Third biological	
	RNA	Purity	RNA	Purity	RNA	Purity
DMSO 1%	210.8	1.98	441.1	2.03	231.5	1.98
EDTA 0.1 mM	131.5	1.99	303.8	2.02	154.3	1.98
Formulation and 0.1 mM EDTA	155.8	1.98	206.7	1.99	329.5	1.99

Table 9.21. Extracted RNAs of samples treated with the formulation and 0.1 mM EDTA at 120 minutes

Treatment condition	First biological		Second biological		Third biological	
	RNA	Purity	RNA	Purity	RNA	Purity
DMSO 1%	591.4	2.04	640.4	2.03	297.7	1.98
EDTA 0.1 mM	225.0	1.99	420.4	2.03	267.6	1.97
Formulation and 0.1 mM EDTA	152.6	2.01	278.3	2.03	78.9	1.98

Table 9.22. Extracted RNAs of samples treated with the formulation and 0.1 mM EDTA at 360 minutes

Treatment condition	First biological		Second biological		Third biological	
	RNA	Purity	RNA	Purity	RNA	Purity
DMSO 1%	288	2.07	401.3	2.06	262	1.99
EDTA 0.1 mM	165.2	1.98	389.8	2.00	373.9	2.09
Formulation and 0.1 mM EDTA	184.1	1.99	256.0	1.98	58.3	1.99