The Antimicrobial Effect of Orange, Lemon and Bergamot Essential Oils Against *Enterococcus* sp.

Submitted for the Degree of Doctor of Philosophy
At the University of Northampton

2008

Katie T. Fisher BSc. (Hons)

Acknowledgements

The author would like to thank AMPHORA (Bristol, UK) for the essential oils and technical information, Judith Sheldon of Oxford Brookes University for carrying out the TEM work, Linda McWatt for the use of the sensory suite at Lincoln University and her expertise, Robert Blake of Leicester University for his advice and the use of the Mass – spectrometry equipment and Dr Gareth Eaton at Leicester University for the GC-MS analysis.

I would also like to thank my supervisors Professor Carol Phillips for her guidance throughout my research and Dr Stuart Raleigh for his advice.

I would like to thank all my friends and family for their support through the ups and downs of the last three years especially Nanny, Gandy and Lynne. Special thanks to Maurice, whose strength and encouragement has made this journey smoother and more enjoyable.

Finally, I would like to dedicate this thesis to my Mum as her help, support and faith in me has made this research project possible.

Abstract

Citrus essential oils (EO) are potential antimicrobials, first described as such in 1949 by Piacentini. Due to their acceptability in terms of fragrance and flavour characteristics, they lend themselves to use both in food and in clinical practice. Lemon (*Citrus limon*) or sweet orange (*Citrus sinensis*) or bergamot (*Citrus bergamia*) essential oils and their components (limonene, linalool, citral, hersipidin and neoericitrin) and vapours at different temperatures and pHs were tested for their anti-microbial activity against vancomycin-sensitive *Enterococcus faecium* and *Enterococcus faecalis.* Lemon essential oil alone or blended and orange EO alone were not as effective as citral or linalool alone or bergamot blends with inhibition diameters of > 9cm. The ranges established for differences in inhibition of growth were; 5-15°C, 20-25°C, 30-45°C and 50°C ($p = 0.005$) and pHs 4.5-6.5, pH 7.5, and pHs 8.5 –10.5 ($p \leq 0.001$). A blend of 1:1 (v/v) orange/bergamot EO was the most effective with MICs at 25°C and pH 5.5 of 0.25% - 0.5% (v/v) and an MID of 50mg/l at 50°C at pH 7.5, under these conditions viable counts were reduced by 5.5 – 10 $\log_{10}$ cfu/ml ($p \leq 0.001$). The mechanisms by which a blend of orange/bergamot EO and its vapour bring about their antimicrobial effect were assessed. Transmission electron microscope (TEM) images established morphological changes as well as suggesting EO blend uptake into the cell. The permeability of the cell increased by x2 after being subjected to the EO and by x40 after exposure to the vapour. There were decreases of 1.5 in intracellular pH, 20 a.u. in membrane potential and 18 pmol/mg protein of intracellular ATP. The application of the EO blend impregnated into wipes reduced surface microbial load of vancomycin resistant and vancomycin susceptible *E. faecium* and *E. faecalis* by up to 4 $\log_{10}$ compared with control wipes ($p \leq 0.001$). The use of the EO blend vapour on surfaces resulted in a 2.5 $\log_{10}$ reduction over 24 hours, whilst the vapours (15mg/L air) showed a microbial reduction of up to 4 $\log_{10}$ on cucumber skin and lettuce leaf after 45 seconds exposure via a heat diffuser at 25°C ($p \leq 0.001$). Sensory tests demonstrated that there were no changes to the taste of the foodstuff after treatment with the EO blend vapour compared with untreated controls. Investigations into the applications of the EO blend showed no significant difference in effect of EO blend between vancomycin resistant and vancomycin susceptible strains. The results of this study suggest that the use of a citrus essential blend both in oil and vapour form could be a potential alternative to chemical based antimicrobial.

Contents

Chapter 1

Introduction

For many years *Enterococcus* sp. was believed to be harmless to humans and considered unimportant medically and, due to its bacteriocin producing properties in the last decade has been used widely in the food industry as probiotics or as a starter culture (Foulquie Moreno *et al.*, 2006). Recently enterococci have become one of the most common nosocomial pathogens having a high mortality rate of up to 61% (De Fatima Silva Lopes *et al.*, 2005).

In 2005 in the UK there were 7066 reported cases of *Enterococcus* sp. bacteraemia, an 8% increase from 2004, with the Health Protection Agency (2007) stating "an increase in bacteraemia causing pathogen like this has not been observed in some time." 28% of all cases were antibiotic resistant (Health Protection Agency, 2007). The risk of death from vancomycin resistant enterococci (VRE) is 75% compared with 45% for those infected with a susceptible strain (Bearman and Wenzel, 2005). These figures are mirrored in the USA. In a fifteen year period there was a 20-fold increase in VRE associated with nosocomial infections reported to CDC's National Nosocomial Infections Surveillance (NNIS, (National Nosocomial Infection Surveillance, 2004) .

This dramatic increase in antibiotic resistance of *Enterococcus* sp. worldwide only highlights the need to find an alternative for these drugs. This is paramount within the clinical arena as well as within the food industry where the move from processed foods to towards organic minimal processed foods is the fastest growing sector of the market, driven by green consumerism and government legislation to find substitutes for

the chemically based bactericides, but still maintain the quality and safety of perishable foods (Burt, 2004).

The quantity of *Enterococcus* sp. within the intestines of animals is highest in pigs and chickens over 12 weeks old, but it can also be isolated from soil, surface water and plants and vegetables, which has implications for the contamination of foodstuff (Franz *et al.*, 1999). The presence of *Enterococcus* sp. in the gastrointestinal tract of animals can lead to a high potential for the contamination of plant material fertilised with manure. *E. faecium* is predominant in raw milk and lack of hygiene practices in cheese factories has also lead to high levels of contamination of the cheese products produced resulting in deterioration of their sensory properties (Franz *et al.*, 1999).

The healing properties of essential oils (EOs) have been recognised since Greek and Roman times but it was in the 13th century that they were first documented as being used in pharmacies. Among native Australians plant extracts of tea tree have been used medicinally for centuries. The earliest written record of this was in the 18th century during the colonisation of Australia (Burt, 2004). With the introduction of chemical based medicines such as antibiotics the use of EOs diminished and they are now being increasingly used for flavour and aroma rather than medicinal purposes. However, more recently, the use and mechanisms of action of EOs have become an growing area of research, mainly due to the increasing number of antibiotic resistant micro-organisms and the requirement for alternative solutions (Salvat *et al.*, 2004).

Research carried out on the use of EOs against *Enterococcus* sp. is very limited. Basil has been shown to have antimicrobial properties against *E. faecalis* when it has been exposed for 24 hours at an minimum inhibitory concentration (MIC) of 0.05%

(v/v) (Opalchenova and Obreshkova, 2003). Thyme has an MIC of 0.12% (v/v) (Bruni *et al.*, 2004) and *Origanum majorana* having an MIC of 2.3 mg/ml against *E. faecalis* (Busatta *et al.*, 2008). No on-food investigations were carried out in any of these studies, so the potential application as a food poisoning antimicrobial was not demonstrated. Cinnamon and Australian herb extracts have also been tested for their antimicrobial properties against *Enterococcus* sp. but again only MICs were established and no on-food studies carried out (Chang *et al.*, 2001, Dupont *et al.*, 2006). When tea tree oil was investigated for its action against a selection of bacteria and *Candidia* sp. *E. faecium* was found to be the most resistant requiring an MIC and MIB of >8% (v/v) (Banes-Marshall *et al.*, 2001). Basil has a inhibitory effect on multidrug resistant *Enterococcus* sp., with bacteriostaic effect resulting in the population not being completely inactivated (Opalchenova and Obreshkova, 2003). The inhibitory activity of *Carlina acanthifolia* root EO when compared to ampicillin was more effective at 2% with 18 and 16mm inhibition zones respectively (Dordevic *et al.*, 2007). Berries such as blueberry, raspberry and strawberry have been shown to have an inhibitory effect on *E. faecalis*. The compounds within the berries that inhibited growth were the flavonol myricetin and the flavone luteolin, both having a bacteriostatic effect (Puupponen-Pimia *et al.*, 2001). Citral and linalool had inhibition zones of 21.6 ± 0.1 and 16.7 ± 1.1 (mm) respectively on *E. faecalis*, with carvacrol, eugenol, geraniol and α-pinene also exhibiting inhibition but limonene, menthone and nerol were found to have no effect (Dorman and Deans, 2000).

Little research has been carried out on the use of citrus EOs as antimicrobials but their oils are generally recognised as safe (GRAS) suggesting that they may have potential usage within both the clinical and food arena. The main concern with applying

EOs to foodstuff is possible changes in organoleptic properties. This could be addressed by the fact that EOs are composed of volatile components and these vapours may be the solution that is needed. Again the research carried out on this aspect of EOs is limited. Preliminary studies have shown bergamot, lemon and orange in both oil and vapour form to have antimicrobial properties against a range of Gram- negative and Gram-positive bacteria including *Campylobater jejuni*, *Escherichia coli* O157, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* (Fisher and Phillips, 2006).

Enterococcus sp. especially *E. faecium* and *E. faecalis* with increasing incidence of infection, seriousness of the resulting illnesses and lack of effective treatment due to antibiotic resistance, is one of the most important emerging pathogens (Poh *et al.*, 2006). Infection with *Enterococcus* sp. is mainly thought to be due to endogenous microflora but increasing awareness of the possible transmission route from food is emerging. Although, it is not a foodborne pathogen *per se,* the food chain has been established as an important source of *Enterococcus* sp., especially with foods being carriers of the plasmids for antibiotic resistance (Franz *et al.*, 1999). Due to this antibiotic resistance an alternative treatment needs to be found which will address the needs of both the clinical arena and the food the industry. Could citrus essential oils be the answer? This project aims to answer this questions by assessing the antimicrobial ability of orange, bergamot and lemon citrus EOs and their vapours, the mechanisms of action they bring about their antimicrobial effect and possible applications both on food and surfaces.

Chapter 2

Literature Review

2.1. *Enterococcus* Species

2.1.1 Taxonomy

The genus *Enterococcus* sp. consists of Gram–positive, catalase-negative, non-spore forming, facultative anaerobic bacteria that can occur both as single cocci and/or in chains. *Enterococcus* sp. belong to a group of organisms known as lactic acid bacteria (LAB) that produce bacteriocins (Health Protection Agency, 2005). The genera of LAB with which *Enterococcus* sp. are grouped is identified by a low G + C content of <50% (Klein *et al.*, 1998). There are no phenotypic characteristics to distinguish *Enterococcus* sp. from other Gram–positive, catalase-negative cocci bacteria, so identification is usually established by reverse identification (elimination of other species traits first). This genus of bacteria has been reviewed on many occasions (Klein, 2003). As a genus it has been recognised since Thiercelin who, in 1899, identified *Enterococcus* sp. as an intestinal organism (Stiles and Holzapfel, 1997). Many attempts have been made to distinguish *Enterococcus* sp. from *Streptococcus* sp. In 1937 Sherman sub classified *Streptococcus* into four groups: faecal (enterococci), dairy streptococci, viridans group and pyogenous streptococci (Klein, 2003). Sherman noted that the enterococci subgroup included the Lancefield group D streptococci and suggested that this could be differentiated by haemolytic and proteolytic reactions although this is inappropriate as haemolysis is determined by the plasmid of the bacteria (Stiles and Holzapfel, 1997). Traditional methods such as biotyping, serotyping and

phage typing left questions as to which of the streptococci species actually belonged to the *Enterococcus* genus (Baharak *et al.*, 2002).

In 1984, through the use of DNA hybridisation and 16S rRNA sequencing it was established that the species *Streptococcus faecium* and *Streptococcus faecalis* were different enough from the other streptococci to be called by another genus i.e. *Enterococcus* (Foulquie Moreno *et al.*, 2006). This means that the D group antigen is found in both streptococci and enterococci. Nine species were transferred from the *Streptococcus* groups and now *Enterococcus* includes 28 species (Foulquie Moreno *et al.*, 2006). The molecular data that was collected using 16S rRNA sequencing of *Streptococcus* enabled the construction of 16S rRNA-dendrogram to be drawn up showing the relationship between *Streptococcus* sp., *Enterococcus* sp. and *Lactococcus* sp. (Figure 2.1). This method also allowed the grouping of *Enterococcus* sp. The *E. faecalis* species group includes *E. faecalis, Enterococcus haemoperoxidus* and *Enterococcus moraviensis* whilst *E. faecium* species group includes *E. faecium, Enterococcus durans, Enterococcus hirae, Enterococcus mundtii, Enterococcus porcinus* and *Enterococcus villorum* (Klein, 2003). The identification of enterocooci from the genus streptococci is mainly established by Lancefield group D antigen as only *S. bovis, S. alactolyticus* and *Sc. equines* have the serogroup D. These groups can be distinguished from *Enterococcus* sp. by the lack of growth in 6.5% sodium chloride at 10 °C. It is harder to distinguish *Enterococcus* sp. from other cocci that do not express the D group antigen such as *Pediococcus* sp., *Lactococcus* sp. or *Tetragenococcus* sp. because no other phenotypical differences have been reported that allow distinction from other genuses. Thus the use of fermentation patterns, enzyme activities (pyroglutamyl aminopeptidase (PYRase) activity) (Domig *et al.*, 2003), growth at

defined temperatures and physiological characteristics are essential in the identification of *Enterococcus* sp. (Shanks *et al.*, 2006)

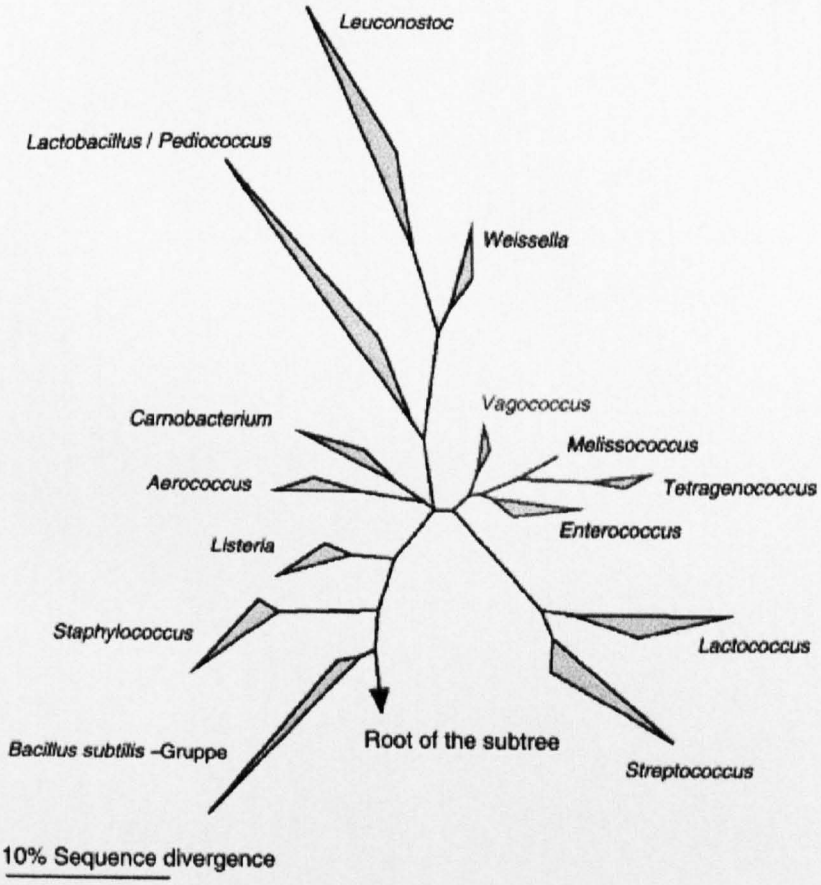


Figure 2.1: 16S rRNA-dendrogram of phylogentic position of *Enterococcus* sp. (Adapted from Klein, 2003).

The differences in the genomes of *E. faecalis* and *E. faecium* have been assessed in a study using competitive DNA hybridisation (Shanks *et al.*, 2006). *E. faecalis* specific sequences were mainly those encoding for surface-exposed proteins. Overall 6.4% of the *Enterococcus* sp. genome is associated with cell surface proteins and 22.6% of the differences between the two species are found in these genes. Thus one of the major differences between *E. faecalis* and *E. faecium* is the proteins associated with the cell membrane. This variation is thought to have implications in avoiding different host immune responses (Shanks, *et al.* 2006).

2.1.2 Growth

Enterococcus sp. will grow at a range of temperatures from 5 to 50°C. The optimum, minimum and maximum temperatures, according to the Rosso model, are 42.7, 6.5 and 47.8 °C respectively on Brain Heart Infusion (BHI) agar in aerobic conditions (Van den Berghe *et al.*, 2006), although growth will also occur in anaerobic atmospheres (Domig *et al.*, 2003). *E. faecalis* has the ability to reduce triphenyltetazolium-chloride (TTC) strongly whereas *E. faecium* only reduces it weakly. Should TTC be added to selective media it is an effective method of distinguishing between the species with red and pink colonies being produced respectively. They can also be distinguished by different growth patterns at certain temperatures i.e. 50°C (Klein, 2003). This was illustrated in a study when different media were used to grow *Enterococcus* sp. Both *E. faecalis* and *E. faecium* can survive heating at 60°C for 30mins, making the *Enterococcus* sp. distinguishable from other closely related genus (Foulquie Moreno *et al.*, 2006). The use of Trypticase soy agar and Columbia agar with 5% (v/v) defibrinated sheep blood can assess the haemolysis produced by

enterococci, although if human or horse blood is used haemolysis is based on cytolysin activity and causes a β-haemolytic reaction (Domig *et al.*, 2003). *E. faecalis* and *E. faecium* will grow in a wide range of pH (4.5-10.5) with optimum, minimum and maximum being 7.5, 4.6 and 9.9 respectively (Van den Berghe *et al.*, 2006) and also at 40% (w/v) bile salts. *E. faecalis* is able to grow in 6.5% NaCl and has a cation homeostasis which is thought to contribute to its resistance to pH, salt, metal, and desiccation. Both species have a large range of antibiotic resistance (Eaton and Gasson, 2002). More than a hundred modifications of selective media for isolation of *Enterococcus* sp. from the environment have been described, but being able to distinguish between the other group D streptococci species is the problem that is often incurred. No medium has been found to be able to do this to date. The use of two media in parallel such as M-*Enterococcus*-agar (ME) and kanamycin-aesculin-azide-agar (KAA) or genetic testing must be carried out to distinguish between the group D streptococci and enterococci (Reuter, 1992).

2.1.3 Temperature and pH Resistance

When assessing growth using optical densities the most important variable on growth parameters is pH with temperature and salt concentration having a lesser effect (Gardini *et al.*, 2001). During lag phase, temperature is the most important factor influencing growth, with stationary cells being the most resistant to heat (Gardini *et al.*, 2001, Martinez *et al.*, 2003). The resilience of *E. faecalis* to a range of pH is thought to be due to its membrane durability and impermeabilty to acid and alkali, although some studies have suggested it may be due to cell membrane-bound H^+-ATPase activity (Nakajo *et al.*, 2005). Temperature resistance is also associated with the membrane

structure and has been related to lipid and fatty acid content. The membrane has been demonstrated to be more stable near the minimal temperature for growth, which is a specific mechanism associated with enterococci (Ivanov *et al.*, 1999). At higher temperatures *Enterococcus* sp. is less resilient, with the membrane fatty acid content increasing and the saturated fatty acid levels decreasing. Findings indicate that the heat resistance of enterococci is not only dependent on the temperature but the phase of growth as well (Martinez *et al.*, 2003).

Studies have shown that when *E. faecalis* is grown at non-stress temperatures subsequent subcultured cells do not have the resilience to warm and cold environments that would occur if the first generation were grown at stressful temperatures (Ivanov *et al.*, 1999). There are three distinct temperature groups (10-13 °C, 17-22 °C and 42-47 °C) established for *E. faecalis* at which permeability of the membrane is different (using 3% NaCl). This has significant implications with regards to biotechnology and food science, the fact that *E. faecalis* withstand a range of temperatures under high salt concentrations makes it ideal for use within the food processing environment for such usages as a bacteriocin or probiotic (Ivanov *et al.*, 1999).

The production of amines is also closely related to the growth temperature and pH. The production of decarboxylases is optimum at acid pH, whereas *E. faecalis* EF37 biogenic amine production decreases at low pHs. Temperature does not have a significant effect on amine production itself, but the effect that temperature has on cell yield does alter the quantity of amines being produced (Gardini *et al.*, 2001). Other products of *Enterococcus* sp. that are affected by pH are bacteriocins. Bacteriocin production is favoured in stressful growth conditions; this is thought to be due to lower

growth rates, which results in better utilisation of energy and greater availability of metabolites for the synthesis of bacteriocins. Under optimal growth conditions and thus high growth rates there is a lack of amino acids available for bacteriocin production (Van den Berghe *et al.*, 2006).

2.1.4 Ecology and Epidemiology

The origins of *Enterococcus* sp. vary from environmental to animal and human sources. As enterococci are an essential part of the microflora of both humans and animals its distribution is very similar in these sources. *E. faecium* and *E. faecalis* are the most common in the human GI tract; *E. faecium* in production animals and *E. mundtii* and *E. casseliflavus* in plant sources (Klein, 2003). Due to the occurrence of *Enterococcus* sp. in the GI tract of animals, the isolation of it from foods is associated with faecal contamination (Poeta *et al.*, 2006) and if found in water it can be used as a indicator species of faecal contamination (Kuhn *et al.*, 2003). The numbers of *E. faecalis* in human faeces range from 10^5 to 10^7 per gram with *E. faecium* being 10^4 to 10^5 per gram. *E. faecalis* has also been shown to be present in the faeces of neonates. The isolation of *E. faecium* and *E. faecalis* is less prevalent in livestock than human faeces (Franz *et al.*, 1999). Faecal materials can enter the food chain via urban sewage, recipient water and soils being treated with fertilisers of animal origin. The reason *Enterococcus* sp. is used as an indicator organism is because of its ability to colonise diverse niches and its insensitivity to pH and salt changes, which is why enterococci are not only associated with warm-blooded animals, but occur in water, soil and on plants and vegetables (Giraffa, 2002).

Studies of the ecology and epidemiology of *Enterococcus* sp. have reported *E. faecalis* and *E. faecium* regularly isolated from cheese, fish, sausages, minced beef and pork (Klein, 2003, Foulquie Moreno *et al.*, 2006). Foods such as sausages and cheese that are of animal origin (where it can cause spoilage even when cooked or processed), are often associated with *Enterococcus* sp. contamination, as it is able to survive the heating process (*E. faecium* can survive 68°C for 30 min). In one study in the UK, samples taken from urban sewage, farmland using pig manure and crops generated from this land, were found to be 100% positive for *Enterococcus* sp. In crops which did not use animal fertilisers, incidences of *Enterococcus* sp., were reduced to 33%. Of 1991 samples taken from animal origin and 355 mixed animal/human origin 77% of the samples showed growth of *Enterococcus* sp. Out of the 17, 157 samples taken within this study the most common species were *E. faecium* (33%) and *E. faecalis* (29%) (Kuhn *et al.*, 2003). A similar study in Germany showed that 416 strains of *Enterococcus* sp. were isolated from 166 samples *E. faecalis* (72%) and *E. faecium* (13%) (Peters *et al.*, 2003).

The distribution of species of *Enterococcus* sp. varies throughout Europe. In Spain and the UK *E. faecalis* and *E. faecium* are the most commonly isolated species from both clinical and environmental sources. Sweden has a lesser incidence of *E. faecium* and a higher isolation rate of *E. hirae*, whereas in Denmark *E. hirae* is the dominating species and is mainly isolated from slaughtered animals (Kuhn *et al.*, 2003).

Clinical isolates of enterococci show a lower diversity than those obtained from the environment and other human sources, with *E. facealis* being the dominant species (Kuhn *et al.*, 2003). The reason for this lack of diversity may be linked with the

virulence factors associated with this strain. The fact that *Enterococcus* sp. are opportunistic pathogens was highlighted by a study in Denmark which showed that hospitalised patients have a 57% isolation rate of *E. faecalis* whereas healthy individuals only show a 39-40% occurrence (Mutnick *et al.*, 2003). Sweden and Spain also show a high *E. faecalis* incidence in hospital patients with rates of 80% and 93% respectively (Kuhn *et al.*, 2003). Hospitalised patients may have a greater incidence of enterococcal infection not only because of virulence, but due to the hospital itself being a hub, illustrated by a report for the Department of Health in the UK which highlighted the fact that enterococci may contaminate and survive around the patient for several days. The reservoir is the human bowels and cross contamination occurs via staff hands and the environment (Brown *et al.*, 2006). One study has shown that, out of ten species inoculated from a hospital environment including, *Staph. aureus, Enterobacter* sp., *Acinetobacter* sp., and *Pseudomonas aeruginosa* and *Enterococcus* sp. the latter was found to survive for the longest with *E. faecuim* having a median survival of 60 days and *E. faecalis* of 30 days. Over the year that the samples were taken *E. faecium* was found to be genetically less diverse than *E. faecalis* (Gastmeier *et al.*, 2006).

2.1.5 Symptoms of Infection

Enterococcal infections include: urinary tract infection, hepatobiliary sepsis, endocarditis, surgical wound infection, bacteraemia and neonatal sepsis (Poh *et al.*, 2006). In Europe infection with *Enterococcus* sp. were considered harmless to humans for a long time, however in the last decade it has been reported as the second most common cause of wound and urinary tract infection and the third most common cause of bacteraemia (De Fatima Silva Lopes *et al.*, 2005). Between 2000 and 2004

Enterococcus sp. was the second most common pathogen associated with skin and soft tissue infection across the three continents of North America, Latin America and Europe (Fritsche *et al.*, 2005). In 2005 in the UK there were 7066 reported cases of *Enterococcus* sp. bacteraemia, 63% of these cases were due to *E. faecalis* and 28% to *E. faecium,* both of which have increasing antibiotic resistance (Health Protection Agency, 2007). In the USA approximately 12% of the hospital acquired infections are from *Enterococcus* sp. *E. faecalis* is the most common species associated with clinical infection however *E. faecium* poses the most antibiotic resistant threat (Giraffa, 2002).

A study in Singapore demonstrated that, out of 23 patients with enterococcal infection, 55% acquired it within a hospital setting and 45% from the community. In the same acute care hospital 31% of the patients infected with *Enterococcus* sp. bacteraemia died (Poh *et al.*, 2006). In the USA although differences have been noted between bacteraemia acquired from hospital environments and from the community, in the hospital acquired bacteraemia *Enterococcus* sp. account for 15% of isolated organisms whereas in the community acquired isolates it is insignificant (Bearman and Wenzel, 2005). This may be due to a reporting bias where community acquired infections are not reported as frequently as the hospital acquired ones, highlighting the point that over the last twenty years there has been a steady decrease in the number of community acquired infections and a three-fold increase in hospital acquired infections (Franz *et al.*, 1999). The increase in hospital acquired infections is thought to be due to the increased use of broad spectrum antibiotics (Giraffa, 2002).

Overall high mortality rates of between 30-61% are associated with enterococcal bacteraemia (Poh *et al.*, 2006). It has been observed that 24% of enterococcal

bacteraemias originate from urinary tract infection (Giraffa, 2002). The majority of clinical isolates represent colonisation rather than an infection (Brown *et al.*, 2006). This was also found to be the case in a study at rehabilitation facility taking part in the National Nosocomial Infection Surveillance System (NNISS) where of the 55 patients with enterococcal urinary tract infections, the organism had not been passed from patient to patient but was endogenous (Lloyd *et al.*, 1998). These findings are surprising due to the increasing levels of hospital acquired enterococcal infection.

The problem with assessing the clinical importance of *Enterococcus* sp., is in being able to identify the species. As described previously it is difficult to distinguish between *Enterococcus* sp. and other closely related genus in particular *Streptococcus.* This is highlighted by the fact that in the UK between 1990-1998, 15% *E. faecalis* species were identified as being ampicillin/amoxicillin resistant and only 12% in 2000, which suggest that some species identified as *E. faecalis* were actually *E. faecium* because *E. faecium* is more commonly antibiotic resistant and there have been no confirmed cases in the UK of *E. faecalis* (Brown *et al.*, 2006). This illustrates the fact that DNA sequencing is essential in the identification of *Enterococcus* sp.

Infection in hospitals from *Enterococcus* sp. leads to extended hospital stays, costs (as much as £6 billion in 2005 and 35% of doctors consultancy time) and greater opportunity for antibiotic resistant strains to emerge (Health Protection Agency, 2006).

2.1.6 Antibiotic resistance

The antibiotic resistance of *Enterococcus* sp. is well documented. Its resistance includes that to glycopeptides (vancomycin and teicoplanin are licensed in the UK) and

aminoglycosides (Kacmaz and Aksoy, 2005). Antibiotic resistance has been of growing concern for a number of years. Vancomycin was first used in the clinical arena in 1972 and the first vancoymycin-resistant enterococci was recognised only fifteen years later in 1987. National Nosocomial Infections Surveillance (NNIS) reported an increase of 7.6% in VRE between 1989 and 1993 (Metan *et al.*, 2005). Due to the increased resistance of *Enterococcus* sp. the Department of Health in the USA in 2003 introduced the glyocopeptide-resistant enterococcal (GRE) bacteraemia surveillance group. Their reports have shown that if GRE is present in an infected patient rather than an antibiotic susceptible strain, clinical treatment failure is increased by 20% and mortality is increased from 27% to 52% (Brown *et al.*, 2006). A high incidence of enterococcal strains ampicillin resistant is also found (as great as 75% in one hospital) which is a concern, as penicillins are the main treatment for enterococcal infection and the emerging resistance to this group of antibiotics limits effective treatment options (Mannu *et al.*, 2003). When assessing the studies carried out on enterococcal antibiotic resistance, the pattern that is emerging is the possible occurrence of multidrug resistant strains (Peters *et al.*, 2003).

A study in an intensive care unit has shown that out of all the patients that contracted enterococcal infections whilst in hospital, 35% of the *E. faecalis* and 57% of the *E. faecium* isolated strains had high levels of resistance to gentamicin and streptomycin (Rodriguez-Bano *et al.*, 2005). In 2003, 60-80% of *E. faecium* strains were ampicillin resistant where as only 0.5-1% of *E. faecalis* showed resistance (Klare *et al.*, 2003). In both the Surveillance and Control of Pathogens of Epidemiological Importance (SCOPE) and SENTRY (Antimicrobial Resistance Surveillance Program) databases, figures show that, of enterococcal isolates from the bloodstream, 2% *E.*

faecalis and 60% *E. faecium* are resistant to vancomycin. Fortunately only 10% of bacteraemia infection in the UK is caused by *E. faecium* (Bearman and Wenzel, 2005). Resistance rates of *Enterococcus* sp. have reached endemic/epidemic in North America with Europe having lower, but increasing, levels (Mutnick *et al.*, 2003).

Enterococcal antibiotic resistance is not exclusive to the clinical arena but is also prevalent in the food industry. Individuals that have been hospitalised with VRE when they have not previously been in hospital or taken antibiotics, suggests that it may have been contracted through the food chain. VRE may emerge in the food chain through use of avoparcin in animal feed (Mannu *et al.*, 2003). GRE has also been emerging in animal faeces; again this is also thought to be due to the use of avoparcin. At least some of these resistant bacteria will enter the food chain and potentially colonise humans (Brown *et al.*, 2006).

Poultry faeces in Spain have been shown to have large numbers of aminoglycoside resistant *Enterococcus* sp. (34.5%), 40% of which were *E. faecium.* The same strains have shown susceptibility to antibiotics associated with the clinical environment such as vancomycin and ampicillin (Tejedor-Junco *et al.*, 2005). High levels of antibiotic resistant enterococci have also been observed in poultry in Portugal, 10 times greater than that isolated from domestic animals (Poeta *et al.*, 2006). 70% of enterococci in Swedish retail chickens were found to be resistant to one or more antibiotics (Giraffa, 2002).

In a study of European cheeses no VRE was isolated between 1980-1990 whereas recently 4% of *E. faecium* isolated from the same cheese sources overall

proved to be vancomycin resistant, with higher numbers found in meat products (De Vuyst *et al.*, 2003, Omar *et al.*, 2004). When assessing both clinical and dairy isolates of *Enterococcus* sp.overall high resistance to meticillin (91%), linocomycin (92%), colistin (97%) were found in dairy isolates, illustrating differences between the resistance and isolation source i.e. the lack of selective pressure. Isolates from dairy sources show low vancomycin resistance. A possible reason is that this antibiotic is not used in feed, whereas the clinical isolates have higher resistance to vancomycin and this is known to be used frequently in hospitals, thus causing selective pressure for vancomycin resistance strains in the hospital setting (De Fatima Silva Lopes *et al.*, 2005, Canzek Majhenic *et al.*, 2005).

A study assessing the occurrence of *Enterococcus* sp. in retail foods including meat, dairy produce and vegetables, demonstrated that all samples tested positive for *Enterococcus* sp., ranging from 10^3 CFU/g in meat and vegetables to 10^4 CFU/g in cheeses. All isolates were susceptible to ampicillin, penicillin and streptomycin whereas the majority were resistant to erythromycin and rifampicin. This study not only highlights the different routes of contamination but how selection pressures determine different antibiotic resistance (Omar *et al.*, 2004). High levels of resistant *Enterococcus* sp. are also found in raw, fermented and unfermented foods. Glycopeptide resistant *E. faecium* obtained from pig or chicken sources has been shown to survive the GI tract and multiply and could be isolated from faeces up to 14 days later, posing a threat to human health (Emborg *et al.*, 2003).

The reductions of antibiotic use in animal feed is reflected in a decrease in the antibiotic resistance *Enterococcus* sp. isolates from animal sources. In a study of broiler

meat from Denmark, resistance to avilamycin decreased in proportion to the amount consumed by the animal from 1400 – 2740 kg in 1995/96 to 670 kg in 1997. In 1998 antibiotic growth promoters were withdrawn from all Danish broiler production, resulting in a further reduction in resistance, so by 2001 less than 20% of *E. faecium* was resistant (Emborg *et al.*, 2003). The use of antibiotics as growth promoters creates a reservoir of antibiotic resistant enterococci and allows a spread to other ecological habitats and humans (Klare *et al.*, 2003, Giraffa, 2002).

Combination drugs are used for treatment of infections from animal origin combinations of penicillin and aminoglycosides are usually used (Peters *et al.*, 2003). Quniupristin and dalfopristin work against both resistant *Enterococcus* sp. and MRSA (Wilcox and Hodgson, 2005) and tigecycline against VRE (Fritsche *et al.*, 2005) in hospital acquired infection.

2.1.7 Virulence

Sources of *Enterococcus* sp. with the highest virulence factors are medical isolates followed by food isolates then starter strains (Omar *et al.*, 2004, Busani *et al.*, 2004). Many factors determine the virulence of *Enterococcus* sp., for example, its ability to colonise the GI tract, which is its normal habitat, its ability to adhere to a range of extracellular matrix proteins including thrombospondin, lactoferrin and vitronrctin and to adhere to urinary tract epithelial and human embryo kidney cells. Most infection is thought to be endogenous, so translocation of the bacteria through the epithelial cells of the intestine which then cause infection via lymph nodes and thus spread to other cells within the body (Franz *et al.*, 1999). The aggregation substance

(Agg) on the surface of *E. faecalis,* has been shown *in vivo* to form large aggregates and hence may contribute to its pathogenesis. The presence of Agg increases the hydrophobicity of enterococcal cell surface, which in turn induces localisation of cholesterol to the phagosomes and is thought to delay or prevent fusion with lysosomal vesicles. Although Agg is only found in *E. faecalis* its incidence in food isolates is high (Eaton and Gasson, 2002). Extracellular surface protein (Esp) was first described in *Enterococcus* sp. in 1999 by Shankar *et al.* The Esp gene consists of 5622 nucleotides and is found in high numbers in infection derived isolates. It is thought to promote adhesion, evasion of the immune system and play some role in antibiotic resistance (Foulquie Moreno *et al.*, 2006). Esp also contributes to enterococcal biofilm formation, which could lead to resistance to environmental stresses and adhesion to eukaryotic cells such as the urinary tract (Borgmann *et al.*, 2004). Twenty one out of 28 clinical isolates of *E. faecium* were found to have primers that were specific for the Esp gene, this goes some way to confirming that the Esp gene does have a role in pathogenicity as this gene was absent from dairy isolates, this would also suggest that dairy *E. faecium* is not responsible for enterococcal infections in humans (Mannu *et al.*, 2003).

The ability for *Enterococcus* sp. to become resistant to antibiotics is probably its greatest virulence factor. There are three main types of resistance VanC (Intrinsic) VanA (acquired) and VanB resistance. VanC-type resistance is specific to *E. gallinarum, E. casseliflavus* and *E. flavescens,* the *vanc* operon is chromosomally located and is not transferable. These species do not posses cytochrome enzymes and thus can not produce the energy needed to up take the anitibiotics into the cell, this means they have resistance to aminoglycosides at low levels, *E faecalis* has this trait towards streptogramins (Klare *et al.*, 2003). VanA-type resistance is commonly

resistance against glycopeptides, it was first detected in *E. faecium*. VanB type-resistance is the second most common type resistance to glycopeptides in enterococci (Courvalin, 2005, Metan *et al.*, 2005). Overall the distribution of the genes encoding for antibiotic resistance from clinical strains was found to be higher in *E. faecium* (Kolodjieva *et al.*, 2006).

Acquired resistance requires two prerequisites, the genetic potential of the microorganism and antibiotic selective pressure (Klare *et al.*, 2003). This type of resistance can be transferred by pheromone mediated, conjugative plasmids or transposons. These resistant mutations in the DNA cannot only be passed on to antibiotic susceptible enterococci but other pathogens as well (Giraffa, 2002). The VRE phenotype is of most concern in the clinical arena as if this resistance is passed onto species such as the already virulent *Staph. aureus* there are major health implications (Mannu *et al.*, 2003). Due to this transfer of resistance all starter cultures must be assessed before being added to food to ensure that either they are antibiotic susceptible or that they do not have acquired resistance (Klein, 2003). The other possible transfer that could occur is between environmental/husbandry strains and human strains.

2.2 *Enterococcus* Species and Food

2.2.1 Lactic Acid Bacteria

Enterococcus sp. belongs to the lactic acid bacteria group as their metabolism is fermentative using the homofermentative Embden-Meyerhof-Parnas pathway producing lactic acid. They also obtain energy from the degradation of amino acids (Stiles and Holzapfel, 1997). Other homofermentors first classified by Orla and Jenson in 1942 (Carr *et al.*, 2002) include *Streptococcus* and *Pediococcus.* Most of the *Enterococcus* sp. with the exception of *E. faecalis* have a peptidoglycan containing lysine-D-asparagine type linkages with *E. faecalis* having a lysine-alanine 2-3 type (Domig *et al.*, 2003).

Enterococcus sp. are identified as a LAB through their morphology, amount of lactic acid produced during growth at 6.5% NaCl and optimum growth temperature of 37°C. Variation of pH is often used as to identify LAB using a general purpose agar and then either use of a homofermenative heterofermentative differential medium (HHD) (Carr *et al.*, 2002).

The use of LAB within food for beneficial reasons is on the increase but due to the potential harm, virulence factors and antibiotic properties that *Enterococcus* sp. possess, the World Health Organisation (WHO) have stated that it is the responsibility of the producer to ensure that its use of microbials in food are safe for human consumption (Tsai *et al.*, 2004). Guidelines have been produced jointly by WHO and the Food and Agriculture Organisation (FAO) in 2002 (Food and Agriculture

Organisation and World Health Organisation, 2002) but with increasing use of probiotics there may be a need for it to be reviewed to provide more detailed guidlines.

2.2.2 Probiotics

Probiotics are live microbial supplements, which have beneficial effects on the host including prevention of lactose intolerance and rotavirus diarrhoea, atopy and improvement in the intestinal microflora balance (Foulquie Moreno *et al.*, 2006). LAB are often used for probiotics (Tsai *et al.*, 2004) but they must have the ability to adhere to cells, to exclude or reduce pathogenic adherence, to persist and multiply in the intestines, produce acids, hydrogen peroxide and/or bacteriocins that are antagonistic to pathogens, be safe to humans and coaggregate to form a healthy microflora (Foulquie Moreno *et al.*, 2006). The ability of LAB to reduce the pH of their environment reduces the array of other bacteria that are unable to survive under more acidic conditions. Low pH has also been shown to permeabilise the membrane of Gram-negative bacteria (Guerra *et al.*, 2006). The application of probiotics can lead to the spread of antibiotic resistant bacteria into the gut and/or the environment (Klein *et al.*, 1998). This may occur with the combined use of probiotics and antibiotics in animal feed to promote better growth performance. Eventually in any environment where an antimicrobial is present selection will occur for those strains that are resistant and antibiotic species can emerge such as VRE (Chenn and Hoover, 2003). Also the onset of gastrointestinal disease is caused by the same pathogens used as probiotics such as *Enterococcus* sp. and *Staphylococcus* therefore raising questions over the effects of probiotics on health and disease (Timmerman *et al.*, 2004). Five strains of *Enterococcus* sp. are being used as probiotics at present, which include *E. faecalis* and *E. faecium*. For the use of

Enterococcus sp. as a probiotic it is essential to remove the *vanA* gene. This gene has been shown to express the resistance protein (39kDa), which is present in both *E. faecalis* and *E. faecium* (Klein *et al.*, 1998).

E. faecium, when used as probiotic in pig feed, increases the body weight and promotes feed conversion. It survives gastric transit (180min of treatment) with a survival of 2 x 10^6 cfu/mL and significantly decreases the number of other coliforms in the piglets faeces over 42 days by 1.4 –1.8 Log_{10} (Guerra *et al.*). The results of a study in Slovakia, using *Enterococcus* sp. as a probiotic in pig feed, has also shown a reduction of staphylococci and *E. coli* in the piglets tested after seven days of supplements (Strompfova *et al.*, 2006). Two strains of *E. faecalis*, two strains of *E. mundii* and seven strains of *E. faecium* isolated from pigs gastrointestinal tracts have a high antagonistic effect of >80% against *E. coli* K88, showing potential for probiotic use (Garcia-Galaz *et al.*, 2004). The growth performance of chickens was also increased with the use of *E. faecium* and reduced faecal *E. coli* O157:H7 shedding in lambs was observed (Timmerman *et al.*, 2004). The possible problem of using probiotics in feed is the contamination of the meat and subsequent affect on humans from its consumption (Hugas *et al.*, 2003).

The Food Standards Agency (FSA) concluded in a report on the use of probiotics for human consumption that LAB are not able to survive the whole of the digestive tract of humans and little variation was noted in the indigenous microflora (FSA, 2006). Another investigation shows that after dysbiosis treatment the consumption of probiotic *E. faecium* SF68 did not correct the procedure but actually enhanced it (Timmerman *et al.*, 2004). Although the evidence for the beneficial use of

Enterococcus sp. as probiotics in humans is controversial, it has still been suggested as a supplement for the prevention and treatment of various gastrointestinal infections and acute enteritis (Tsai *et al.*, 2004).

2.2.3 Starter Cultures

E. faecium K77D has been approved as a starter culture (ripening) in dairy products by the UK Advisory Committee on Novel Foods and Processes (ACNFP 1996) and is used widely by the International Dairy Federation (Hugas *et al.*, 2003).

Enterococcus sp. are used as starter cultures in dairy products due to their effective acidifying (*E. faecalis* is a stronger acidifier than *E. faecium*) and proteolytic properties. *E. faecium* and *E. faecalis* are important in the production of dairy products, due to their ability to survive a range of temperatures, substrates and pasteurisation, as well as being able to increase in number in refrigerated milk (Franz *et al.*, 1999). Although the positive influence of *E. faecium* and *E. faecalis* in the ripening of cheese is well documented it has also been stated that its presence may be the result of poor hygiene and manufacturing practices, which leads to cross contamination of products and deterioration of the sensory quality of the products (Giraffa, 2003). Cheddar cheese ripening has been found to occur more efficiently with the presence of LAB as it leads to a greater presence of free fatty acids (Hickey *et al.*, 2006). This has also been shown to be the case in feta and cebreiro cheeses (Foulquie Moreno *et al.*, 2006). *Enterococcus* sp. produce typical flavour components i.e. acetaldehyde, acetoin and diacetyl, therefore *E. faecalis* is used in mozzarella and *E. durans* in feta cheese due to their aroma, taste, colour and structure developments (Franz *et al.*, 1999, Foulquie

Moreno *et al.*, 2006). The use of *Enterococcus* sp. in green olive fermentation produces effective acidification, consumption of carbohydrates and decrease in pH of the brine, hence speeding up the fermentation process (De Castro *et al.*, 2002). A example of inappropriate use of starter cultures with detrimental effects was the use of one strain of Lactobacillus as a starter culture in yoghurt which was subsequently found to be streptomycin resistant with an MIC of > 1024 μg/ml, potentially leading to the spread of antibiotic resistance to other pathogens (Katla *et al.*, 2001). Out of twenty nine isolates of *E. faecium* from probiotic products, 90% were found to be resistant to kanamycin, 97% to erythromycin and between 24 – 41% were resistant to tetracycline, penicillin, chloramphenicol and vancomycin (Temmerman *et al.*, 2003). A study in Brazil also showed high levels of antibiotic resistance in 80 isolates of *E. faecalis* and 139 *E. faecium* from foods (meat, cheeses, milks and vegetables) 65% of the *E. faecium* isolates had at least one virulence gene and all *E. faecalis* isolates had three or more, thus demonstrating the safety problems of using *Enterococcus* sp. in foods (Gomes *et al.*, 2008).

2.2.3 Bacteriocins

Ribosomally synthesised antimicrobial peptides (AMPs), are widespread in nature and are produced by mammals, birds, amphibians, insects, plant and bacteria, one of which is *Enterococcus* sp. The peptides produced by bacteria are referred to as bacteriocins (Papagianni, 2003). Bacteriocins have antimicrobial properties, which destroy bacteria by permeablising the cell membrane. From an ecological point of view the ability to destroy other organisms would limit competition which was first noted in 1676 by Leeuwenhoek and again in 1877 by Pasteur with *Bacillus anthracis* (Chenn and Hoover, 2003).

The only bacteriocins used commercially are nisin and pediocin PA-1 produced by *Lactobaccilus lactis* and *Pediococcus acidilactici* respectively (Deegan *et al.*, 2006). Bacteriocins can be used in the food industry as natural preservatives in variety of products to prevent spoilage and thus extend shelf life, e.g. nisin in cottage cheese, liquid egg, beer and wine fermentations and some vegetable fermentations (O'Sullivan *et al.*, 2002). The application to food can take two routes, either the use of bacteriocin producing LAB or direct application of bacteriocin preparations (Hugas *et al.*, 2003). LAB bacteriocins have also been shown to reduce *Staph. aureus* viable counts by 99% in foods like ham sandwich spread, chicken gravy and ground beef (O'Sullivan *et al.*, 2002). Enterocins also have potential as they are recognised to prevent spoilage in meat products including chicken breast, ham, pork etc. against *L. monocytogenes,* via the introduction of the non-pathogenic bacteriocin producing strains (Papagianni, 2003).

Enterococcus sp. are known to produce a range of enterocins including enterocin A, B, I and L or P, which are active against *Listeria* sp., *Clostridum* sp. and *Staph. aureus* (Campos *et al.*, 2006). Most of the bacteriocins produced by *E. feacalis* and *E. feacium* are identical to enterocins A and B first described from *E. faecium* CTC492 and *E. faecium* T136 (Table 2.1) (De Kwaadsteniet *et al.*, 2005).

TEXT
CUT OFF IN THE
ORIGINAL

Table 2.1: Bacteriocins produced by *Enterococcus* sp.

Bacteriocin	Produced by	Isolated from	Size (Da)
Enterocins A and B	*E. faecium* P21	Chorizo (dry-fermented sausage)	
Enterocin EJ97	*E. faecalis* S-47		
No name	*E. faecium* A2000	Cheese	
Enterocin CRL35	*E. faecium* CRL35	Cheese	3500
Bacteriocin N15	*E. faecium* N15	Nuka (Japanese rice-bran paste)	3000–5000
Enterocins A and B	*E. faecium* WHE81	Cheese	4833 and 546
No name	*E. faecium* RZS C5		
	E. faecium DPC 1146		
AS-48	*E. faecalis* subsp. *liquefaciens* S-48	Intestinal tract of porcine	
Enterocin 012	*E. gallinarum*	Duodenum of ostrich	3400
No name	*E. faecium* CRL 1385	Free-range chicken	
Enterocin P	*E. faecium* P13	Dry-fermented sausage	
Enterocins 1071A and 1071	*E. faecalis* BFE 1071	Faeces of minipigs	4285 and 389
Mundticin ATO6	*E. mundtii* ATO6	Vegetables	4287
Mundticin KS	*E. mundtii* NFRI 7393	Grass silage	4290

Adapted from De Kwaadsteniet *et al.* (2005)

E. faecium RZS C5 is a natural cheese isolate, which is lacking in virulence factors and has antilisterial properties. It is used in cheese and meat fermentation (Leroy *et al.*, 2003). Enterocin EJ97 from *E. faecalis* S-47 has also shown antilisterial properties with 1.6 $\log_{(10)}$ reduction after six hours and complete inhibition after 24 hours at a concentration of 20 AU/ml (Garcia *et al.*, 2004). The enterocin AS-48 (produced by *E. faecium* A-48-32) inhibits growth of *Bacillus coagulans* vegetative cells, both at refrigeration and high temperatures in canned fruits and vegetables (Lucas *et al.*). The AS-48 bacteriocin from *E. faecalis* A- used in fermented sausages prevented growth of *Listeria* sp. over a nine day period, whereas in the untreated control sausages *Listeria* sp. grew from 7.8×10^2 to 3.12×10^4 cfu/g (Ananou *et al.*, 2005). Bacteriocin activity against Gram-negative bacteria is unusual, but *E. mundtii's* bacteriocin ST15 has been shown to be effective against a range of Gram-positive and Gram-negative bacteria including *Acinetobactor, Bacillus, Clostridium, Klebsiella, Lactobacillus* and *Pseudomonas* (De Kwaadsteniet *et al.*, 2005). Although enterocins have beneficial effects in preserving foods, for example speeding up fermentation and extending shelf life by inhibiting organisms causing spoilage, there are undesirable affects caused by the target strains used for their bacteriocins i.e. they could potentially cause disease or spoilage of the food product, as well as the threat of the development of antibiotic resistance (Kuipers *et al.*, 2000).

The use of LAB and bacteriocins as biopreservatives is increasing due to changes in consumer demands and greater awareness of the dangers of foodborne pathogens (Hugas *et al.*, 2003). The use of *Enterococcus* sp. in a beneficial role in food is questionable due to its increasing resistance to antibiotics. It is paramount before bacteria are used in food that any antibiotic resistant gene should be removed or non-

pathogenic strains ascertained (Giraffa, 2003). These pitfalls in the use of *Enterococcus* sp. in food illustrates the importance of finding alternative natural antimicrobials, without the potential danger of illness to the consumer.

2.3 Antimicrobial Properties of Essential Oils

Plant extracts from around the world have shown antibacterial properties. For example, out of 39 plants from northern Argentina tested against a range of pathogens, 25% of the extracts showed some type of inhibitory activity with all inhibiting *Staph. aureus* (Salvat *et al.*, 2004). Thai medicinal plants are effective against a range of enteropathogens including Salmonella sp. *E. coli* O157:H7, *Camp. jejuni and Clostridium perfringes* (Wannissorn *et al.*, 2005).

In a study assessing 21 plant EOs for their antimicrobial properties against five food-borne pathogens (*C. jejuni, Salmonella enteritidis, E. coli, Staph. aureus* and *L. monocytogenes*) it was demonstrated that bay, cinnamon, clove and thyme were most inhibitory with bacteriostatic concentrations of 0.075% (v/v) or less (Smith-Palmer *et al.*, 1998). Thyme also has an inhibitory effect on *L. monocytogenes*, greater than that of electric shocks in combination with nisin, which is proven to be a very effective treatment (Rasooli *et al.*, 2006b). *Origanum vulgare* has inhibitory effects against *Helicobacter pylori* (Chun *et al.*, 2005), while three other oregano species (*Origanum majorana, O. heracleoticum and O. compactum*) are effective against *E. coli, Salmonella, Staph. aureus* and *L. monocytogenes* (Oussalah *et al.*, 2007). The effect of oregano against *H. pylori* is thought to be due to the high concentration of phenolics present, this was also shown to be the case with cranberry

extracts (Vattem *et al.*, 2005). In a comparative study of the antimicrobial effects of dill, coriander and eucalyptus against a range of Gram-positive and Gram-negative bacteria, dill was found to be the least effect, with cilantro being the most effective at the lowest concentration against *L. monocytogenes*, which, it was suggested, is due to the long chain alcohols and aldehydes present in cilantro (Delaquis *et al.*, 2002). Phlomis EOs commonly found in Turkey have antimicrobial activity against a range of bacteria being most effective against *C. perfringens* at a low concentration of 125 μg/ml with *E. coli* being completely resistant.

It has been established that a bioflim of *Staph. aureus* can be prevented with the use of a topical EO based formula containing eucalyptus, tea tree, thyme, clove and citrus species at MIC varying from 0.6 – 20 000 ppm. This has significant implications not only for the food industry but also in the clinical arena, especially with its possible use against meticillin-resistant *Staphylococcus aureus* (MRSA). Using tea tree oil an MIC of 0.32% (v/v) was effective against MRSA (Halcon and Milkus, 2004, Al-Shuneigat *et al.*, 2005, Banes-Marshall *et al.*, 2001), while mint also has an antimicrobial inhibition at 1.2% (v/v) against *Staph. aureus* with a 6-7 $\log_{(10)}$ of cfu/ml reduction when tested in growth medium (Tassou *et al.*, 2000).

The antimicrobial activity of essential oils is not fully understood but it is thought to be due to the complex mix of components and not the result of a single chemical *per se* (Burt, 2004). It is has been suggested that the combination of components within the EOs have either a synergistic or antagonistic relationship, which may cause the differences in the antimicrobial properties within the same genus and even species (under different growth conditions). Hence oils with the same overall

components but at different percentage concentrations can have different inhibitory effects (Hili *et al.*, 1997, Burt, 2004, Delaquis *et al.*, 2002). Throughout history, blends of herbs have been used to cure aliments and disease, yet the study of EOs as potential antimicrobials on the whole has been studied at an individual oil level. The antimicrobial synergism and antagonism of the component salicyladehdye was assessed in the essential oil *Filipendula vulgaris* against a range of bacteria including *E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, S. enteritidis, Staph. aureus* and the fungi *Aspergillus niger* and *Candida albicans.* It was noted when carrying out a disc diffusion method, that a synergistic effect occurred between salicyladehdye and linalool where inhibition zones were increased from between 33-40 mm for individual components to no growth per plate when the components were used in combination. An antagonistic effect between methyl salicylate and salicyladehdye was observed and what was interesting was that these interactions were not selective between bacteria species or fungi (Radulovic *et al.*, 2007). The EOs of *Thymus vulgaris* and *Pimpinella anisum* and their methanol extracts have been investigated for their synergistic antimicrobial effect against an array of Gram-negative and Gram-positive bacteria. Overall the combination of these EOs reduced the MIC required to inhibit the bacteria with the greatest effect being against *Proteus vulgaris* with an MIC of 15.6 μg/ml compared to 31.2 μg/ml for *Thymus vulgaris* and 62.5 μg/ml for *Pimpinella anisum* individually and *P. aeuruginosa* where the EOs separately showed no inhibition but in combination had an antimicrobial effect (Al-Bayati, 2008).

Overall there is no one EO that can be used as a universal antimicrobial, it is dependent not only on the EO itself whilst taking into considerations variations of composition of the oil dependent on geographical area of growth, seasonal variations in

terms of harvest and extraction method of the oil (Celiktas *et al.*, 2007) but also the organism it is acting upon.

2.4 Citrus Essential Oils

Although EOs *per se* and their antimicrobial properties have been reviewed extensively (Burt, 2004, Smith-Palmer *et al.*, 1998, Lanciotti *et al.*, 2004, Inouye *et al.*, 2001, Tassou and Nychas, 1995, Tassou *et al.*, 2000) the antimicrobial properties of citrus EOs have been relatively unexplored until recently.

The genus *Citrus* has approximately 16 species in the family *Rutaceae* and are mainly cultivated in subtropical regions. Citrus EOs make up the largest sector of the world production of essential oils (Tirado *et al.*, 1995). The oils are stored in cavities in the fruits lined with secretory cells that are continually enlarging and differences in the antimicrobial effect of the same type of citrus oil may be attributed to factors such as fruit quality, stage of growth, when extracted and ecological conditions. Over 4000 species of lemon scented plants are harvested each year, with a total of 750 000 tonnes being collected from the wild (Moreira *et al.*, 2005).

2.4.1 Composition

The components of EOs are important as their qualitative and quantative composition determines the characteristics of the oils, which in turn could have an effect on their antimicrobial potential (Dugo *et al.*, 2000). Citrus EOs contain 85-99% volatile and 1-15% non-volatile components. The volatile constituents are a mixture of

monoterpene (limonene) and sesquiterpene hydrocarbons and their oxygenated derivatives including: aldehydes (citral), ketones acids, alcohols (linalool) and esters (Table 2.2) (Borgmann *et al.*, 2004, Banes-Marshall *et al.*, 2001, Smith *et al.*, 2001, Flamini *et al.*, 2007).

Table 2.2: Volatile compounds of citrus fruits, adapted from (Moufida and Marzouk, 2003)

Volitile Compounds	Sweet Orange %	Lemon %	Bergamot %
Methanol	0.37	0.20	0.35
Isopropanol	0.91	0.12	0.28
α -Pinene	6.37	0.27	1.39
Butylacetate	0.00	1.47	4.97
3-Heptanone	0.00	0.34	0.94
Limonene	88.21	78.84	72.88
Ocimene	0.00	3.85	0.00
Nonanol	0.00	0.26	0.00
Linalool	0.02	0.02	10.23
α -Terpineol	0.71	1.30	0.00
Valencene	0.00	3.34	0.00
α Terpinene	0.00	0.46	0.23
β -Ionone	0.00	0.35	0.11
β - Pinene	0.00	0.02	0.14
Terpinen-4-ol	0.00	0.00	0.00
p-Cymene	2.37	1.75	5.62
Citral	3.00	0.10	0.70

The overall composition of citrus oils can alter the interactions of the components and qualitatively and quantitatively change the evaporation rate profiles (Saiyasombati and Kasting, 2003). For components to evaporate and be classed as volatile it is imperative that there is a loss of weight over a time or temperature course. The activation energies for lemon oil, limonene and linalool are 33.2, 37.87 and 65.64 kJ mol^{-1} respectively (Hazra *et al.*, 2002) meaning that they are highly volatile especially above room temperature. Evaporation of the oils is affected by external factors such as temperature, humidity, concentration and pressure (Aumo *et al.*, 2006) i.e. diffusion across a homogenous membrane increases with increasing temperature (Clarys *et al.*, 1998), as does the evaporation of aldehydes such as citral (Maki-Arvela *et al.*, 2006).

By 1970, 200 different chemical compounds from orange oil had been described of which 100 had been identified (Wolford *et al.*, 1971). Monoterpenes make up 97% of the citrus oil composition with alcohols, aldehydes and esters being the lowest percentage components, ranging from 1.8 to 2.2% (Moufida and Marzouk, 2003). The major chemical component of citrus oils is limonene, ranging from between 32 - 98%, with sweet orange containing 68-98%, lemon 45-76% and bergamot 32-45% (Svoboda and Greenaway, 2003). Linalool has concentrations of 0.018, 0.015 and 10.231% (v/v) in sweet orange, lemon and bergamot respectively (Moufida and Marzouk, 2003). The main component extracted from orange blossom EO is linalool with a concentration of 40% (w/v) (Jeannot *et al.*, 2005). The aldehyde citral has been shown to be present in lemon, orange and bergamot essential oils often in the form of the stereoisomers neral and geranial (Benvenuti *et al.*, 2001, Stashenko *et al.*, 1996). Citral and linalool are

thought to be the most potent aroma compounds in citrus fruits but do not exceed 3% in Colombian citrus peel oils including lemon, mandarin and orange (Tirado *et al.*, 1995).

Fatty acids make up a negligible percentage (about 0.2% v/v) of citrus oils, with the major fatty acids being linolenic in sweet orange, linoleic in lemon and oleic in bergamot (Moufida and Marzouk, 2003). Flavanoids are another group of components that are present in citrus species and are useful in differentiating between species (Mouly *et al.*, 1998). There are six classes of flavonoids: flavanones, flavonols, isoflavonoids, anthocyanins and flavans (Peterson and Dwyer, 1998), which make up the non-volatile part of the oils (Buiarelli *et al.*, 1996). It has been demonstrated that lemons and limes have flavanone profiles similar to sweet orange including two major flavanone glycosides, hesperidin and eriocitrin, which are almost exclusive to citrus fruits (Peterson *et al.*, 2005). Bergamot has an unusual flavonoid composition with large amounts of neoeriocitrin and little hesperidin (Nogata *et al.*, 2006).

The analysis and extraction of the components in EOs varies with seasonal variation, ripeness of the fruit and geographical region, hence percentages may vary (Smith *et al.*, 2001). The importance of the effects that these variations have on components has been demonstrated by growing two lemongrass species under different water stresses for 45 and 90 days when significant differences in the levels of the components geraniol and citral, dependant on stress and time, were reported (Hili *et al.*, 1997). It has also been noted that oil content of oranges do not meet their maximum until they are fully mature and that a decrease in oil content occurs after rainfall (Wolford *et al.*, 1971). Although more recently it has been observed that, in lemon peel, the maximum concentration of volatile compounds is when the fruit is at

intermediate maturation stage (Tirado *et al.*, 1995). The composition of the blend of components in specific ratios determines the flavour and also the possible antimicrobial effects of citrus oils which is unique to each species or hybrid (Shaw, 1979)

2.4.2 Antimicrobial Effects

The first record of citrus fruits having medicinal uses was by Theopharustus in 4th century B.C. when they were used as antidotes to poisons and inhaled to ease the throat. The first mention of their use against nausea and stomach cramps as a result of food consumption (possible food poisoning) was by Pliny in 23-72 A.D. (Arias and Ramon-Laca, 2005).

With consumer trends for natural alternatives to chemical-based bactericides together with changes in legislation, citrus EOs may provide a solution for both industry and consumers, as their components are GRAS (Food and Drug Administration, 2005) and their scent and flavours lend themselves to the use in food (Svoboda and Greenaway, 2003). Piacentini first noted the antimicrobial properties of citrus oils in 1949, when it was reported that citrus essences in aqueous solutions were more powerful as disinfectants than phenol. A study by Subba *et al,* (1967) demonstrated that orange and lemon oils at the concentration of 2000 ppm inhibited outgrowth of spores of *B. subtilis* (at cell concentrations of 400 000 per ml) in nutrient agar. At 2000 ppm orange oil produced complete inhibition and lemon 99.9% inhibition of *Streptococcus faecalis* (now reclassified as *Enterococcus faecalis*) and *Lactobacillus plantarum* at concentrations of 10^6cells/ml. Later Deans and Ritchie (1987) showed citrus oils to have antimicrobial properties against not only yeast, moulds and spore forming bacteria but also food-poisoning bacteria.

More recently *Citrus limonum* was found to be effective against four strains of *E. coli* with a MIC of 2.5 ml/100ml and a Minimum Bactericidal Concentration (MBC) of 2.8 ml/100ml in Brain Heart Infusion Broth (BHI) at 37°C (Moreira *et al.*, 2005) although the results of another study suggested that there was a less than 50% inhibition against *E. coli* and *S. typhimurium* from lemon oil and citral, but > 80% inhibition for geraniol in TBS medium. When the pH of the media was reduced from pH 7 to pH 2 geraniol (300μg ml^{-1}) exhibited a lesser effect of 33-13% inhibition (Si *et al.*, 2006). Lemon, sweet orange and bergamot and their components, linalool and citral, but not limonene, were found to have antimicrobial effects both in direct oil and vapour form against *Camp. jejuni, E. coli* O157, *L. monocytogenes, Bacillus cereus* and *Staph. aureus.* When MICs were established *in vitro* only bergamot (1 - 0.125% v/v), linalool (0.06 –0.125% v/v) and citral (0.03 – 0.06% v/v) had concentrations that were acceptable to food application (Fisher and Phillips, 2006). Similar results were found by Smith-Palmer *et al.*, (1998) using the agar well method with lime oil being most effective against *S. enteritidis* and lemon oil against *Staph. aureus* with inhibition zones of 7.2 mm. MICs of 12.8 mg/ml for lemon and orange oils and 6.4 mg/ml (excluding *Staph. aureus*) for lime oil are required against *Staph. aureus, B. subtilis, K. pneumoniae* and *P. aeruginosa*, (Prabuseenivasan *et al.*, 2006, Palhano *et al.*, 2004). However, these high MICs would be unacceptable for the application to food products due to the possible changes in organoleptic properties especially aroma. This factor must be considered when assessing the potential of citrus oils *in vitro* for the food industry.

Citral has been highlighted as an active compound in citrus fruits against decay caused by *Penicillium digitatum.* One study assessed the antifungal activity of the

following citrus oils: *Citrus sinensis, C. aurantium, C. deliciosa, C. paradises* and *C. limon* and found their effective doses to be 2180, 1015.4, 713.3, 910.3, 1056.4 ppm respectively against *Penicillium digitatum,* (Caccioni *et al.*, 1998). Orange oil also inhibits the yeast *Saccharomyces cerevisiae* (10^4 cells/ml) with an MIC of 2.8 μg/ml (Schelz *et al.*, 2006). It has been shown that in liquid medium the oils containing the higher concentrations of terpenes such as citral are more effective against this yeast, (Belletti *et al.*, 2004). The possible use of citral against growth of *Aspergillus flavus* was assessed at pH 3.5 and 4.5 at 25°C in water activities of 0.99 or 0.95 (a_w), pH did not effect the MIC, but water activity did, with 1800 ppm needed for a_w 0.99 and 1400-1600 ppm for a_w 0.95 (Lopez-Malo *et al.*, 2005).

Citrus peel oils also reduce the levels of *Aspergillus parasiticus* at concentrations of 1.6% over a 10 day incubation period (Karapinar, 1985). *Citrus sinensis* is active against *A. niger* and demonstrated fungistatic activity at 1.5 μg/ml with 79% growth inhibition over 7 days (Stashenko *et al.*, 1996). Citral can also delay growth of *S. typhimurium* (10^6 cells/ml) in BHI broth by 1.86 hours and reduce growth by 10.69% (Nazer *et al.*, 2005), an MIC of 3.3 μg/ml of citral was needed for inhibition in Trypticase Soya Broth (Kim *et al.*, 1995a).

The antimicrobial intensity of the oils decrease over time due to the most volatile components, e.g. linalool and limonene, evaporating first (Saiyasombati and Kasting, 2003). Investigations with citral have shown, due to its high volatilisation rate, antimicrobial activity occurs rapidly in the first three hours and then diminishes just as rapidly thereafter (Inouye *et al.*, 2003). Linalool is another component of citrus oils which exhibits antimicrobial properties. Inhibition zones against *Shigella sonnei* and

Shigella flexneri (10^5 cfu/plate) range from 0.5mm at 0.1% concentration to 0.5–2 mm at 10% concentration, highlights the fact that there is no linear or logarithmic relationship between the zone of inhibition and the concentration of linalool applied in the agar diffusion method (El-Sharoud, 2005). However, the results of an investigation by Nazar *et al* (2004) do show a linear relationship between increased MIC and reduction in percentage growth of *Salmonella* i.e 0.5% v/v citral reduces growth by approximately 10% whereas at 3.5% v/v reduction in growth is 100% (Nazer *et al.*, 2005).

It has been noted in fruits subjected to volatiles that there are no changes in colour (Tzortzakis, 2007). The volatile components of EOs that quickly evaporate lend themselves as a possible source of antimicrobial agents, although this potential is relatively unresearched, The first reports of antimicrobial activity from vapours of EOs was in 1960 (Maruzzella and Sicurella, 1960). A grapefruit extract called Citricidal™ vapour was evaluated against three strains of *Staphylococcus* including meticillin-resistant *Staph. aureus* (MRSA). It was assessed both *in vitro* on sensitivity test agar and in a gauze dressing. It showed inhibitory effects only in direct contact but not in vapour form however, when combined with geranium or tea tree oil the zones of inhibition were increased from that of the single oil alone in both agar and gauze with combined zones of inhibition of 45-9mm and 22.5-14.5mm (susceptible strain only) respectively (Edwards-Jones *et al.*, 2004).

MIDs of EOs have also been shown to be lower than MICs using an agar dilution method against *E. coli,* with MICs (μg/ml) and MIDs (μg/cm^2) respectively of lemongrass >3200, 100, citral >800, >12.5; linalool 3200, 50 and limonene >3200,

>800. This same pattern was also observed in *Staph. aureus* (Inouye, 2003). These findings have great implications for the use of citrus oils in food, as the reduced concentrations needed when using vapours, compared with oils, would limit any potential adverse organoleptic effects. It is thought that the lipophilic molecules in the aqueous medium associate to form micelles and thus suppress that attachment of the oils to the organism, whereas the vapour state of the oils allows free attachment (Inouye *et al.*, 2003). Although it was also noted that actual vapour concentration was less than the nominal concentration indicating only part of the vapour is effective against the organisms and, over long incubation periods, the active components may be lost differentially changing the anti-microbial effect over time (Inouye *et al.*, 2003).

Essential oils, containing citrus EO components, have been evaporated via candles underneath incense evaporators into the air of offices and homes. The airborne bacterial load was reduced within 30 mins with the greatest reduction being from 2500 to 1250 (cfu/m^3). The volatile organic compounds in citrus EOs found in the air during this reduction time were linalool 496-987 ($\mu g\ m^{-3}$) and *D*-limonene 13-69 ($\mu g\ m^{-3}$) (Su *et al.*, 2007). The addition of orange oil to a candle can increase the reduction of *E .coli* over a 5-hour period from 55% viability in the control to less than 20% and of *Staph. aureus* from 100% to 30% in a air tight acrylic booth. The combination of candle and orange oil produced ionised antibacterial vapours (Gaunt *et al.*, 2005). Airwashers have been used to evaporate citral and citronellal into the air of a room. An hour later the bacterial counts in the air were calculated and while the control of just water increased counts by 35%, reductions by citral and citronellal were 30% and 17% respectively (Sato *et al.*, 2006). The effect of the evaporation rate has an effect on the MID (mg/L air) values of oils. In an assessment of lemongrass oil, the differences between fast and

slow evaporation against *Staph. aureus* and *E. coli* were 12.5 compared with >100 and 100 to >100 respectively (Inouye *et al.*, 2001). This illustrates the need for investigations into the most effective method of dispersing oil vapours.

2.4 Aims

Citrus EOs and their components have been shown to exert antimicrobial properties against a variety of Gram- positive and Gram-negative bacteria both in oil and vapour form. This study aimed to investigate the antimicrobial effects of citrus EO against *Enterococcus faecalis* and *Enterococcus faecium*

Objectives:

- To investigate the growth and survival of *E. faecium* and *E. faecalis* at a range of temperatures and pHs.

- To determine the effectiveness of a range of citrus essential oils, blends, components and their vapours against *E. faecium* and *E. faecalis in vitro*

- To establish potential applications of the citrus essential oils identified as active in food systems and on surfaces.

- To investigate the mechanisms of action of citrus essential oils identified as active against *E. faecium* and *E. faecalis.*

Chapter 3

Materials and Methods

3.1. Universal Methods.

3.1.1 Microrganisms and culture methods

All media were obtained from Oxoid Ltd (Basingstoke, Hampshire, UK) unless otherwise stated. Test organisms included: *Enterococcus faecalis* NCTC 12697, *Enterococcus faecium* NCTC 07171. Vancomycin resistant *Enterococcus faecalis* NCTC 12203, vancomycin resistant *Enterococcus faecium* NCTC 12202 (Health Protection Agency, London, UK).

Strains were stored on beads (Microbank PL.160/M, Pro-lab diagnostics, Cheshire, UK) and stored at -80°C. They were also phenotypically typed using Biolog 2 (Biolog, CA, USA)

Organisms were grown on Brain Heart Infusion (BHI) solid media then one colony per 100ml Brain Heart Infusion (BHI) broth (CM225) was grown aerobically for 6hr (exponential phase cells) or 24hr (stationary phase cells) at 37°C. BHI agar (CM0375) was used to culture the bacteria for enumeration of viable counts.

3.1.2 Essential Oils and Oil Products

Orange (sweet) (*Citrus sinensis*), lemon (*Citrus limon)* and bergamot (*Citrus bergamia)* were obtained from AMPHORA, (Bristol. UK). Limonene 97%, (18, 316-4) linalool 97% (W26, 350-8), citral 95% (C8, 300-7), hersipidin 90% (52040) and neoericitrin 95% (72129) were obtained from Sigma-Aldrich Co. Ltd. (Dorset, UK). All other chemicals were obtained from Merck (Eurolab Ltd., Leicester, UK) unless otherwise specified.

3.1.3 Blends

Combinations of the oils of orange/lemon, lemon/bergamot, bergamot/orange (1:1 v/v) and a combination of all three in equal volumes 1:1:1 v/v) were prepared before each experiment.

3.1.4 Growth Curves

100mls of BHI broth was inoculated with one colony of either *E. faecalis* or *E. faecium* and incubated at 37°C. Samples were taken hourly for the first ten hours and then again at 24 and at 48 hours. These were plated using a spiral plater (Don-Whitley, West Yorks, UK) onto BHI agar and incubated for 24 hours at 37°C before viable counts were enumerated.

The optical density of the culture was read every two hours at 600nm using a spectrophotometer (CE1011, Cecil, Cambridge, UK), against a sterile BHI broth blank.

3.1.5 Statistical analysis

All statistical analysis was carried out using SPSS version 11.5 for Windows with significance set at $p = 0.05$, (unless otherwise stated). Assumption of normality was tested using a Kolmorgorov-Smirnov test and variances of homogeneity were checked using Levine's test, if all assumptions were met one-way analysis of variance (ANOVA, *t*-test) was carried out. Tukey post-hoc tests were used to investigate where significant differences between group means occurred. If any parametric assumptions were violated a Mann-Whitney U or Kruskal-Wallis test was performed. Effect size (r) was also calculated where appropriate. An example of ANOVA output can be found in Appendix I.

All investigations were carried out in triplicate on at least two separate occasions.

Chapter 4

Growth of *Enterococcus* sp. Under Different Conditions

4.1 Introduction

4.1.1 Methods for Assessment of Anti-microbial Effectiveness of EOs

The anti-microbial effect of EOs is mainly assessed by an initial *in vitro* screening method usually that of the disc diffusion method, where a filter disc is impregnated with the antimicrobial agent, placed on the surface of inoculated agar plates and inhibition of growth is observed, adapted from the standard antibiotic screening method (Deans and Ritchie, 1987, Smith-Palmer *et al.*, 1998, Fisher and Phillips, 2006, Wannissorn *et al.*, 2005, Skandamis and Nychas, 2001, Bauer *et al.*, 1966). In the late 1950s this method was adapted for screening EO vapours by placing the filter disc on the lid of the petri dish (Maruzzella and Sicurella, 1960). Another method of assessing antimicrobial activity is to make a well in the inoculated agar and add the test substance (Deans and Ritchie, 1987, Dorman and Deans, 2000). None of the screening methods are quantitative and only give an indication of the EOs having antimicrobial properties.

A recent study has assessed the contribution of the vapours to the antimicrobial effect in the direct disc diffusion method. It indicated that only the water-soluble components diffused across the agar whilst the re-deposition of the vapourised components on the surface of the agar accounted for the remainder of the inhibition. In the study the disc was in contact with the agar and sealed with a ring. A delta value (100 $\approx$ 70) was calculated to assess the contribution of the vapour activity. In citrus oils the

delta values ranged from 19 for lemongrass to 84 for limonene. It was found that for oils containing alcohol, ketone, ester, oxide and hydrocardons the major inhibition came from the vapours whereas for oils containing greater volumes of aldehydes inhibition came from diffusion (Inouye *et al.*, 2006).

Minimum Inhibitory Concentrations (MIC) of EOs can be assessed by two protocols: the agar dilution method, with visible growth observed, or broth dilution with visible growth, optical density or viable counts measured (Burt, 2004). The MIC is determined as the lowest concentration at which growth is inhibited. The major problem with determination of strength of the EOs antibacterial properties in this way is their hydrophobic nature, which makes them insoluble in water-based media. This may be overcome with the use of emulsifiers such as Tween 20 or Tween 80 either alone or in combination with acetone, polyethylene glycol or ethanol (Tassou *et al.*, 2000, Smith-Palmer *et al.*, 1998, Deans and Ritchie, 1987, Fisher and Phillips, 2006, Burt, 2004). The other consideration when determining the MIC of an EO is that the absolute concentration for inhibition can be anywhere between the lowest MIC and the next concentration where growth is observed.

Minimum Inhibitory Doses (MID), where the minimum dose of the gaseous state to inhibit growth is determined are a parallel method to the MIC. This is carried out in a sealed container and the EO can either be placed on the surface of the container (slow evaporation) or an impregnated disc (quick evaporation) can be used (Inouye *et al.*, 2003, Fisher and Phillips, 2006). The incubation temperature can have an effect on inhibition when using this method as the MID value is dependent on the evaporation of the volatile components. The other factor to be considered is that loss of vapour can

occur through absorption into the media as chamber leakage and also spontaneous decomposition has been known to occur (Inouye *et al.*, 2001).

The differences between MIC/MID in studies against the same organism might be due to differences in the oil composition, which come about because of regional differences temperature, humidity or growth maturity of the fruit when oils were extracted and the different method of extraction (Burt, 2004).

4.1.2 Hurdle Technology

The microbiological safety of processed food is based on the combination of preservative technologies (hurdles), allowing the nutritional and sensory quality as well as safety of the food to be maintained. The main factors considered as hurdles are: temperature; water activity; pH; redox potential; preservatives and the use of competitive microrganisms as probiotics. A combination of 'hurdles' such as low temperature, low pH and presence of preservative aims to bring about microbial stability, whilst maintaining economical value. Sixty potential hurdles including non-thermal technologies such as high hydrostatic pressure, pulsed electric fields, ultrasonification, ionising energy and carbon dioxide have been described and this list is still expanding (Leistner, 2000). These can be used in combination with each other or with an antimicrobial agent to reduce microbial load (Ross *et al.*, 2003). Combining hurdles brings about an overall preservation strategy and has shown promising results on fresh cut fruit and vegetables (Allende *et al.*, 2006, Valero *et al.*, 2003) and meat products (Thomas *et al.*, 2008a, Thomas *et al.*, 2008b). By subjecting bacteria to a number of sublethal stresses or hurdles, which act on the same element of the cell the

synergistic effect of combining hurdles, which act simultaneously disturbing several different functions of the cell (cell membrane, DNA and enzyme systems) an additive inhibitory effect occurs. Whether the effect of the combined technologies results in an additive or synergistic effect on the bacteria both can lead to cell death (Ross *et al.*, 2003). The use of combined technologies as a multitarget preservation system can act on homeostasis of the cell or produce metabolic exhaustion and therefore autosterilisation. This is caused by inducing stress reactions which can lead to tolerance of the cells environment but also causes the cells to synthesis stress proteins which require high energy input and thus indirectly cause metabolic exhaustion due to the lack of energy available (Leistner, 2000). The use of natural antimicrobials in hurdle technology is well recognised but when assessing the synergistic effect of combined technologies for reducing microbial load in food, certain parameters of the organisms should be taken into consideration such as, extension of the lag phase, reduction in culture density after 24hours and residue viability at 24 hours, to establish if the combined hurdles have inhibited the microrganisms to such an extent that the potential for food spoilage or disease is sufficiently limited (Dufour *et al.*, 2003). Hurdle technology has been used for centuries although only in the last 20 years has it been studied in any detail (Leistner, 2000).

The application of 'hurdle technology' may allow the use of low levels of EOs in combination with other food preservation techniques such as refrigeration and/or modified atmospheres to bring about the desired effect i.e. reduction in microbial load. Low temperature in combination with cinnamaldehyde and thymol is effective in the case of *B. cereus* (Valero and Frances, 2006) and the combination of low temperature, modified atmospheres and oregano EO is effective against *S. typhimurium* (Skandamis

et al., 2002). Combinations of temperature, pH and carvacrol/nisin against *B. cereus,* showed that in combination carvacrol and nisin had a greater effect at the optimum growth conditions (pH 7 and 30°C) than individually. When the pH was lowered (6.3 and 5.75) nisin had an even greater effect reducing the cell counts by approximately 5 $\log_{(10)}$. In combination with lowered temperature (8°C) the effect of nisin was reduced, illustrating that some combinations can have a negative effect (Periago and Moezelaar, 2001). The use of tea tree oil together with low pH, low water activity and vacuum packaging in pork sausages resulted in *Staph. aureus* counts being maintained at levels at less than 2 $\log_{(10)}$ cfu/g over six days at 37 °C and at undetectable levels in refrigerated samples (Thomas *et al.*, 2008a). However another study which assessed the use of storage temperatures (3 °C - 20 °C) and the EOs oregano and nutmeg against *E. coli* on barbecued chicken showed no significant difference in bacterial populations between treated and untreated samples (Shekarforoush *et al.*, 2007). The use of bacteriocins at 8°C and pHs between 5.5 - 6 have been used to inhibit microbial growth in combination with other techniques such as nisin, pulsed electric field (PEF) and metal chelators (Cleveland *et al.*, 2001). When temperature, pH and bacteriocins were assessed for their combined effect on growth of *Enterococcus* sp., ranges of temperatures (4-25 °C, 30-37 °C and 45-60°C) and pHs (2-4, 6-8 and 10) were established. Lower and higher temperatures had the same effect on bacteriocin absorbance, whilst lower pHs had a negative effect on uptake (Todorov *et al.*, 2006).

The use of lemon juice to stimulate a mild acidification (pH 5) in combination with refrigeration was demonstrated to have a significant additive/synergistic effect on the growth of *B. cereus* in carrot broth and zucchini (Valero *et al.*, 2003). *B. cereus* growth was also assessed in carrot puree in the presence of cinnamadehyde over a

range of temperatures (5 – 16°C) and pHs (5 – 5.5 at 0.1 intervals). It was found for cinnamadehyde to inhibit growth that as the pH rose the refrigeration temperature also had to increase i.e. 5.3°C & 8°C and 5.4 – 5.5 & 5°C (Valero *et al.*, 2003).

Due to *Enterococcus* sp. increasing antibiotic resistance both in the clinical and food arena, alternative antimicrobials need to be found. The use of citrus essential oils/vapours in combination with temperature and pH (hurdle technology) may be the solution that is required; using combination of hurdles may also go some way in limiting further emergence of resistance/tolerance within the species.

4.1.3 Aims and Objectives

The aim of the investigation was to determine the antimicrobial properties of orange, bergamot and lemon EOs and their components in both oil and vapour form in combination with different temperatures and pHs against vancomycin resistant and susceptible strains of *E. faecium* and *E. faecalis.*

The objectives are:

- To determine antimicrobial activity of the citrus oils, vapours, blends and components at a range of different temperatures (5 °C to 50 °C) and pHs (4.5 – 10.5).
- To establish minimum inhibitory concentrations (MIC) and minimum inhibitory doses (MID).
- To assess the effect of the most inhibitory combination of EO, pH and temperature on cell size and DNA content.

4.2 Methods

4.2.1 Growth at Different Temperatures

Bacterial cultures of vancomycin resistant or vancomycin susceptible *E. faecalis* or *E. faecium* were grown as described previously (section 3.1.1) and incubated at 5°C intervals from 5-50°C (5, 10, 15, 20, 25, 30, 37, 40, 45 and 50°C), growth curves were then carried out (section 3.1.4). Generation times and D values were calculated.

4.2.2 Growth at Different pHs

Bacterial cultures of vancomycin resistant and susceptible *E. faecalis* or *E. faecium* were grown as described previously (section 3.1.1) with the pH adjusted with either HCl or KOH to 4.5 - 10.5 (4.5, 5.5, 6.5, 7.5, 8.5, 9.5 and 10.5). The cultures were then incubated at 37°C, and samples taken at 6 and 24 hours and plated using a spiral plater (Don-Whitley, West Yorks, UK) onto BHI agar and incubated for 24 hours at 37°C. This (method 4.2.2) was repeated at 25°C and 50°C. Generation times and D values were calculated.

4.2.3 Screening of Essential Oils and Oil Components

For the following experiments *E. faecalis* and *E. faecium* vancomycin susceptible strains were tested at both exponential and stationary growth phases, cultured as section 3.1.1, unless otherwise stated.

Aliquots (0.1 ml) of each of the oils/components were spotted onto 2 cm diameter filter paper discs. Three discs were then placed onto the surface of an agar plate (pH 7.5) previously spread with either a *E. faecalis* or *E. faecium* culture at a concentration of 10^8 cfu/ml (adapted from Kirby-Bauer 1966).

In order to test the effect of the vapour, each of the bacterial cultures was exposed to the vapour of the oils/components/blends by placing one impregnated disc onto the lid of the petri dish at approx 8mm from bacteria (Edwards-Jones *et al.*, 2004).

All plates were incubated at 37°C for 24 hours and the zones of inhibition were then measured (diameter in cm) using Vernier calipers. Controls were plates that had not been exposed to any oils, components or vapour. This (method 4.2.3) was repeated at 5°C intervals from 5-50°C.

All further experiments were carried out at 25, 37 and 50°C in combination with pHs of 5.5, 7.5 and 9.5 on stationary cells.

4.2.4 Determination of Minimum Inhibitory Concentrations (MIC)

MICs for the essential oils/components/blends that were found to be inhibitory by the screening method, were established using an agar dilution method (Banes-Marshall *et al.*, 2001). The antimicrobials were added to the appropriate agar plates at concentrations of 0.03%, 0.06%, 0.12%, 0.5%, 1%, 2% and 4% (v/v), 4% is the highest concentration able to solubilise in agar. 0.5% (v/v) of Tween 20 was added and pH was adjusted to either 5.5, 7.5 or 9.5. Controls had no oil and 0.5% (v/v) of Tween 20,

previously shown not to have an inhibitory effect, incorporated in the agar. Plates were then spread plated with the organism (10^8 cfu/ml) and incubated at either 25°C, 37°C or 50°C for 24 hours. The MIC was defined as the lowest concentration of the oil inhibiting visible growth.

MICs of the vancomycin resistant stains was only established for orange: bergamot blend 1:1 (v/v) at either pH 5.5, 7.5 or 9.5, at 25°C, 37°C or 50°C.

4.2.5 Determination of Minimum Inhibitory Dose (MID)

The absorption of oil/component/blend vapour into BHI agar was assessed before commencing the MID experiments to ensure any uptake of vapours into the agar had no antimicrobial effect. Un-inoculated plates were placed in vapour chambers at concentrations of 1600, 800, 400, 200, 100 and 50mg/L air and incubated for 24 hours. Plates were removed from the chambers and spread with 0.1ml of bacteria (10^8 cfu/ml) and incubated at 37°C for 24 hours. Growth on plates was assessed to establish if any uptake of the oils into the agar would have an inhibitory effect.

MIDs for the oils/blends/components vapours were established using a vapour chamber method (Inouye *et al.*, 2001). Aliquots (0.1 ml) of each potential antimicrobial in twofold dilutions of 1600, 800, 400, 200, 100 and 50mg/L air, were spotted onto 3 cm diameter filter paper discs (quick evaporation). The discs and inoculated plates (0.1ml spread plated with 10^8 cfu/ml on agar adjusted to either pH 5.5, 7.5 or 9.5) were placed in a 1.3L airtight beaker (vapour chamber) and incubated at either 25°C, 37°C or

50°C for 24 hours. The MID was the lowest concentration of EO/blend/component that inhibited bacterial growth. A control was a vapour chamber with no paper disc added.

MICs of the vancomycin resistant stains was only established for orange: bergamot blend 1:1 (v/v) at either pH 5.5, 7.5 or 9.5, at 25°C, 37°C and 50°C.

4.2.6 Assessment of Antagonistic or Synergistic Effect of a Combination of Linalool and Citral

A microdilution method (Schelz *et al.*, 2006) was used to assess the inhibition of a combination of components. Dilutions of linalool and citral were added to BHI broth at final concentrations of 0.03%, 0.06%, 0.12%, 0.5%, 1%, 2% and 4% (v/v), with 0.5% (v/v) of Tween 20 also added. Combinations of these varying concentrations of components (200µl) and 20µl of vancomycin susceptible strains of *E. faecalis* or *E. faecium* stationary phase cells at 10^8 cells (grown at 37°C) were added to 96 well plates and mixed using the shaking mechanism of a water bath at 25°C (Grant OLS200, Cambridge, SG8 6GB) for two minutes at 80 rpm. The optical densities of the samples were obtained using a microplate reader (Bio-Rad 680XR, Hertfordshire HP2 7DX). The plates were incubated at either 25, 37 or 50°C for 24hours, the optical density was read again and growth calculated. The MIC for the combination of components was established and the Fractional Inhibitory Concentrations (FIC) calculated: $FIC = FIC_A + FIC_B$. (FIC_A = component$_A$ MIC of combination/MIC alone and FIC_B = component$_B$ MIC of combination/MIC alone). Where $FIC < 0.5$ = synergy, $0.5 \leq FIC \leq 1$ = addition, $1 < FIC \leq 4$ = indifference and $FIC > 4$ = antagonistic (Gutierrez *et al.*, 2008). The negative controls were wells with no bacterial culture added to the BHI broth with EO

and Tween 20 incorporated and the positive controls were wells with no EO added to the BHI broth with bacterial culture and Tween 20.

The assessment of the corresponding vapours was carried out using the vapour chamber (section 4.2.5) set up using filter paper disks impregnated with aliquots of (0.1ml) of combinations of linalool and citral at concentrations of 1600, 800, 400, 200, 100 and 50mg/L air. Inoculated plates (spread with 10^7 cells) were placed in the chambers and incubated at either 25°C, 37°C or 50°C for 24hours. FIC for linalool and citral was calculated: $FIC = FIC_A + FIC_B$.

4.2.7 Assessment of Growth Using the Most Inhibitory Combination of EO, Temperature and pH

100ml of BHI broth was pH adjusted with either HCl or KOH as appropriate was inoculated as method 3.1.1. The MIC of a combination of orange/bergamot EO was added to the cultures with 0.5% (v/v) of Tween 20. Both vancomycin resistant strains were grown at 37°C at pH 5.5 and an MIC of 2% (v/v). An MIC of 2% (v/v) was also used for vancomycin susceptible *E. faecium* at 25°C and 37°C at pH 7.5 and 9.5 Vancomycin susceptible *E. faecalis* was grown at 25°C at pH 9.5 and 37°C at pH 7.5 with an MIC of 2% (v/v). Both vancomycin susceptible strains were also tested at 25°C at pH 5.5 with an MIC of 0.25% (v/v) and at 37°C at pH 5.5 with an MIC of 0.5% (v/v) and 1% (v/v) for *E. faecalis* and *E. faecium* respectively. Assessment of the effect of the vapours on growth was carried out in the vapour chambers at the MID of 50 mg/L air. Both vancomycin resistant strains were grown at 50 °C and pH 7.5 susceptible *E. faecium* at pH 7.5 and pH 9.5 and *E. faecalis* at pH 9.5. Method 3.1.4 was carried out to

assess cell growth in the presence of EOs/vapours. Controls were cell growth without any EOs/vapours present.

The most inhibitory combinations of pH, temperature and MIC/MID of orange/bergamot blend as determined (Table 4.3) were used for all experiments against the vancomycin susceptible strains of *E. faecium* and *E. faecalis*, with samples taken at 6, 24 and 48 hours unless otherwise stated. Controls were samples grown in optimum conditions with no oils/vapours added.

4.2.8 Bacteriostatic/Bactericidal Assessment

Aliquots of 5ml were taken from growing cultures of method 4.2.7 at 24 and 48 hours. The samples were centrifuged (Rota, F 45-30-11, Eppendorf 5864R, Hamburg, Germany) for 20 minutes at 25°C at 10 000 rpm. The resulting pellet was washed twice in 10ml of sterile deionised water for 10 minutes. The cells were added to 100ml of BHI broth and after 24 hours incubation at 37°C, plated using a spiral plater onto BHI agar plates, incubated for a further 24 hours at 37°C and colonies enumerated.

4.2.9 Live/Dead Cells After Exposure to EO, Temperature and pH

In order to assess live/dead cell ratios for the vancomycin susceptible strains after treatment with the EO blend at pH 5.5, 37°C and MIC 0.5% (v/v) for *E. faecalis* and pH 9.5, 37°C and MIC 2% for *E. faecium* and with vapour 50mg/L air at 50 °C and pH 7.5 for both *E. faecium* and *E. faecalis* a live/dead Baclight kit (Live/Dead Baclight, bacterial viability Kit L7007, Molecular Probes, Paisley, UK) was used.

Aliquots of 10ml were taken from growing cultures at 6, 24 and 48 hours, concentrated by centrifugation (Rota, F 45-30-11, Eppendorf 5864R, Hamburg, Germany) for 20 minutes, at 25°C, at 8 000 rpm. The pellet was then washed twice with 10 ml of sterile deionised water for 10 minutes. The cells were re-suspended in 1ml of sterile deionised water (for live cells), or 1ml of isopropyl alcohol (for comparative dead cells). The samples were then incubated at room temperature for one hour and mixed every five minutes. A 1:1 v/v mixture of component A (SYTO 9 dye: - 1.67mM/ Propidium Iodide: 1.67mM) and Component B (SYTO 9 dye: - 1.67mM/ Propidium Iodide: 18.3mM) (Live/Dead Baclight, bacterial viability Kit L7007, Molecular Probes, Paisley, UK) was made in micro centrifuge tubes. 3µl of the dye was required for each 1 ml of suspension. After the addition of the dye the samples were incubated at room temperature in the dark for 15 minutes.

Aliquots of 5µl of samples were placed on glass slides and observed under a fluorescence microscope (Zeiss MC63, W. Germany) using a long-range filter e.g. Omega filter XF25, XF26 or XF115. Comparisons were made between red (dead) and green (live) cells.

Live/Dead Ratios:

To produce a standard curve 1 ml of five different proportions of 24hr live control bacterial suspensions and 3µl of dye were added to 1 cm acrylic fluorescence cuvettes and incubated at room temperature in the dark for 15 minutes, this was repeated with 1ml from the same sample being treated with isopropyl alcohol

(comparative dead cell standard curve). The fluorescence emission spectrum was measured (excitation 470 nm, emission 510-540 nm for live cells and emission 620-650 nm for dead cells) on a spectrofluorometer (Fluorimeter 6200, Jenway Essex England).

Test samples as prepared previously were then measured under both live and dead emission spectra and ratios of live/dead cells calculated.

4.2.10 Cell Size Evaluation

In order to assess cell size after treatment with the EO blend at pH 5.5, 37°C and MIC 0.5% (v/v) for vancomycin susceptible *E. faecalis* and pH 9.5, 37°C and MIC 2% for vancomycin susceptible *E. faecium* and with vapour 50mg/L air at 50 °C and pH 7.5 for both vancomycin susceptible *E. faecium* and vancomycin susceptible *E. faecalis* a coulter counter (Meritics Ltd. W. Yorks, UK) was used.

Samples of 10 ml were taken from growing cultures at 6 and 24 hours and concentrated by centrifugation (Rota, F 45-30-11, Eppendorf 5864R, Hamburg, Germany) for 20 minutes, at 25°C, at 8 000 rpm. The pellet was then washed twice with 10 ml of DH_2O for 10 minutes, and then re-suspended in 1ml of PBS. Cell size was then measured with a Multisizer 3 Coulter Counter (Meritics Ltd. W. Yorks, UK). An aperture of 20µl was used to assess the frequency distribution of particles according to particle size.

4.2.11 UV Spectrophotometric Determination of DNA

In order to determine the quantity of DNA in the vancomycin susceptible strains after treatment with the EO blend at pH 5.5, 37°C and MIC 0.5% (v/v) for *E. faecalis* and pH 9.5, 37°C and MIC 2% for *E. faecium* and with vapour 50mg/L air at 50 °C and pH 7.5 for both *E. faecium* and *E. faecalis* UV spectrometry (UV1101 photometer, Biotech Cambs, UK) was used.

An absorption spectrum was obtained using a UV spectrophotometer (UV1101 photometer, Biotech Cambs, UK) for either a solution of EO in Tris-EDTA buffer, lysed cells in Tris-EDTA buffer or Tris-EDTA buffer alone to determine suitable wavelengths for analysis of quantities of either DNA, protein or EO. Ensuring that individual components can be identified from a solution containing a mixture of cell components and EO. Standard curves were then established for DNA (260 nm) and EO (321 nm).

Aliquots of 5ml were taken from growing cultures at 6 and 24 hours, concentrated by centrifugation for 20 minutes, at 25°C, at 8 000 rpm (Rota F 45-30-11, Eppendorf 5864R, Hamburg, Germany). The pellet was then washed twice with 10 ml of DH_2O for 10 minutes (to remove any EO externally attached to the cells), dissolved in 2ml of Tris-EDTA buffer with lysozome to a half concentration of 1mg/ml (Fluka 62971) and incubated at 37°C for 1 hour. Samples were then centrifuged for 10 minutes at 25°C at 8 000 rpm. The supernatant was pippetted into a quartz curvette and EO quantified in a UV spectrophotometer (UV1101 photometer, Biotech Cambs, UK). Using a multi component function the solution was scanned at different absorption

wavelengths for protein (280 nm), EO (321 nm) and DNA (260 nm). Using the line equations of the standard curves the quantity of DNA was calculated from the multi component solution using simultaneous equations.

4.2.12 Statistical Analysis

The statistics were carried out as described previously (section 3.1.3). The following were assessed

Growth at different temperatures: Doubling times and D values were calculated. These results plus the screening of essential oil results were used to establish if any significant differences in *Enterococcus* sp. growth/inhibition between temperatures occurred.

Growth at different pHs: Any significant differences in *Enterococcus* sp. growth between pHs at each temperature were established

Oil screening: Possible significant differences between inhibition of *Enterococcus* sp. by oils/components/blends and vapours between each temperature and growth phase were determined.

Growth with EO: It was established in what time period there were any significant differences between growth when being subjected to direct EO or vapour.

Cell size: To determine if there were any significant changes in *Enterococcus* sp. cell sizes over 24 hours.

DNA content: To assess any significant differences in the amount of DNA present in *Enterococcus sp.* over 24 hours after treatment with EO.

4.3 Results

4.3.1 Growth and Screening Assessment

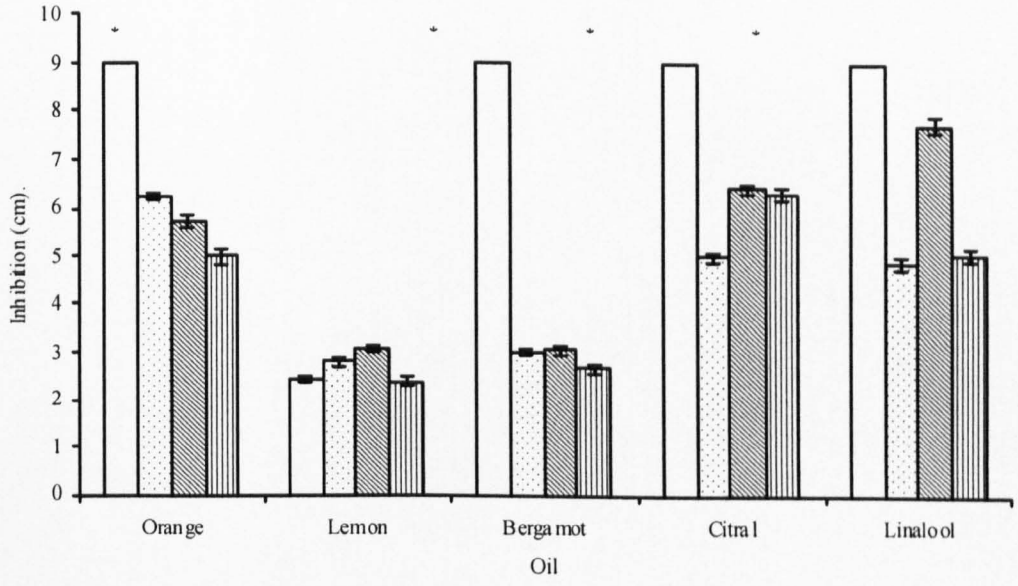
From the screening of the oils/vapours and their components via disc diffusion method (Fig. 4.1, 4.2, 4.3, 4.4 & 4.5) and generation times for exponential growth (Tables 4.1 & 4.2) ranges were established for differences in inhibition of growth (Appendix II: growth curves). These were; 5-15°C, 20-25°C, 30-45°C and 50°C, assumptions of normality and variance of homogeneity were met and therefore ANOVA was carried out $F(46) = 46.284, p \leq 0.001$ for temperature ranges and $F(32) = 75.581$, $p \leq 0.001$ for pH ranges. Post hoc analysis showed that within each range there was no significant difference of inhibition by the oils/components e.g. 30-45°C but there was a significant difference in inhibition between the ranges, 5-15°C and 20-25°C, $t(46) = 1.5$, $p \leq 0.001$, 20-25°C and 30-45°C, $t(46) = 0.2251$, $p \leq 0.001$ and between 30-45°C and 50°C $t(46) = 0.9915$, $p = 0.005$. Significance values between the ranges of the pHs were 4.5-6.5 and 7.5, $t(32) = -0.4633$, $p \leq 0.001$ and between pH 7.5 and pHs 8.5 –10.5, $t(32) = -0.5060$, $p \leq 0.001$. Mid points of pH ranges i.e. pH 5.5, 7.5 and 9.5 at 25°C and 37°C were carried forward for testing the effect of direct oils, while 50°C was used for vapour investigations, as this was the only temperature tested at which vapours were inhibitory (Fig. 4.1, 4.2, 4.3, 4.4 & 4.5 & Table 4.3).

Table 4.1 Generation times and D value for exponential growth of vancomycin susceptible *E. faecalis* and *E. faecium* at a range of temperatures from 5-50°C at pH 7.5 (n = 6).

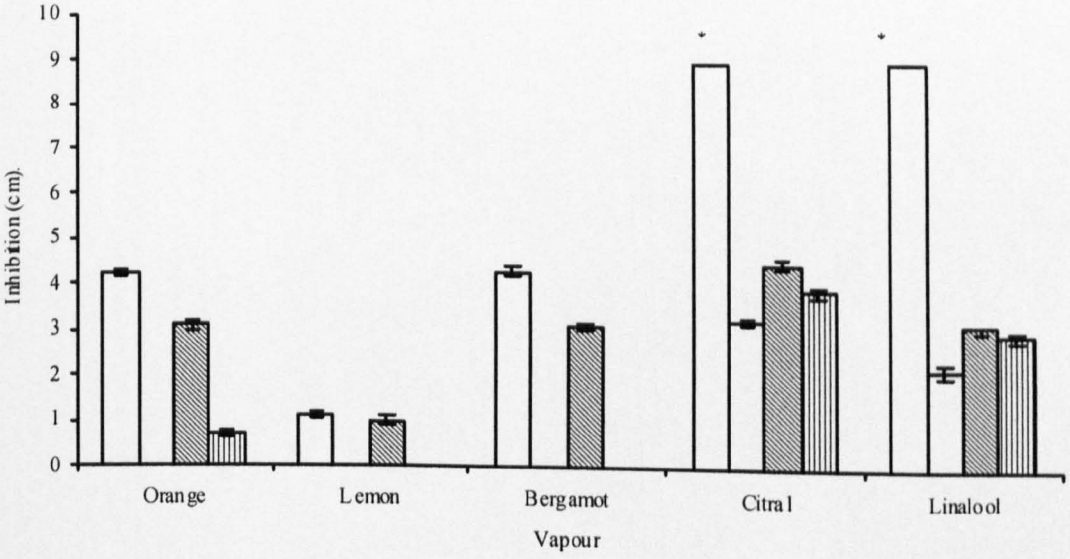
	E. faecalis		*E. faecium*	
Temp (°C)	Generation/hour	D value (hours)	Generation/hour	D value (hours)
5	0.37	8.93	0.52	7.24
10	0.45	6.9	0.72	10.61
15	1.04	2.2	0.71	2.95
20	0.81	2.39	0.79	3.12
25	0.97	2.2	1.41	2.26
30	1.25	1.68	1.12	1.51
37	2.16	0.99	1.75	1.14
45	1.90	0.96	2.09	0.48
50	4.03	0.61	4.12	0.54

No significant difference was observed between the inhibition of growth by the EOs/vapours and their components for stationary phase cells (oil *mdn* = 4.8, vapour *mdn* = 2.6) or exponential phase cells (oil *mdn* = 5.4, vapour *mdn* = 3.2) for either *E. faecium* or *E. faecalis* (oils, $U = 12809$, $p < 0.077$ or vapours $U = 12957$, $p < 0.06$), with the effect size being $r = -0.096$ and $r = -0.12$ respectively (Fig. 4.1, 4.2, 4.3, 4.4 & 4.5). The oils/vapours/components showed complete inhibition (>9cm) at 5°C and 10°C. The components limonene, hersipidin and neoericitrin had no inhibitory effect against *E. faecium* or *E. faecalis* (results not shown)

a)



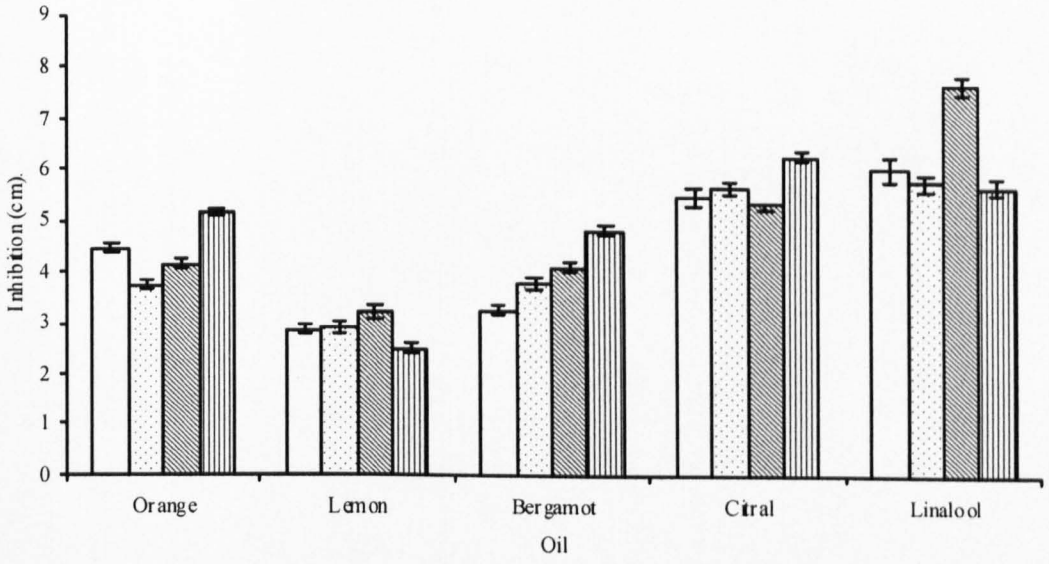
b)



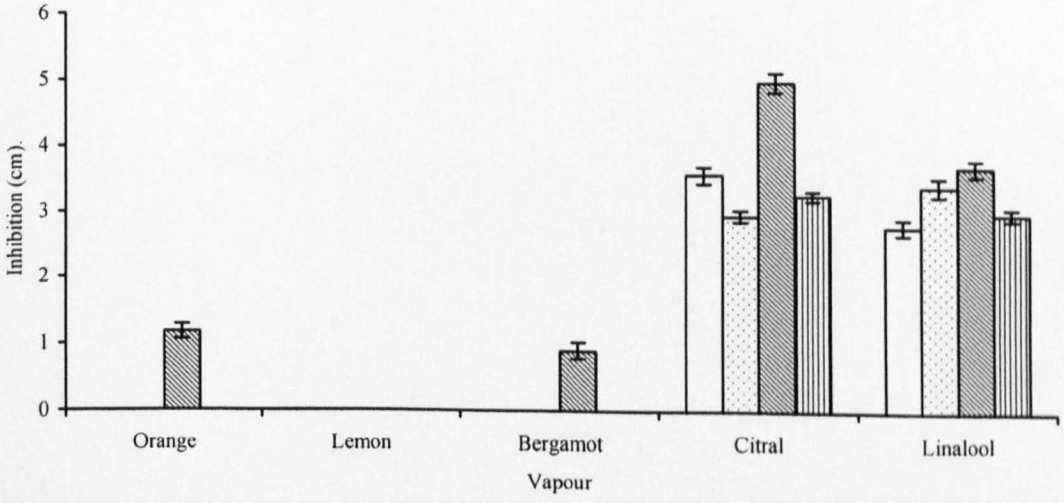
* Inhibition was limited by petri dish size therefore inhibition was > 9cm

Figure 4.1: Mean diameter of inhibition of growth of exponential and stationary phase cells of vancomycin susceptible *E. faecium* or *E. faecalis,* by citrus EO and their components a) oil, b) vapours at 20°C(± SD, n= 6)
Exponential *E. faecalis* □ , Stationary *E. faecalis* ⊡, exponential *E. faecium* ▨ and Stationary *E. faecium* ▥

a)



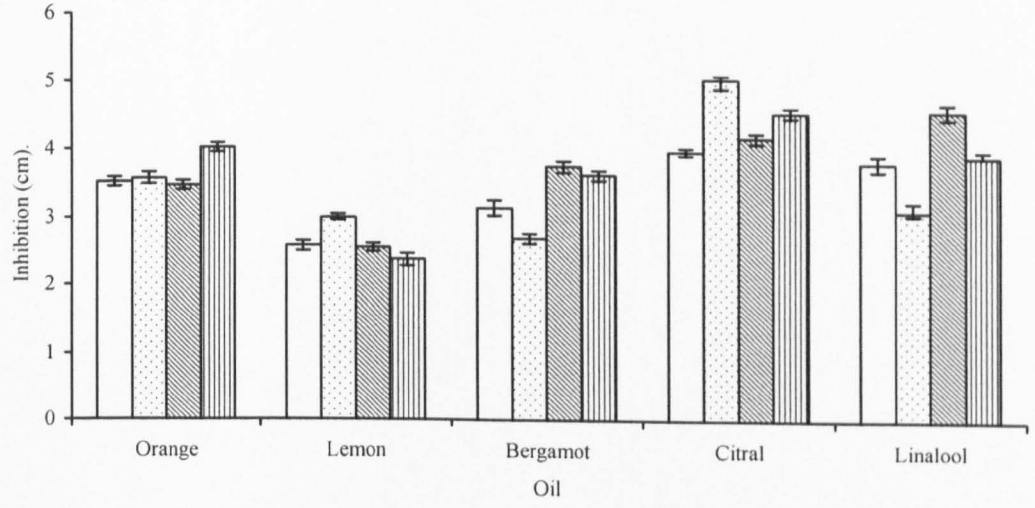
b)



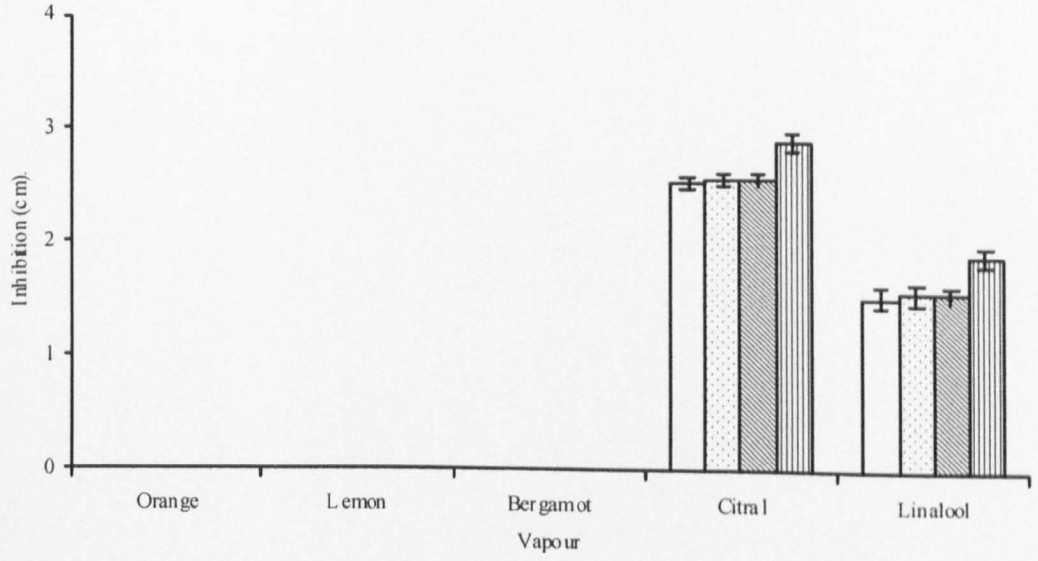
* Inhibition was limited by petri dish size therefore inhibition was > 9cm

Figure 4.2: Mean diameter of inhibition of growth of exponential and stationary phase cells of vancomycin susceptible *E. faecium* or *E. faecalis,* by citrus EO and their components a) oil, b) vapours at 25°C(± SD, n= 6)
Exponential *E. faecalis* □ , Stationary *E. faecalis* ⊡, exponential *E. faecium* ▨ and Stationary *E. faecium* ▥.

a)



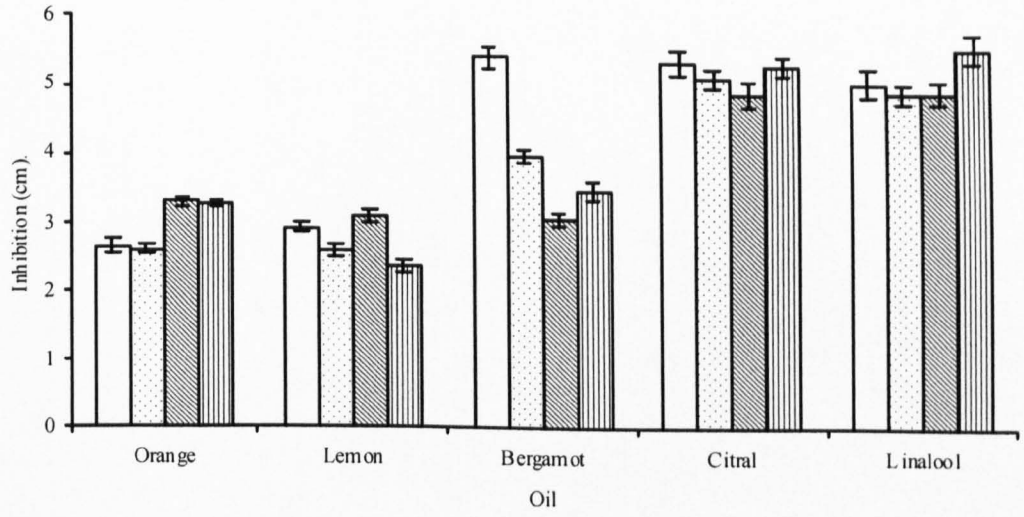
b)



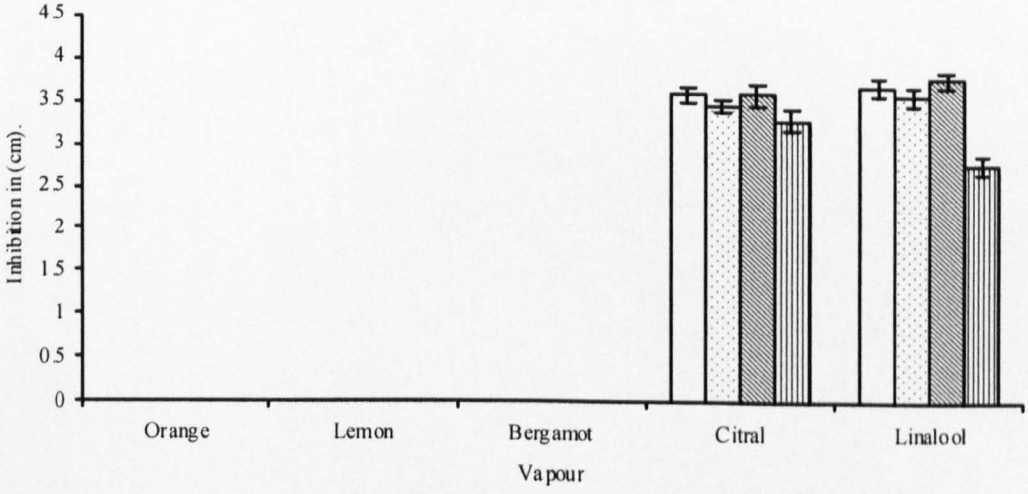
* Inhibition was limited by petri dish size therefore inhibition was > 9cm

Figure 4.3: Mean diameter of inhibition of growth of exponential and stationary phase cells of vancomycin susceptible *E. faecium* or *E. faecalis,* by citrus EO and their components a) oil, b) vapours at 30°C(± SD, n= 6)
Exponential *E. faecalis* □ , Stationary *E. faecalis* ⊡, exponential *E. faecium* ▨ and Stationary *E. faecium* ▥.

a)



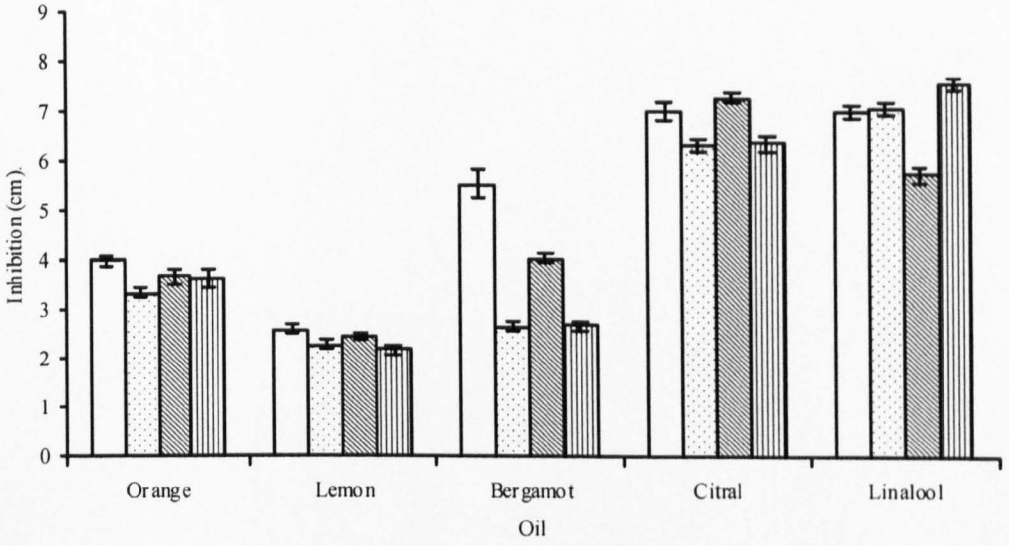
b)



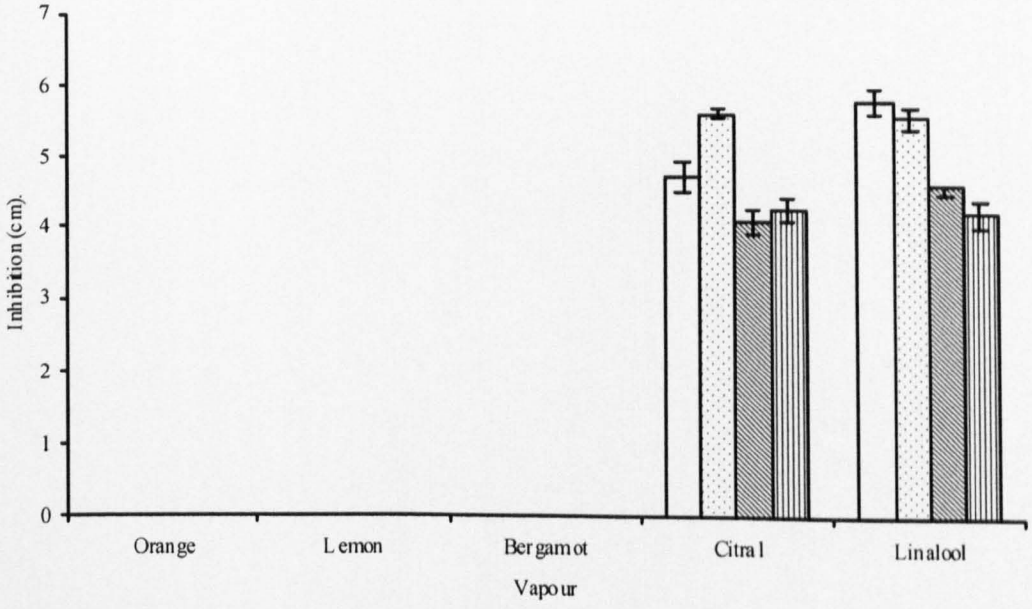
* Inhibition was limited by petri dish size therefore inhibition was > 9cm

Figure 4.4: Mean diameter of inhibition of growth of exponential and stationary phase cells of vancomycin susceptible *E. faecium* or *E. faecalis,* by citrus EO and their components a) oil, b) vapours at 37°C(± SD, n= 6)
Exponential *E. faecalis* □ , Stationary *E. faecalis* ⊡ , exponential *E. faecium* ▨ and Stationary *E. faecium* ▥

a)



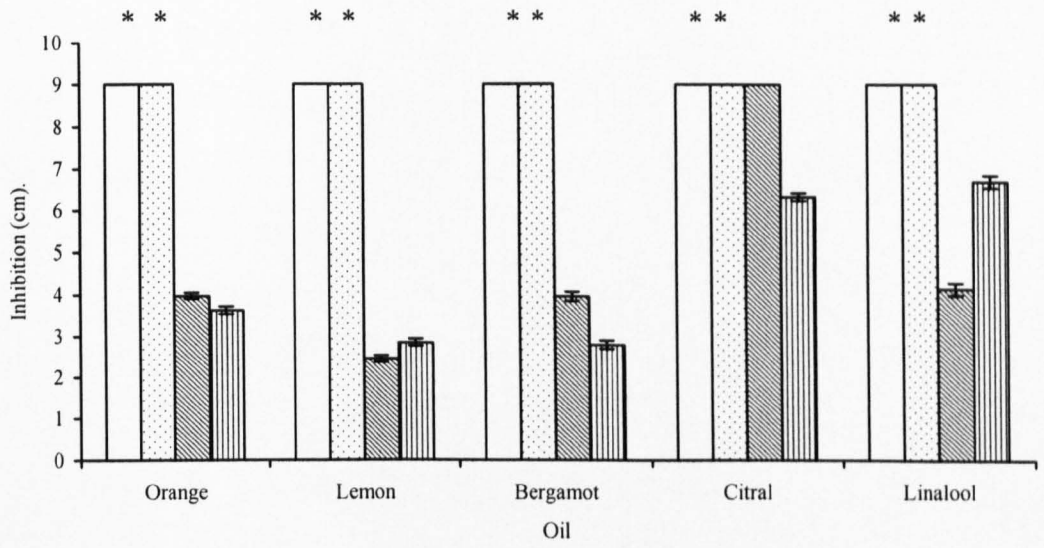
b)



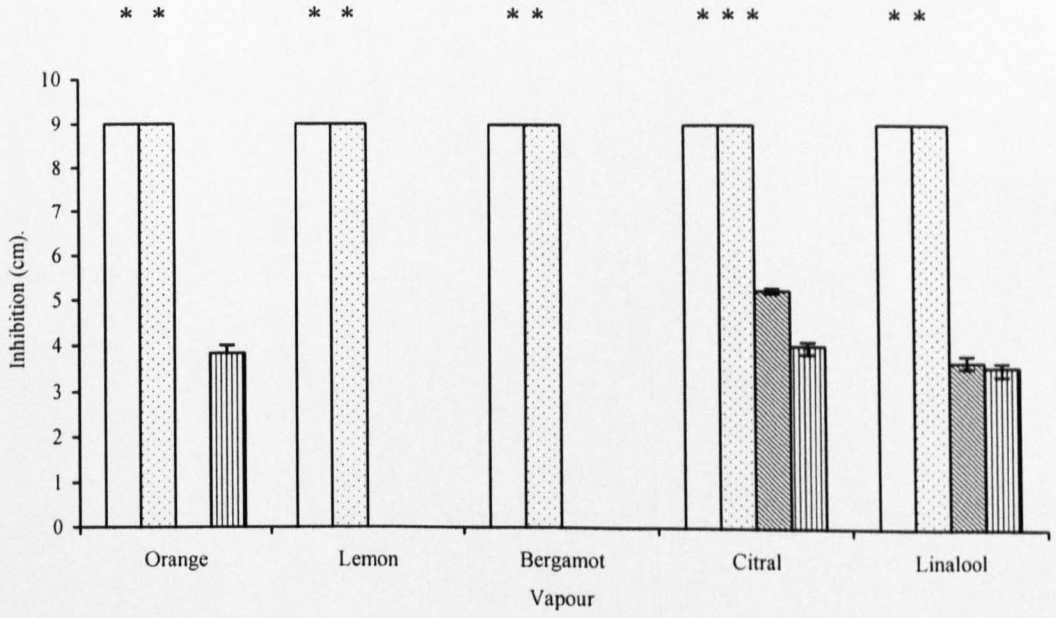
* Inhibition was limited by petri dish size therefore inhibition was > 9cm

Figure 4.5: Mean diameter of inhibition of growth of exponential and stationary phase cells of vancomycin susceptible *E. faecium* or *E. faecalis,* by citrus EO and their components a) oil, b) vapours at 45°C(± SD, n= 6)
Exponential *E. faecalis* □ , Stationary *E. faecalis* ⬚, exponential *E. faecium* ▨ and Stationary *E. faecium* ▥

a)



b)



* Inhibition was limited by petri dish size therefore inhibition was > 9cm

Figure 4.6: Mean diameter of inhibition of growth of exponential and stationary phase cells of vancomycin susceptible *E. faecium* or *E. faecalis,* by citrus EO and their components a) oil, b) vapours at 50°C(± SD, n= 6)
Exponential *E. faecalis* □ , Stationary *E. faecalis* ⬚, exponential *E. faecium* ▨ and Stationary *E. faecium* ▥.

Growth of *E. faecalis* and *E. faecium* at 37°C was completely inhibited by altering the pH to 9.5 or 10.5 and at 50°C *E. faecalis* was inhibited at pHs 4.5, 5.5, 6.5, 8.5, 9.5 and 10.5, whereas *E. faecium* was only inhibited at pHs 4.5, 5.5, 6.5 and 10.5, therefore showing greater tolerance to higher pHs up to pH 9.5. Table 4.2 shows the generation times of the organisms at the pHs and temperatures that were carried forward to establish MICs/MIDs.

Table 4.2: Generation times and D value of vancomycin susceptible *E. faecalis* or vancomycin susceptible *E. faecium* at pH 5.5 or 9.5 at 25°C or 37°C (n = 6).

		E. faecalis		*E. faecium*	
	pH	Generation/hour	D value (hrs)	Generation/hour	D value (hrs)
25°C	5.5	0.34	9.69	0.48	6.94
	9.5	0.38	8.84	0.40	8.35
37°C	5.5	1.38	1.61	1.76	1.76
	9.5	NG	NG	1.04	3.18

NG = no growth < 20 cfu/ml

Although screening gives an indication of the effectiveness of the antimicrobial agent it is not quantitative therefore determination of concentrations/dosages of inhibition of growth is essential. As demonstrated by the MICs for the vancomycin susceptible strains lemon EO alone or blended and orange EO alone were not as effective as citral or linalool alone or bergamot blends (Table 4.3). Only citral and linalool vapours inhibited growth at 25°C or 37°C with MIDs between 100- >1600 mg/L air at 25°C and 37°C. Overall at 50°C oils/blends vapours had greater inhibition than at 25°C or 37°C, with the blends of orange/bergamot and orange/ lemon being effective

against *E. faecium* with MIDs between 50 and 400 mg/L air. Only orange/lemon blend was ineffective against *E. faecalis* with all other oils/blends having MIDs of between 50-100 mg/L air (Table 4.3). Overall the blend of orange/bergamot was more effective in combination than orange, lemon or bergamot alone, although linalool and citral were most effective with MICs an range from 0.5 – 2%, 0.5 – 4% and 1- 2% at 25°C, 37°C and 50°C respectively compared with next most effective oil (bergamot) with MICs of 1 - >4%, 1 - >4%, and 1 – 4% at 25°C, 37°C and 50°C respectively across all the pHs. The orange/bergamot blend vapour also had the lowest MID 50mg/L air at 50°C to inhibit both *E. faecalis* and *E. faecium.* Orange and lemon vapours alone showed no inhibitory effect and bergamot vapours alone only had an effect at 50°C at pH 9.5 against *E. faecalis* with an MID of 50 mg/L air (Table 4.3).

Table 4.3: Mean MIC (%v/v) and MID mg/L air of Citrus essential oils/components/blends against vancomycin susceptible strains of *E. faecalis* and *E. faecium* using the agar dilution method (n = 4).

		E. faecalis						*E. faecium*					
	pH	5.5		7.5		9.5		5.5		7.5		9.5	
		Oil	Vapour	Oil	Vapour	Oil	Vapour	Oil	Vapour	Oil	Vapour	Oil	Vapour
	Orange	1	NE	>4	NE	1	NE	2	NE	>4	NE	2	NE
	Lemon	>4	NE	>4	NE	4	NE	>4	NE	>4	NE	>4	NE
	Bergamot	1	NE	>4	NE	1	NE	2	NE	>4	NE	2	NE
	Citral	0.06	>1600	0.5	100	0.125	>1600	0.125	>1600	0.25	>1600	0.125	800
25°C	Linalool	0.25	>1600	1	400	0.25	>1600	0.25	>1600	0.5	>1600	0.25	800
	*O/L	1	NE	>4	NE	>4	NE	2	NE	>4	NE	2	NE
	*O/B	0.25	NE	1	NE	2	NE	0.25	NE	2	NE	0.5	NE
	*B/L	0.5	NE	4	NE	4	NE	0.5	NE	>4	NE	1	NE
	*O/L/B	0.25	NE	2	NE	2	NE	1	NE	2	NE	1	NE
	Orange	>4	NE	>4	NE	1	NE	2	NE	>4	NE	2	NE
	Lemon	>4	NE	>4	NE	4	NE	>4	NE	>4	NE	4	NE
	Bergamot	1	NE	>4	NE	1	NE	2	NE	3	NE	2	NE
	Citral	0.06	400	0.125	1600	0.06	NE	0.125	800	0.125	>1600	0.125	400
37°C	Linalool	0.125	100	0.5	1600	0.25	NE	0.5	200	0.25	>1600	0.25	100
	*O/L	1	NE	>4	NE	>4	NE	4	NE	>4	NE	>4	NE
	*O/B	0.5	NE	2	NE	4	NE	1	NE	2	NE	2	NE
	*B/L	>4	NE	4	NE	4	NE	1	NE	>4	NE	4	NE
	*O/L/B	2	NE	4	NE	4	NE	4	NE	4	NE	4	NE
	Orange	_	_	1	50	_	_	_	_	2	NE	4	NE
	Lemon	_	_	>4	50	_	_	_	_	>4	NE	4	NE
	Bergamot	_	_	1	50	_	_	_	_	4	NE	4	NE
	Citral	_	_	0.125	50	_	_	_	_	0.125	100	0.06	50
50°C	Linalool	_	_	0.125	50	_	_	_	_	0.125	100	0.06	50
	*O/L	_	_	4	NE	_	_	_	_	>4	NE	>4	NE
	*O/B	_	_	1	50	_	_	_	_	2	50	1	50
	*B/L	_	_	4	100	_	_	_	_	4	400	4	100
	*O/L/B	_	_	2	50	_	_	_	_	4	NE	1	NE

*- = Not assessed, no growth < 20cfu/ml at the specific temperature and pH combination, NE = No effect, O/L = orange/lemon blend, O/B = orange/bergamot blend and O/L/B = orange/lemon/beragmot blend.

Since the orange/bergamot blend was the most effective against vancomycin susceptible *E. faecium* and *E. faecalis*, the blend was tested against the vancomycin resistant strains. The MICs were lower against the vancomycin susceptible strains with only those at pH 5.5 and 25°C or 37°C being below 4% (Table 4.4). The MIDs (50 mg/L air) were the same for both resistant and susceptible strains, with the exception of *E. faecium* at pH 7.5 at 50°C when the MID was 200 mg/L air (Tables 4.3 and 4.4). Oils/blends/components with MICs of 4% (v/v) and MIDs of 50mg/L air or above were not used in succeeding experiments because at these high levels there would be possible organoleptic effects on food or allergic effects in humans.

Table 4.4: Mean MIC (%v/v) for the oil and MID (mg/L air) for the vapour of orange/bergamot blend against vancomycin resistant strains of *E. faecalis* (12203) and *E. faecium* (12202) using the agar dilution and vapour chamber method (n = 4)

	MIC (% v/v)						MID (mg/L air)		
	25°C			37°C			50°C		
pH	5.5	7.5	9.5	5.5	7.5	9.5	5.5	7.5	9.5
E. faecalis	0.5	>4	4	2	>4	4	NG	50	NG
E. faecium	0.5	4	>4	2	4	4	NG	50	50

NG = No growth < 20 cfu/ml, NE = No effect

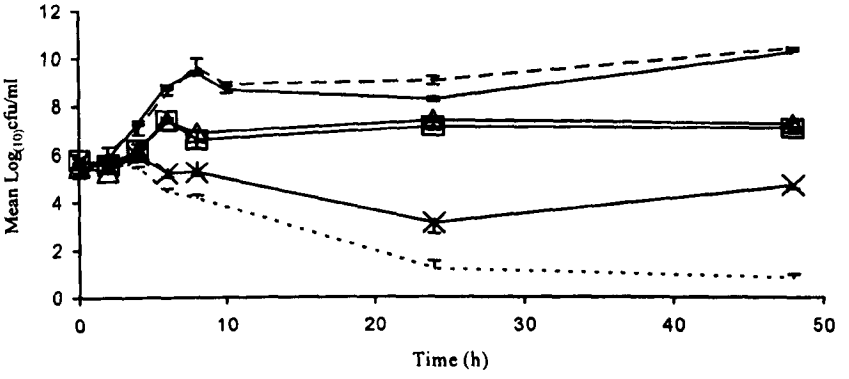
In vapour form citral and linalool had an FIC of between 0.5 and 1 therefore the inhibition of *Enterococcus* sp. required the same dosages in combination as the components alone (Fig. 4.7) to inhibit both vancomycin susceptible *E. faecium* and *E. faecalis.* Table 4.5 demonstrates the data output from the combination of the oils of citral and linalool, with the shaded area demonstrating where growth occurred. Overall the combination of citral and linalool at pH 7.5 had an antagonistic effect with FICs against vancomycin susceptible *E. faecium* and *E. faecalis* being 3 and 12 at 25°C, 33 and 9 at 37°C and 32 and 20 at 50°C respectively.

Table 4.5: Checkerboard inhibitory combinations of the oils citral and linalool against vancomycin susceptible *E. faecium* at 37°C at pH 7.5 (n = 6).

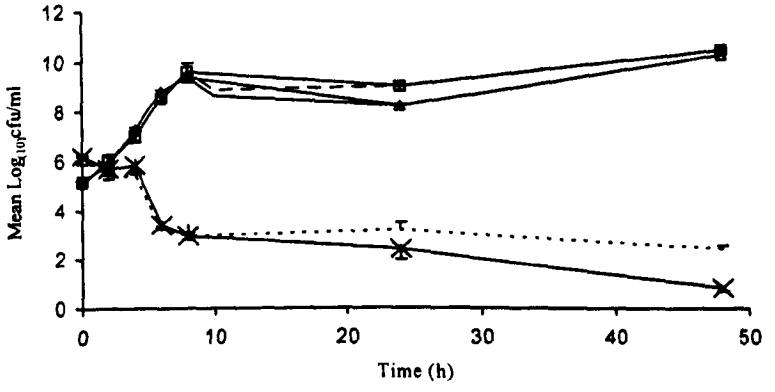
Concentration		Citral					
(% v/v)		4	2	1	0.5	0.25	0.125	0.06
	4	-0.028	-0.054	-0.044	0.023	0.24	0.329	0.34
	2	-0.306	-0.223	-0.036	-0.016	0.152	0.029	0.096
	1	-0.009	0.045	-0.062	-0.058	0.111	0.159	0.168
Linalool	0.5	-0.043	-0.061	0.213	0.278	0.261	0.341	0.414
	0.25	-0.045	-0.004	0.287	0.264	0.333	0.379	0.453
	0.125	-0.028	0.036	0.261	0.199	0.461	0.452	0.477
	0.06	-0.037	0.202	0.268	0.194	0.335	0.489	0.598

The inhibition of growth by orange/bergamot blend of vancomycin susceptible strains ranged from 5.5 to 10 $\log_{(10)}$ at 37°C (Fig. 4.6 a, b & c), compared to a reduction at 25°C of approximately 5.5 $\log_{(10)}$ at pHs 5.5, 7.5 and 9.5 (Fig. 4.7). No growth was detectable in the vapour experiments at 50°C and pH 7.5 or pH 9.5 after 48 h (results not shown). $\text{Log}_{(10)}$ reductions in growth with oils/vapours in optimum conditions (pH 7.5/37°C) became significantly different between 2-6 h $F(10) = 13.793$, $p = 0.004$ under all conditions tested $t(10) = 6.455$, $p \leq 0.001$ (Fig. 4.6, 4.7 & 4.8). The antibiotic resistant strains were also less susceptible at 25°C compared with 37°C with a 5-6 and 8.5-10 $\log_{(10)}$ reduction in growth respectively. The vapours only inhibited growth of the resistant strains by 4.5 $\log_{(10)}$ compared with that observed with the susceptible strains of 10 $\log_{(10)}$ (Fig. 4.8b). The inhibition was a bacteriostatic effect in that, when removed from the EO and grown under optimal conditions, cell population numbers increased from $\log_{(10)}$ 1-3 in the presence of orange/bergamot blend to $\log_{(10)}$ 6.5-7.5 in optimum conditions and from undetectable levels (< 20 cfu/ml) in the presence of the vapours to a population of 4.5 $\log_{(10)}$ cfu/ml.

a)



b)



c)

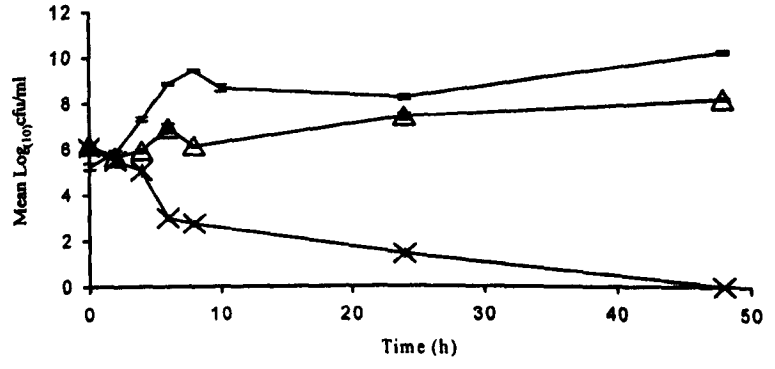
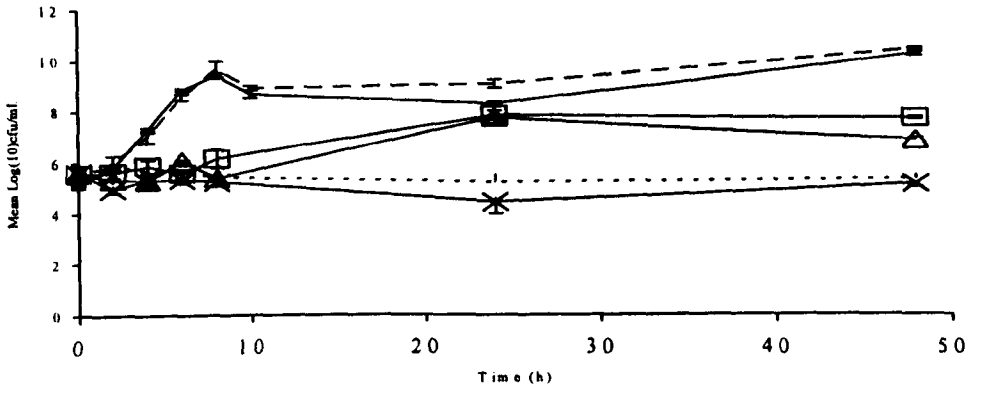
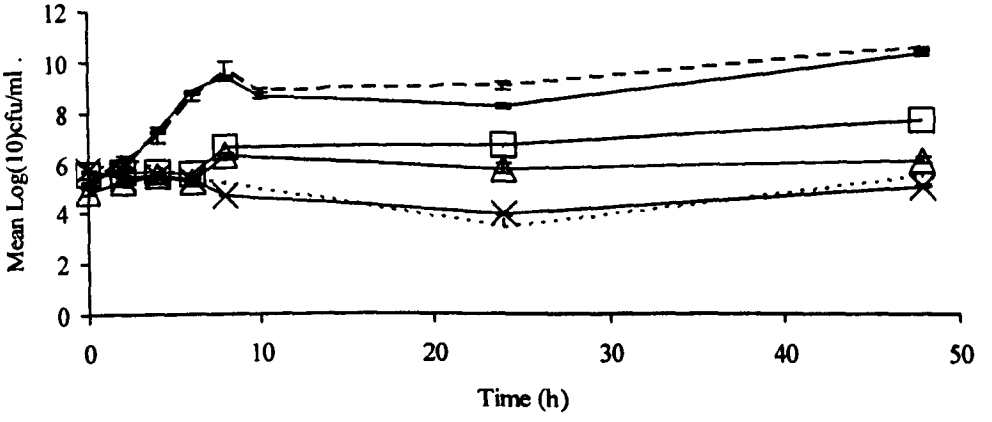


Figure 4.6: Growth over 48 h with orange/bergamot blend at 37°C of vancomycin susceptible strains a) pH 5.5, MIC 0.25% *E. faecalis*, 1% *E. faecium* b) pH 7.5, MIC 2% *E. faecalis* and *E. faecium* c) pH 9.5, MIC 2% *E. faecium* (mean ± SE, n = 6).
E. faecalis with oil/vapour (·····), *E. faecalis* with no oil/vapour (-□-), *E. faecalis* optimum conditions (----), *E. faecium* with oil/vapour (-×-), *E. faecium* with no oil/vapour (-Δ-), *E. faecium* optimum conditions (___)

a)



b)



c)

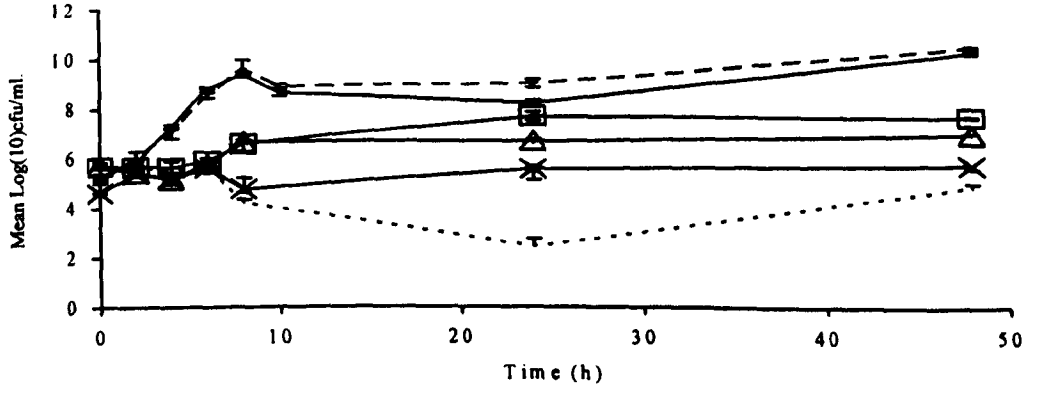
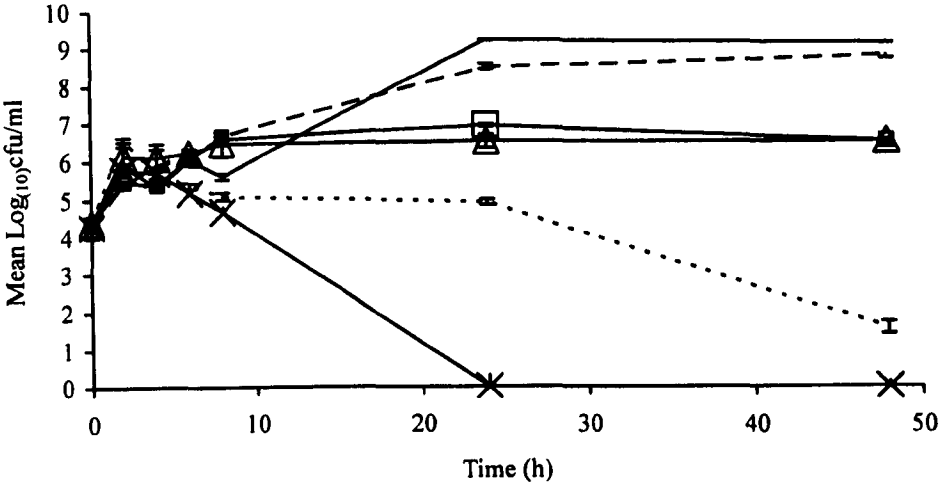


Figure 4.7: Growth over 48 h with orange/bergamot blend at 25°C of vancomycin susceptible strains a) pH 5.5, MIC 0.25% *E. faecalis* and *E. faecium* b) pH 7.5, MIC 1% *E. faecalis*, 2% *E. faecium* c) pH 9.5, MIC 2% *E. faecalis* and *E. faecium* (mean ± SE, n = 6). *E. faecalis* with oil/vapour (·····), *E. faecalis* with no oil/vapour (-□-), *E. faecalis* optimum conditions (----), *E. faecium* with oil/vapour (-×-), *E. faecium* with no oil/vapour (-Δ-), *E. faecium* optimum conditions (——)

a)



b)

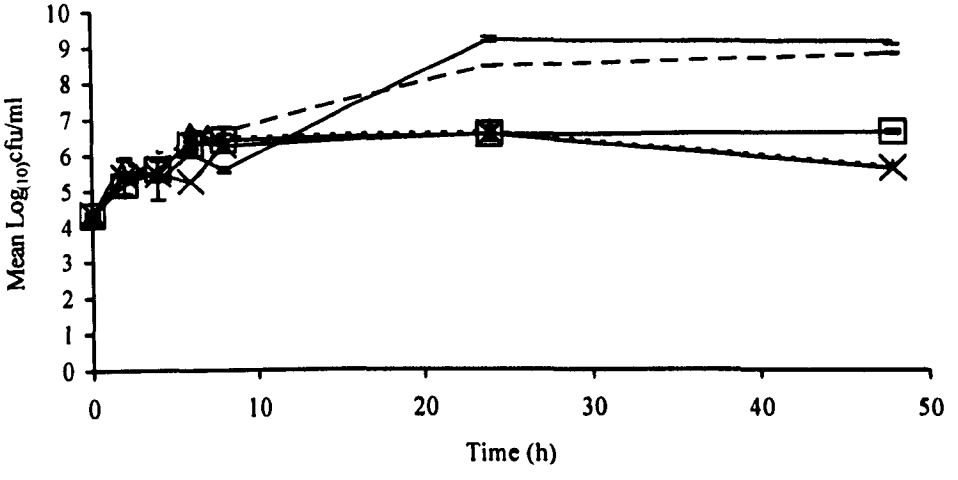


Figure 4.8: Growth of vancomycin resistant strains over 48 h with orange/bergamot blend a) 37°C, pH 5.5 and MIC of 2% b) 50°C, pH 7.5 and MID of 50 mg/L air (mean ± SE, n = 6). *E. faecalis* (12203) with oil/vapour (.....), *E. faecalis* (12203) with no oil/vapour (-□-), *E. faecalis* (12203) optimum conditions (----), *E. faecium* (12202) with oil/vapour (-×-) *E. faecium* (12202) with no oil/vapour (-Δ-), *E. faecium* (12202) optimum conditions(——).

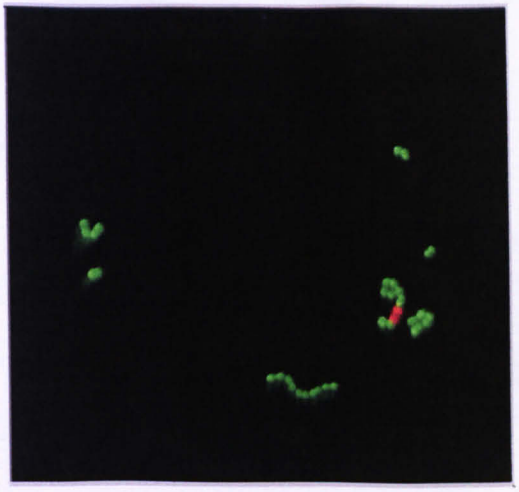
4.3.2 Effect of the Combination of EO, Temperature and pH on Enterococcus sp.

The re-growth of cells after being subjected to the combination of temperature, pH and EO blend showed a bacteriostatic effect. Florescence images after exposure to the EO blend (Fig. 4.9) demonstrated that even after a large reduction in viable counts (Figs. 4.6 & 4.7) many of the cells were still live (Fig. 4.9b). Ratios of live to dead cells were calculated (Table 4.6) the results showed that the blend vapour had the greatest bactericidal effect against *E. faecalis* with a live: dead ratio of 1.16:1 at 6 hours and 1.6:1 at 24 hours compared to the control of 21.77:1 and 22.87:1 respectively. Whilst the oil of the blend was most effective against *E. faecium* compared with *E. faecalis* with ratios of 0.93:1 and 5.16:1 at 6 and 24 hours compared with untreated cultures of 24.15:1 and 21.23:1. Overall cells of both strains were recovering from the effect of both the oil and the vapour over the 48 hour period where the ratio of live:dead cells being approximately the same as the controls by 48 hours (Table 4.6).

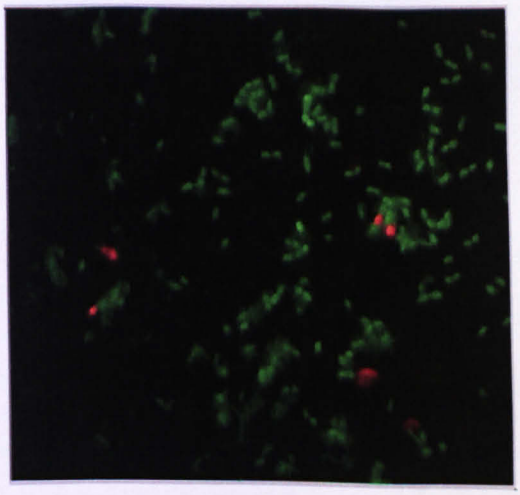
Table 4.6: Ratio of live:dead cells of vancomycin susceptible *E. faecalis* and *E. faecium* under optimum conditions (37°C, pH 7.5) or with EO blend at pH 5.5, 37°C and MIC 0.5% for *E. faecalis* and pH 9.5, 37°C and MIC 2% for *E. faecium* and with vapour 50mg/L air at 50 °C and pH 7.5 for both (± SE, n = 4).

	Hours	Optimum	Oil	Vapour
	6	21.77 ± 1.1	6.5 ± 0.9	1.16 ± 0.9
E. faecalis	24	22.87 ± 0.4	18.44 ± 1.3	1.6 ± 1.1
	48	15.68 ± 0.3	17.14 ± 0.4	12.22 ± 1.2
	6	24.15 ± 1.2	0.93 ± 1.2	2.05 ± 1.2
E. faecium	24	21.23 ± 0.2	5.16 ± 0.2	5.46 ± 0.4
	48	23.23 ± 0.3	26.93 ± 1.3	23.56 ± 0.02

a)



b)



c)

Figure 4.9: Baclight images of dead/live cells of vancomycin susceptible a) *E. faecalis* under optimum conditions (37˚C, pH 7.5) for 24 hours, b) *E. faecalis* exposed to 48 hours at pH 5.5 and MIC 0.5% orange/bergamot oil at 37˚C and c) *E. faecium* exposed to 6 hours of 50mg/L air of orange/bergamot vapours at pH 7.5, at 50°C.

The effect of the EO blend on cell size and DNA content was monitored over 24 hours. Results showed that, although cell viability as measured by growth curves and Baclight had been reduced, the size of individual cells had not been affected, with no significant difference between the size of control cells and those being subjected to EO oil or vapour (Table 4.8). Assumptions of normality and variance of homogeneity were met and *t*-tests were carried out, at 24 hours for oil $t(15) = 1.967$, $p = 0.188$ and vapour $t(15) = 0.223$, $p = 0.844$ against *E. faecium* and oil $t(15) = 7.812$, $p = 0.055$ and vapour $t(15) = 1.623$, $p = 0.246$ against *E. faecalis* (Fig 4.6, Appendix III shows an example of the coulter counter output).

However the DNA content per cell was significantly increased after the blend had been applied, with control cells having approximately 0.09 – 0.14 pg and 0.04 to 0.3 pg DNA per cell at 6 and 24 hrs compared with that of those cells subjected to EO oil or vapour of approximately 53 - 59 pg/cell and 19 - 47 pg/cell at 6 hours and 2.63 – 6.76 μg/cell and 18.29 – 40.6 μg/cell respectively (Table 4.7). The mean ratio of the samples at 260/280nm was 1.88, which is within the range (1.8-2.0) desired for purifying nucleic acids when using this method.

Table 4.7: Mean cell DNA content (pg/cell) of vancomycin susceptible *E. faecalis* and *E. faecium* under optimum conditions (37°C, pH 7.5) or with EO blend at pH 5.5, 37°C and MIC 0.5% for *E. faecalis* and pH 9.5, 37°C and MIC 2% for *E. faecium* and with vapour at 50 °C and pH 7.5 for both (n = 4).

Incubation time		6 hrs			24 hrs	
Conditions	37 °C / pH 7.5	Oil	Vapour	37 °C / pH 7.5	Oil	Vapour
E. faecalis	0.14	52.9	19.2	0.037	6762.26	18286.7
E. faecium	0.09	59	47.3	0.295	2625.76	40595.7

Table 4.8: The effect of orange/bergamot blend at 37°C on cell size (modal diameter, μm) of vancomycin susceptible *E. faecalis* and *E. faecium* under optimum conditions (37°C, pH 7.5) or with EO blend at pH 5.5, 37°C and MIC 0.5% for *E. faecalis* and pH 9.5, 37°C and MIC 2% for *E. faecium* and with vapour at 50 °C and pH 7.5 for both (n = 4).

Conditions	37 °C / pH 7.5		With oil		With vapour	
Incubation time	6 h	24 h	6 h	24 h	6 h	24 h
E. faecalis	1.73	1.67	1.38	1.36	1.86	1.17
E. faecium	1.63	1.52	1.3	1.34	1.61	1.43

4.4 Discussion

4.4.1 Growth and Screening Assessment

The ranges of temperature and pH for differences in growth of *Enterococcus* sp. established in this study are similar to those from previous studies assessing the membrane permeability of *Enterococcus* sp. in the presence of 3% NaCl i.e. 10-13°C, 17-22°C and 42-47°C or bacteriocins from *Lactobacillus plantarum* i.e. 4-25°C, 30-37°C and 45-60°C pH 2-4, 6-8, and 10 (Ivanov *et al.*, 1999, Todorov *et al.*, 2006, Koch *et al.*, 1998).

The results of this investigation demonstrate that the blend of orange/bergamot (1:1 v/v) EO has antibacterial properties against both vancomycin resistant and vancomycin susceptible *E. faecalis* and *E. faecium*, although *Enterococcus* sp. has been found in this study to be less susceptible than other Gram-positive bacteria such as *L. monocytogenes* and *B. cereus* to orange or bergamot EOs (Fisher and Phillips, 2006). Citral, linalool and bergamot are the most inhibitory oils with lemon being the least inhibitory with similar results being demonstrated in previous studies (Fisher and Phillips, 2006, Deans and Ritchie, 1987) The reason that citral may have been so effective may be due to its high partition coefficient (2.8 to 3 $\log_{(10)} P_{ow}$) which allows a higher rate of diffusion through the cell membrane and damage and or death to the cell (Raybaudi-Massilia *et al.*, 2008). The use of citral or linalool alone is similar to using a chemical biocide which is reason that these components were not carried forward for further investigation. Citral and linalool are not natural alternatives to chemical based antimicrobials. Limonene, hersipidin and neoericitrin showed no inhibitory effect against *Enterococcus* sp., which was also found to be the case when hersipidin and

neoericitrin MICs were established against a range of bacteria including *E. coli, Salmonella, Bacillus, Listeria* and *Staphylococcus* in all cases the MIC was found to be > 1000 $\mu g/ml^{-1}$ (Mandalari *et al.*, 2007). The concept of blending citrus EOs has yet to have been explored extensively, despite single and poly herbal antimicrobials being used throughout history (Al-Bayati, 2008). The results from this study show that the blending of orange/bergamot results in a lower MIC than those of bergamot and orange individually (Table 4.3). This is similar to a previous study when a combination of EOs *Thymus vulgaris* and *Pimpinella ansium* was assessed against *Staph. aureus, B. cereus, E. coli, Proteus vulgaris, S. typhi, K. pneumoniae* and *P. aeruginosa.* The combination showed antimicrobial activity against most of the bacteria with the greatest inhibition being against *Proteus vulgaris* with an MIC of 15.6 μg/ml, compared with the use of thyme and *Pimpinella* alone of 31.2 μg/ml and 62.5 μg/ml respectively (Al-Bayati, 2008). A study on *B. cereus* showed that the combination of nisin and carvcrol increased antibacterial activity at 8°C whereas nisin alone only had a small effect (Periago and Moezelaar, 2001). It is essential to obtain the lowest MIC/MID possible because of potential changes in organoleptic properties of foodstuff. For example, thymol have been found by a tasting panel to be unacceptable at any level within carrot juice (Valero and Frances, 2006). In the clinical arena possible allergic reactions must also be considered when assessing the use of EOs in humans.

The reduction of growth over 48 h using the EO blend in both vancomycin resistant and vancomycin susceptible strains at 25°C was statistically lower than that at 37°C (Figs. 4.6 & 4.7). This is may be considered unusual as, by subjecting bacteria to a number of sublethal stresses, such as high temperature, high or low pH and high salt concentrations, an additive inhibitory effect is generally observed, such as in

S. typhimurium with combinations of low temperature, modified atmospheres and oregano EO (Skandamis *et al.*, 2002). The antimicrobial effect of nisin against *B. cereus* has also shown to be more effective at 8°C than 15°C and this effect was increased when the pH was reduced from pH 7 to pH 6.3 (Periago and Moezelaar, 2001). The combination of temperature and pH used to inhibit growth of *B. cereus* in vegetable substrates showed that only small changes in pH were required to increase viable cells, at 16°C, pH 5 there was complete inhibited of growth for 60 days but at pH 5.1 and 5.2 a growth rate of 0.012 $hours^{-1}$ was observed. The results of the study also showed that as pH increased the refrigeration temperature also needed to be increased to prevent growth of *B. cereus* i.e. at pH 5.3 the inhibition temperature was 8°C, but at pH 5.4 and 5.5 the temperature required to inhibit growth was 5°C (Valero *et al.*, 2003).

Although tolerance to stresses in *E. faecalis* has been shown after pre-treatment with subleathal stresses for example a 6000-fold increase to heat tolerance was observed at 62°C after adaptation at 50°C and a 1500 – fold increase in tolerance to bile salts at 0.08% after being subjected to 0.3% (Rince *et al.*, 2000). *E. faecalis* cells that have not been cultured at either warm or cold temperatures but cultured under non-stress temperatures were shown to have phenotypic alterations which actually make the cells more sensitive to heat or salinity. When these two stressess were combined the phenotypic modifications became more pronounced and these changes were observed for a number of generations (Ivanov *et al.*, 1999). *Streptococcus*, a closely related species to *Enterococcus* sp., demonstrated adaptation by exposure during exponential stage of growth to acid and heat stressed cells of 1 to 60 000 – fold. Whereas pre-treatment of oxidative and osmotic stresses had a detrimental effect on cell survival, this was thought to be due to the harshness of the stress applied. The results of the study

suggested that the treatment of cells with one type of stress can actually lead to cross-protection to a variety of stress due to the array of stress induced proteins expressed (Zotta *et al.*, 2008).

The results of an investigation on *E. coli* combining temperature and the EOs oregano and nutmeg, agreed with the findings of this present study that the EOs had greater inhibtion at optimal temperatures of 37°C (Figs. 4.2, 4.3 & 4.4). Different concentrations (20µl/ml to 0.04µl/ml) of both EOs at 3°C and 8°C for 72 hours showed no significant difference to the controls ($p > 0.05$), whereas when the temperature was increased to 20°C (nearer optimum conditions) there were significant differences in viable counts 6.61 $\log_{10}$ cfu/ml compared to 8.52 $\log_{10}$ of the control (Shekarforoush *et al.*, 2007). One of the reasons that *Enterococcus* sp. in this study had greater resistance to the EO blend at conditions other than optimum may be because the enterococcal membrane has been demonstrated to be more stable near the minimal growth temperature, which is a specific mechanism associated with *Enterococcus* sp., as a result of a rise in branched-chain fatty acids within the cells at these temperatures (Ivanov *et al.*, 1999). When *L. monocytogenes* was subjected to S-carvone and heat treatments of 35°C and 45°C viable counts are reduced by approximately 1.3 $\log_{10}$, yet those cells that had been heat pre-treated are not susceptible to the combined treatment. What was interesting was the difference in the phospholipid composition of the cytoplasmic membrane of the cells. If cells were grown at 7°C instead of 30°C, increasing the amount of C15:0 fatty acids and reducing the C17:0, whilst increasing the amount of unsaturated fatty acid C18:1, meant that when the cells were exposed to higher temperatures in combination with S-carvone, the membrane had more fluidity and thus allowed the S-carvone to penetrate the membrane resulting in cell death.

Those cells grown at 45°C membranes had low membrane fludity and was not as permeable to S-carvone (Karatzas *et al.*, 2000). An investigation into the effect of pH on the adhesion of *E. faecalis* to collagen, found changes in the membrane proteins that altered cell surface tension, charge and hydrophobicity as well as increasing its ability to adhere to collagen. These changes in cell surface a pH 9.5 at 25°C may be what increased the resistance of the *Enterococcus* sp. cells to that of pH 9.5 at 37°C in this study (Fig. 4.6 & 4.7).

The ability for *Enterococcus* sp. to survive such adverse conditions i.e. its large ranges of growth temperature (5 - 50°C) and pH (4.5 – 10.5) suggests that it has proteins that are able to function at extreme parameters and also able to adapt (Van den Berghe *et al.*, 2006, Klein, 2003, Gardini *et al.*, 2001, Ivanov *et al.*, 1999). The proteins identified as being involved in temperature change response are DnaK and GroEL (Koch *et al.*, 1998). The effect of pH on the inhibition of *Enterococcus* sp. is dependent on the species itself; *E. faecium* is more resistant to pH 9.5 than *E. faecalis,* which under high temperatures does not grow (Table 4.5). It has been noted that unlike the cross-response of alkaline to acid stresses in *E. coli, E. faecalis* does not have this ability, therefore acid adaptation does not then result in alkaline tolerance as it involves different resistance pathways (Flahaut *et al.*, 1996b). The pH level of the growth environment for *E. faecalis* is essential in interactions between amino acid decarboxylase activities, which is thought to be a physiological mechanism to overcome acid environments in bacteria. This was demonstrated in a study on the effects of pH, temperature and NaCl on the growth kinetics, proteolytic activity and biogenic amine production of *E. faecalis.* The effect of temperature on the growth and biogenic amines

was shown to be insignificant, but 5% NaCL minimises the presence of amines, which in turn can be lowered further if the pH is decreased (Gardini *et al.*, 2001).

Although the use of EOs at 50°C has limited application the vapours were only effective at this temperature (Table 4.3). Vapours are needed in lower concentrations than direct oils to produce the same inhibition and they produce a greater bactericidal effect in that recovery, when removed from the EO vapour is less i.e. $\log_{(10)}$ 4.5 compared with $\log_{(10)}$ 7.5 cfu/ml when removed from the oil *per se* (section 4.3.2).

Vancomycin resistant strains are not as susceptible to orange/bergamot citrus EO vapour as vancomycin susceptible strains, with only a $\log_{(10)}$ 4.5 reduction in growth (Fig. 4.8) compared with reduction to undetectable levels from approximately $\log_{(10)}$ 9 in the vancomycin susceptible strains. The application of direct citrus EO has approximately the same inhibitory effect in both strains suggesting the mechanism by which vapours inhibit growth may be different from that of oils. Vancomycin inhibits the peptidoglycan biosynthesis of Gram-positive bacteria, thus when there is resistance to the antibiotic a gene cluster produces an enzyme which alters the D-alanine in the pentapeptide of the murein precursor resulting in vancomycin being unable to bind to the cell (Klare *et al.*, 2003). This may suggest that the EO vapour is using antibiotic docking sites to affect the cell membrane. EOs are believed to disrupt and penetrate the lipid structure of the cell wall of bacteria, leading to denaturing of proteins and destruction of cell membrane leading to cytoplasmic leakage and cell lysis (Oussalah *et al.*, 2006).

The antimicrobial effect of EOs is thought to be due to a delicate balance of all the components of the oil and that no one individual chemical is responsible (Burt, 2004, Bakkali *et al.*, 2007). The assessment of the synergistic/antagonistic effect of individual components has not been reported widely in the literature, mainly thought to be due to the fact that the current tests used epsilometer test, time-kill, checkerboard and microgel diffusion assay are not that well suited to natural antimicrobials. One study tried to develop a method suitable for the assessment of natural antimcrobial synergistic interactions using nisin, lauricidin ™, totarol and lactoperoxidase system (LPS) against *L. monocytogenes, B. cereus, Strep. thermophilus, Staph. aureus, E. coli, S. typhimurium, Y. enterocolitica* and *P. aeruginosa.* A microtiter-based assay was developed, based on observing lag phase growth, reduction of culture density after 24 hours and residual viability at 24 hours, this method identified two novel synergistic combinations being nisin - lauricidin ™, LPS- nisin and LPS- lauricidin ™, the study suggested that this method could be effective in food-based systems (Dufour *et al.*, 2003). The results of this present investigation found that the combination of citral and linalool the most effective components tested had an antagonistic effect in oil form, yet they were indifferent in vapour form (Table 4.5). These results suggest that the blending of these components may result in the chemicals reacting together thus reducing their ability to penetrate the cells. However when the volatile components are evaporated they have different vapour pressure of 0.2 Pa at 23.5°C and a half life of 30 minutes and < 130 Pa at 40°C and a half life of 230 days for the isomer nerol and 106 days for geraniol, linalool and citral respectively (American Chemical Society, 2008). This means that the components in vapour form would be acting on *Enterococcus* sp. in combination much like they did in the individual assessments; with citral having the greater vapour pressure it is more volatile than linalool and thus would evaporate more

quickly from liquid form and act on the enterococci cells first. The assessment of the synergistic effect of salicyladehyde in the EO *Filipendula vulgaris* against a range of bacteria and fungi, showed that the combination of salicyladehyde and linalool demonstrated a synergistic relationship within the range of 60:40 and 80:20 (mol ratio). At a concentration of 1.7μg/disk, no microbial growth was observed; the pure compounds alone were shown to be less active, but the combination of methyl salicylate with salicyladehyde had an antagonistic relationship at a 40:60 (mol ratio), which resulted in a complete loss of activity (Radulovic *et al.*, 2007). Citral was found to reduce growth of *S. enterica* at an MIC of 3.4 (mM) by 20.29%, but when used in combination with thymol (MIC 1mM) and carvacrol (MIC 1mM) or thymol and geraniol (MIC 3.2mM) no growth was observed. Thymol was the compound recognised to have the main effect, thus adding a small amount of thymol is more effective than adding another compound (Nazer *et al.*, 2005). Studies on the synergistic/antagonistic effects of combinations of EOs highlights further the anitmicrobial activity of EOs *per se* is down to the unique combination of components within the oil. Therefore it may be more beneficial to concentrate further studies on the antimicrobial action of combinations of EO, rather than trying to discover the exact combination/ratios of antimicrobial components within the EOs, as the investigation of an orange/bergamot blend has shown them to be more effective in combination than individually and their major components citral and linalool to antagonistic when combined.

4.4.2 Effect of EO, Temperature and pH on Enterococcus sp.

Cell recovery from exposure to *Enterococcus* sp. was between $\log_{(10)}$ 4.5–5.5 dependent on the combination of temperature, pH & EO/vapour/component. The bacteriostatic effect of EOs is well documented and accepted (Helander *et al.*, 1998, Ultee *et al.*, 1999, Oussalah *et al.*, 2006, Burt, 2004, Bakkali *et al.*, 2007, Kim *et al.*, 1995a). The use of florescence in this investigation allowed for greater analysis of the bacteriostatic action and thus the ratio of live to dead cells was established (Table 4.5 & 4.6). A previous study using fluorescent probe carboxyflurescien (cFDA) was used to assess the viability and propidium iodide (PI) for live:dead ratios of lactic acid bacteria after exposure to the EO *Melaleuca armillaris*. The PI probes showed injured cells, which still had esterase activity, the damage to the membrane allowed the penetration of probe. Using 5 μg/ml of the EO against *L. casei* and *L. sakai* for 4 hours only 7.69% did not take the probe up, these levels dropped as the concentration of the EO was reduced, the use of cFDA showed that 97.87% of the cells were still viable. Cells stressed by the EOs were re-grown under optimum conditions and demonstrated a moderate re-growth of 0.027 h^{-1} demonstrating a bacteriostatic effect of the EO (Hayouni *et al.*, 2008).

The cell size of the *Enterococcus* sp. (1.3-1.7 μm) did not change with the application of the citrus blend in either oil or vapour form (Table 4.8), although other studies have shown a membrane swelling in *E. coli* and *Staph. aureus* when being subjected to tea tree oil (Cox *et al.*, 2000). EOs are known to cause damage to the cell wall leading to permeability and loss of cell content (Ultee *et al.*, 2002, Oussalah *et al.*, 2006, Fitzgerald *et al.*, 2004). Although it has been shown that when *L. monocytogenes*

is subjected to an MIC of 125 ppm of thyme EO there is a reduction in cell size this was thought to be associated with cell survival (Rasooli *et al.*, 2006b).

DNA content of *E. coli* varies among individual cells and to some degree is linked with cell size, but is only expected to vary between a two to eightfold increase within one genome, not to the extent observed within this study (Table 4.7) (Skarstad *et al.*, 1986). The increased levels of DNA may also be due to adaptation/survival of the cells against the EO blend as longer DNA fragments are sometimes required for adaptation, this can be limited to a few base pairs and does not explain the increases observed within this study (Townsend *et al.*, 2003). Geranial, an isomer of citral, has been shown to inhibit Caco-2 cell growth by reducing DNA synthesis, cells treated with geraniol for 24, 48 and 72 hours thymidylate synthase mRNA was reduced by at least 2 fold at all time points and reduced the resistance of the cells to 5-fluorouracil (Carnesecchi *et al.*, 2004).

The other possibility is that damage to the cell membrane has led to the cell being unable to replicate hence the lower number of viable cells in the samples (Table 4.6) but the production of DNA for replication has not been affected leading to increase DNA content in the cell. Taking doubling times into account i.e. 0.57 hrs and 0.46 hrs for *E. faecium* and *E. faecalis* respectively (Table 4.1), means that the organisms would have doubled in number under optimum conditions, approximately twelve times in the 6 hour period, equivalent to 3 $\log_{(10)}$ increase. The DNA content of the cells increases from 10^{-14} $\log_{(10)}$ to 10^{-11} $\log_{(10)}$ (Table 4.7) being a three-$\log_{(10)}$ increase, suggesting that this hypothesis may be correct.

In conclusion a blend of orange/bergamot EOs in both oil and vapour form has antimicrobial properties against both vancomycin resistant and susceptible strains of *E. faecium and E. faecalis* tested in this study and this is amplified when used with other hurdles to growth such as high / low pH, whereas stress from temperature appears to make the cells more resistant to the citrus EOs tested. The effect on the cells was bacteriostatic, whilst the size of the cell did not significantly change the DNA content increased when exposed to both oil and vapour, suggesting that the blend of EO had some effect on the metabolic processes within the cells With antibiotic resistant bacteria becoming an increasing issue in both the clinical situation and the food industry the low MICs/MIDs demonstrated by the orange/bergamot blend in this study suggest that orange/ bergamot blend may provide an alternative natural antimicrobial in both arenas.

Chapter 5

On-Food Studies

5.1 Introduction

5.1.1 Use of EOs as Antimicrobials in Food

The combination of antimicrobial properties and the aromas and flavours of EOs lend themselves to their use in food and has also led to research into the uses of EOs as a potential food preservatives although, considering their potential, studies involving citrus oils themselves have not been well documented. For EOs to have an antimicrobial application in food they must not only be safe for consumption but also reduce the initial microbial load during production to extend shelf life of the foodstuffs (Moreira *et al.*, 2005). High doses of EOs (0.05% v/v) have been shown to have a cytotoxic effect on Caco-2 cells and actually increase the damage to the cell population caused by *E. coli,* although medium to low dose (≤ 0.01%) of thyme, oregano and carvacrol over a short period cause no damage (Dusan *et al.*, 2006), which emphasises the necessity to find a balance between the risk of food poisoning and/or food spoilage and the dosage of EOs required to have an antimicrobial effect. There are a some of food preservatives on the market containing EOs. "DMC Base Natural" comprises of 50% EOs of rosemary, sage and citrus and 50% glycerol while "Protecta One and Two" is a solution containing a blend of herbs and EOs in solutions of sodium citrate and sodium chloride (Nedorostova *et al.*, 2008).

Fresh seafood has a short shelf-life of, on average, three days. If this could be extended, the product would be able to reach more distant, new markets. The

microflora of carp skin, gill and intestines has been shown to be reduced by citral and linalool at 20°C for 48 hours. Linalool was demonstrated to be more effective against bacteria isolated from the skin at concentrations of 2% (v/v). Inhibition diameters of 8mm, 2mm, 5mm and 8.3mm against *Acinetobacter* sp., *Enterobacteriaceae, Moraxella* sp. and *Vibrio* sp. respectively were reported whereas citral at 1% (v/v) and 2%(v/v) was effective against a wider range of bacteria isolated from the skin, gill and intestines of the carp (Mahmoud *et al.*, 2004). Citral (0.5-3% wv) has also been shown to be effective in fish cubes against *S. typhimurium* with reduction in the finial population of up to 1.5 $\log_{(10)}$ cfu g^{-1} (Kim *et al.*, 1995b).

A study testing orange and lemon dry powder at 5% (w/w) for their potential as antimicrobials in beef meatballs found them to be very effective against LAB over a 12 day period, both reducing the LAB load to under the detection limit of <1.69 $\log_{(10)}$ cfu/g. The meatballs were also tested for rancidity (TBA) at the end of the incubation period and the product with lemon extracts reached a higher TBA ($p < 0.05$) than the orange extract, thus the lemon extracts did not prevent rancidity as effectively as the orange extracts (Fernandez-Lopez *et al.*, 2005).

Chicken skin inoculated with 8.5 $\log_{(10)}$ cfu per $2cm^2$ with either *L. monocytogenes, B. cereus, Staph. aureus* or *E. coli* exposed to lemon, orange, bergamot EOs and their components citral and linalool for 10 min still has a microbial load of 5-6 $\log_{(10)}$, hence being unsuitable for consumption (Fisher and Phillips, 2006).

Taste panels showed that 1000 µl of orange oil was the maximal acceptable concentration per litre of skimmed milk. Orange, lemon, grapefruit, madrine,

terpeneless lime, orange, *d*-limonene, terpineol and geraniol were tested against *Salmonella senftenberg, E. coli, Staph. aureus* and *Pseudomonas* sp. Taste panels only found orange, lemon and grapefruit as acceptable for the addition to milk. From the screening of all the oils terpineol was deemed the most effective *in vitro* and therefore terpineol and orange oils were incorporated into pasteurised milk at a concentration of 1 000 µl per litre for 52 days at 4°C. The effect of the oil varied with the fat content of the milk, the reductions were: 7 $\log_{(10)}$ cfu/ml in skimmed milk, 4 $\log_{(10)}$ cfu/ml in low butterfat milk and 3 $\log_{(10)}$ cfu/ml in whole milk. In half cream/half milk and chocolate milk there were no differences between the control and test samples. Orange oil did not have as great an effect as the terpineol and was only effective in skimmed milk with a 1-2 $\log_{(10)}$ reduction. (Dabbah *et al.*, 1970). The possible reason that the oils may not be as effective in higher fat products is the higher concentrations of lipids present, creating a layer around the bacteria, thus prevent the oils from being absorbed into the cell (Holley and Patel, 2005).

Washes with either bergamot, linalool or citral to cabbage leaf reduce microbial loads by approximately 5-6 logs cfu/2cm^2 sample on a range of Gram-positive and Gram-negative bacteria including *Camp. jejuni, L. monocytogenes, B. cereus,* and *Staph. aureus.* The vapours of linalool and citral also reduced the microbial load of Gram-positive bacteria on cabbage leaf from 8.5 $\log_{(10)}$ per cfu/2cm^2 sample to 2.5 $\log_{(10)}$ up to 14 hours, although after this time there was no significant further reduction (Fisher and Phillips, 2006). The vapours of bergamot (2-0.125% v/v), linalool (0.25-0.06% v/v) and citral (0.125-0.03% v/v) tested against three isolates of *Arcobacter butzleri* on cabbage leaf reduced the microbial load from 7.5 logs per cfu per 2cm^2 to

undetectable levels in 200µl in both type and water isolates although there was no reduction in the chicken isolate (Fisher *et al.*, 2007).

The FDA's HACCP rule states that prior to packaging fruit juice microorganism levels must be below 5-$\log_{10}$ (Parish *et al.*, 2003). The use of terpeneless orange oil against *Salmonella* sp. at 25°C and 4°C storage temperatures would take 0.04 and 0.03 hours respectively to reduce the microbial load to an acceptable level to enable juice manufactures to comply to the FDA rule (Parish *et al.*, 2003). Unpasteurised fruit juices have a short shelf-life due to microbial enzymatic spoilage. Lemongrass and geraniol have been found to be effective against 10^6 cfu/ml *E. coli, Salmonella* sp. and *Listeria* sp. in apple, pear and melon juices at 35°C. The oils were most effective in apple juice with survival reduced to 3 $\log_{(10)}$ cfu/ml for *E. coli* and *Salmonella* sp. and 1.8 $\log_{(10)}$ for *Listeria* sp. (Raybaudi-Massilis *et al.*, 2006). When lemongrass and citral oils (0.5% w/w) were added to the edible films of aligante-apple and tested against *E. coli* 0157: H7. Citral was more effective than lemongrass with an inhibition zone of 49.8mm compared with 40.8mm, although it was noted that there was a significant reduction in tensile strength of the film with the addition of the EOs (Rojas-Grau *et al.*, 2007).

When 1 kg milk chocolate bars were inoculated with a mixture of exponential cultures of *E. coli* O157:H7, *L. monocytogenes* and *Staph. aureus,* EO added and samples stored at 7°C and a_w 0.450 or 20°C and a_w 0.340 for 24 hours, lemon oil was most effective against *E. coli* and reduced the microbial load by 1.7 – 1.8 $\log_{(10)}$ under both conditions. There was no difference between test and control strains when lemongrass was added, but during prolonged storage (5 days) there was an increased

effect against *Staph. aureus* of a 1.4 $\log_{(10)}$ reduction at 20°C in humidified environment (Kotzekidou *et al.*, 2007). Unlike fats carbohydrates are not thought to protect bacteria from the antimicrobial action of EOs (Shelef *et al.*, 1984).

Many studies have demonstrated that higher concentrations of EOs are required in food systems than *in vitro* investigations. As there are greater nutrient sources available this varies between food groups, leading to concerns about the effect of EOs on the organoleptic properties of the foodstuff (Smith-Palmer *et al.*, 2001, Fisher and Phillips, 2006, Burt, 2004, Holley and Patel, 2005, Fisher *et al.*, 2007).

Dispersion of the vapours other than natural evaporation in food models is another factor that must be addressed. The heating of oils can increase the evaporation rate but can also destroy or alter some of the components of the EOs and thus possibly affect the antimicrobial effect of the oils. It has been observed that the use of candles with different burning temperatures can affect the emission patterns of oils, as well as combustion-related emissions (CO_2 and CO) suggesting the need for fresh air intake during evaporation (Su *et al.*, 2007). The incorporation of the oils into candles as a means of dispersion has a different effect than that of just heating the oils as it is proposed that the ionised vapour molecules have a greater attachment to the bacterial cell membrane, increasing cell membrane permeability and then cell death (Gaunt *et al.*, 2005). The use of water to disperse oils has also been investigated with use of an air washer and citral. It was found that the phenolic compounds showed a lesser antimicrobial activity, thought to be due to their poor solubility in water. The water could also reduce their volatility as compounds with hydroxyl groups may be more solvated and remain in water phase (Sato *et al.*, 2006).

The other factor that must be considered with use of citrus EOs on food is that volatile components such as terpenes, *d*-limonene, hydrocardons, alcohols and ethers can play a role in the formation of secondary aerosols from interaction with oxidants, such as ozone, hydroxyl and nitrate radicals and form pollutants such as formaldehyde (Su *et al.*, 2007).

Peppermint and its component, menthol, have been shown to have an antiplasmid effect and, although this would not be a mechanism for the destruction of the cell, it does disrupt cell efficiency. The anti-plasmid effect means that extra-chromosomal DNA sequence cannot be shared among pathogens via the R-plasmid, to allow for the emergence of antibiotic resistant (EO resistant) strains (Schelz *et al.*, 2006).

6.1.2 Organoleptic properties

The use of citrus oils as antimicrobials in food might affect organoleptic properties of the foodstuff, so that finding an oil/component/vapour that has the greatest effect at the lowest concentration is essential. Thus, sensory tests need to be carried out and, although this can be done using instrumental analysis, ultimately such a test needs to be performed by trained individuals. Odour cross matching tests have been carried out on citrus oils to establish the contributory flavour compound that relate to food aroma (Chida *et al.*, 2006). Some components were found to be neutral including: limonene, l-octanol, dodecanal and therefore have no effect on aroma quality.

Citral is thought to be a potent character-impact compound and a key component in lemon oil, although linalool is the overall aroma compound in orange oil (Chida *et al.*, 2006). As oxygentated compounds have better organoleptic properties it may be beneficial to remove the terpene content (Arce *et al.*, 2007). EOs could be an alternative to spices and herbs, as they have many of the same principal flavourings but without the potential to be carriers of bacterial and fungal spores and, in their case, there probably would be no loss of organoleptic properties (Dorman and Deans, 2000). The use of citrus vapours in food may be a way of combating some of the negative organoleptic implications associated with EOs, as there would be no direct contact between the concentrated oil and the foodstuff, although alterations in aroma would still need to be assessed.

5.1.3 The Future

With demands from consumers to find alternatives to chemical based antimicrobials for food application, due to a trend in "green" consumerism, citrus EOs are potentially an ideal alternative. Comprehensive on-food studies are required to assess the changes of organoleptic properties of foodstuffs after the application of the EOs. The economical viability of EOs must also be reviewed as the mechanisms by which they produce their effects is not clear and there is a potential for resistant pathogens to arise (Hili et al., 1997). Should citrus EOs be applied to food they may be able to inhibit a wide range of organisms, but they could also cause an imbalance in gut microflora (Dorman and Deans, 2000).

Active compounds and toxicity should also be clearly documented before being used within the food industry or clinical arena (Rios and Recio, 2005). As demonstrated

recently by Dusan *et al* (2006) high doses of some EOs can have detrimental effects on intestinal cells and therefore the effect to the whole of the intestinal tract needs to be assessed before safe usage can be achieved. The use of citrus oils, that lend themselves to flavours within the food industry and are GRAS, therefore may be a good starting point for the use of EOs as antimicrobials within the food industry.

5.1.4 Aims and objectives

The overall aim of the investigation is to assess the potential on-food applications of a citrus EO blend (orange: bergamot 1:1 (v/v)) vapour against *E. faecium* and *E. faecalis* vancomycin susceptible and resistant strains.

The objectives are:

- To assess the use of the citrus blends vapours as an antimicrobial on cucumber and lettuce inoculated with *Enterococcus* sp.
- To determine the sensory changes to cucumber and lettuce after being exposed to the citrus blends vapours.
- To establish the components of the citrus blend present in the head space during the investigation.

5.2. Methods

5.2.1 Assessment of Heat and Fan Diffusion of Vapours

The 50:50 (v/v) blend of orange and bergamot at a final concentrations of 5, 10 or 15 mg/L air were diffused via either a heat or fan diffuser (AMPHORA, UK) within a 600L anaerobic unit (gas off) (miniMAC, Don Whitley Scientific, UK). BHI plates adjusted to pH 5.5, 7.5 or 9.5 were spread with 0.1µl of an overnight culture of either vancomycin susceptible or resistant *E. faecium* or *E. faecalis* and placed in the unit for 24 hours at either 37°C or 25°C. Inhibition was assessed by the observation of the plates with the least growth and the most effective diffuser (heat) and concentration (15mg/L air) were used in the following experiments.

100mls of BHI broth (pH 7) was inoculated (method 3.1.1) with either vancomycin susceptible or resistant *E. faecium* or *E. faecalis* and incubated at 37°C or 25°C in the 600L vapour chamber with 15mg/L air of the EO orange:bergamot blend being diffused by the heat diffuser to give a final concentration of 15 mg/L air. Samples were taken every 15 minutes for the first hour and then hourly for the next eight hours and again at 24 hours, plated using a spiral plater onto BHI agar and incubated for 24 hours at 37°C.

The control was the use of the diffusers without any EO blend added. The unit was sterilised with the use of a UV lamp for a 24 hour period prior to use.

5.2.2 On-food Vapour Model using Lettuce and Cucumber

Squares of 2cm x 2cm of iceberg lettuce leaves or cucumber skin were taken and left under UV light for 30 minutes to remove any competing microflora. The samples were inoculated with 50 µl of a overnight culture of either vancomycin susceptible or resistant *E. faecium* or *E. faecalis,* diluted with BHI broth to 10^5 or 10^8 cfu/ml and left to dry for 20 minutes. The inoculated food samples were placed in a 600l vapour chamber and subjected to 15mg/L air final concentration of the orange/bergamot blend vapour via a heat diffuser at 25°C for 15, 30, 45 and 60 seconds after a 15 min accumulation of vapour within the chamber. Food samples were then placed in 10 ml of sterile PBS solution, stomached for 30 seconds, plated onto BHI agar using a spiral plater and incubated for 24 hours at 37°C. Un-inoculated samples after the UV treatment were also placed in 10ml of PBS, stomached and plated to assess the remaining contamination levels. After sensory evaluations had been carried out (method 5.2.4), this experiment was repeated with an initial innoculum on the food-stuff of 10^5 cfu/ml and a final concentration 30 mg/L air of the blend vapour.

5.2.3 Determination of Survival on Lettuce and Cucumber

Food was prepared and inoculated as before with overnight cultures containing 10^5 cfu/ml of either vancomycin susceptible or resistant *E. faecium* or *E. faecalis*. The food was then placed in the vapour chamber after a 15 min accumulation of 15mg/L air (final concentration) of vapour of the orange/bergamot blend for 45 seconds at 25°C.

Food samples were then spiral plated and incubated as previously immediately after being in the vapour chamber and after 6, 8, 10, 24 and 48 hours exposure.

5.2.4 Sensory Evaluation

Cucumber skin and lettuce leaves were exposed to a final concentration of 15mg/L air of the orange:bergamot blend vapour for 45 seconds after 15 minutes accumulation of the vapour. A triangle forced choice procedure was carried out using a sensory panel consisting of 28 participants who were randomly chosen from a college population consisting of both students and staff, all were non experts in sensory properties of food. Participants were given three samples of foodstuff, one of which differed from the others i.e. two controls and one test piece or two test pieces and one control and asked to select the sample that tasted different. In order to determine if the panel could distinguish between the foodstuff that had been subjected to the vapour and the controls.

5.2.5 Mass Spectrometry Analysis of Vapour Component Changes Over Time

Mass spectrometry (MS) was used to assess the components present in the air at a final concentration of 15mg/L air of the blend vapour diffused via a heating element in a 10 L Tedlar bag (232-08, SKC, Dorest UK) over 25 minutes at 25°C ± 2°C. A Kore technologies 500 mass spectrometer system (Cambs, UK) with a scan interval of 5 seconds. The pressure of the MS was maintained at 1.35 mb, and was regulated by a needle value. The sample introduced at a 1 in 10 dilution at 23°C. The pressure of the discharge of the ion source (hollow cathode) was 1.85 mb to allow the water vapour

injected to cause the initial hydronium formation. Changes in quantities of the vapour components were monitored over time.

5.2.6 Gas Chromatography – Mass Spectrometry Identification of Vapour Components:

A 50ml syringe was used to extract air from the headspace of the mass spectrometry analysis method (section 5.2.5) at 15 minutes and dissolved in 10ml of water; 1 μl was injected (split injection 50:1) into a Perkin-Elmer 'Turbomass' GC-MS with an injector temperature of 250°C. The capillary column used was a Zebron ZB-5 with a column length of 30 m and diameter of 0.25 mm i.d., the carrier gas used was helium at a flow rate of 1 ml per min^{-1}. The intial temperature was 40°C for 3 minutes, with a ramp temperature of 8°C min^{-1} and a final temperature of 240°C for 2 minutes, carried out for a total of 30 minutes. For GC/MS detection, an electron impact (EI) ionisation system, with ionisation energy of 70 eV with a M/z range of 50 to 450 Da, with a scan interval of 0.55 seconds, interscan delay of 0.15 seconds and a solvent delay of 3 minutes. NIST05 and AMDIS32 libraries were used for identification.

5.2.7 Statistical Analysis

This was carried out according to section 3.1.3

Growth in the Presence of Vapour: It was established at what time period there was a significant difference in growth of *Enterococcus* sp. when being subjected to 15 mg/L air of vapour via a heat diffuser at 37°C and 25°C.

Growth On-food: Significant differences between reduction of viable counts of *Enterococcus* sp. on lettuce and cucumber over different time periods were established.

Survival On-food: It was determined if there were significant differences in the survival of *Enterococcus* sp. over a 48 hour time period.

Sensory Evaluation: $\propto$ risk was calculated with a significance level set at p = 0.05 to determine if there was any significant difference in taste between treated and untreated lettuce and cucumber.

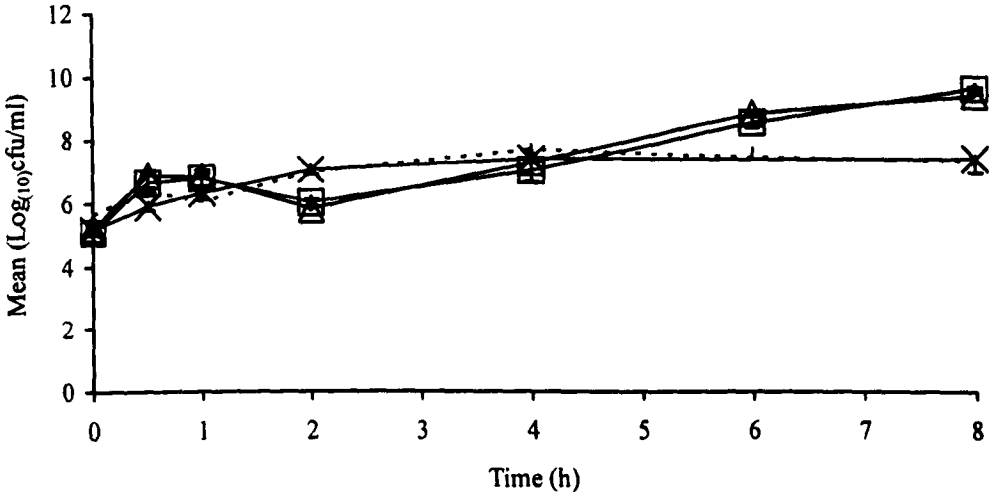
5.3 Results

The vapours demonstrated inhibition of growth against *Enterococcus* sp. but this was only at 50°C (section 4.3.1) which has limited application within the food industry. Therefore diffusers were used to try to reduce the temperature at which the vapours produced their inhibitory effect. There was no inhibition of growth of any of the *Enterococcus* sp. when using 5, 10 and 15 mg/L air of vapour via a fan diffuser or 5 and 10 mg/L air of vapour via a heat diffuser at either 25°C or 37°C at pH 5.5, 7.5 or 9.5. Using 15 mg/L air of the blend vapour at 25°C or 37°C, at pH 7.5 some inhibition was observed. Higher concentrations of the blend were not used as it was deemed that it probably would affect organoleptic properties of the food-stuff.

Growth of *Enterococcus* sp. whilst being exposed to 15 mg/L air blend vapours is shown in Fig. 5.1 and 5.2. At 25°C there was a significant difference in growth compared with that of the controls (those cells not being exposed to the vapour) for 15 – 30 minutes exposure against all strains with a ANOVA statistical value of $F(16) = 5.527$, $p = 0.001$, and post hoc analysis $t(10) = 13.537$, $p \leq 0.001$ for 15 minutes and $t(9) = 3.855$, $p \leq 0.001$ for 30 minutes (Fig. 5.1b & 5.2b) with a reduction in growth from the controls ranging from 0.2 – 1.1 $\log_{10}$ at 15 and 30 minutes respectively. A significant decrease is observed for the first hour in all strains at 37°C, with an ANOVA statistical value of $F(16) = 1.164$, $p = 0.008$ and post hoc analysis of $t(10) = 26.207$, $p \leq 0.001$ in both the vancomycin resistant and susceptable strains (Fig. 5.1b & 5.2b). With reduction in growth of the control cells to those being exposed to the blend vapour ranging from 0.65 – 0.88 $\log_{10}$ at 15 minutes. Overall, both the vancomycin susceptible

and vancomycin resistant *E. faecium* and *E. faecalis* strains had significant changes in growth from 15 minutes, thus accumulation of 15 minutes of vapour was used in the following investigation.

a)



b)

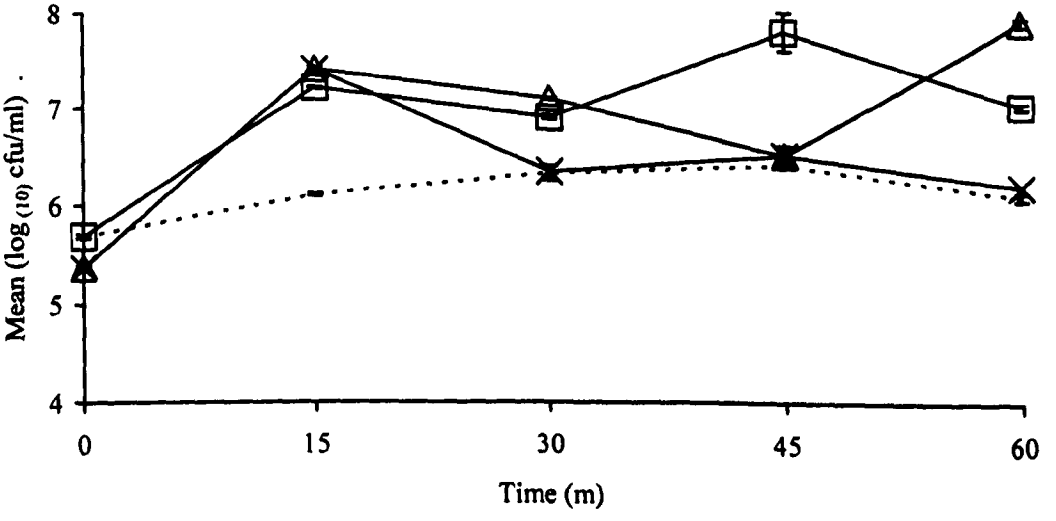
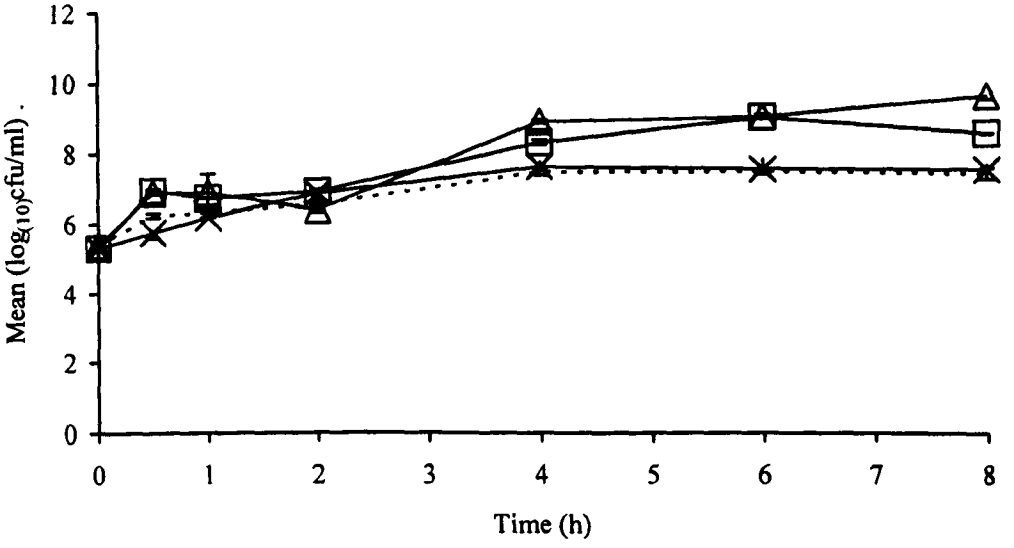


Figure 5.1: Growth of vancomycin susceptible *E. faecalis* and *E. faecium* in the presence of orange/bergamot vapour (15mg/L air) at pH7.5 at a) 37°C and b) 25°C (± SD, n = 6). *E. faecalis* in the presence of vapour (·····), *E. faecium* in the presence of vapour(-✱-), *E. faecalis* no vapour (-□-) and *E. faecium* no vapour (-△-)

a)



b)

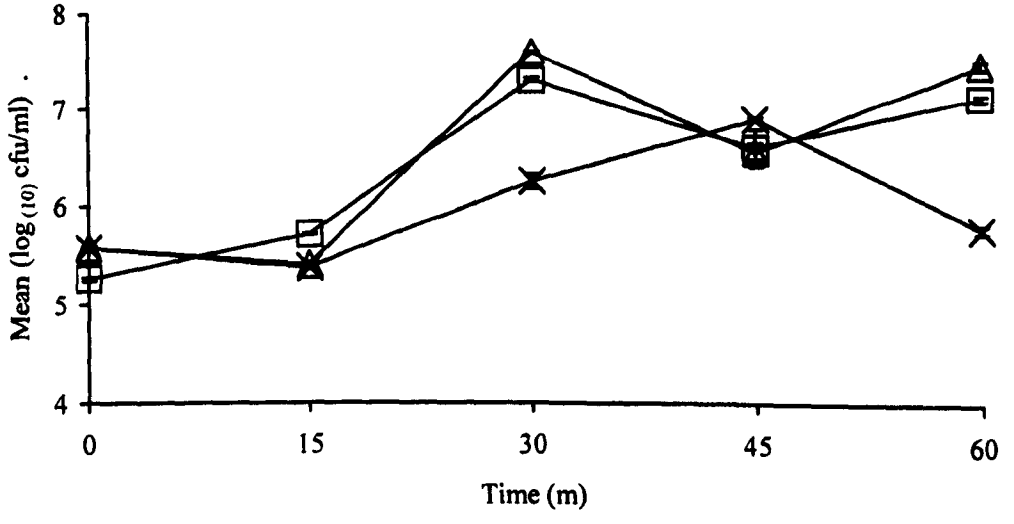
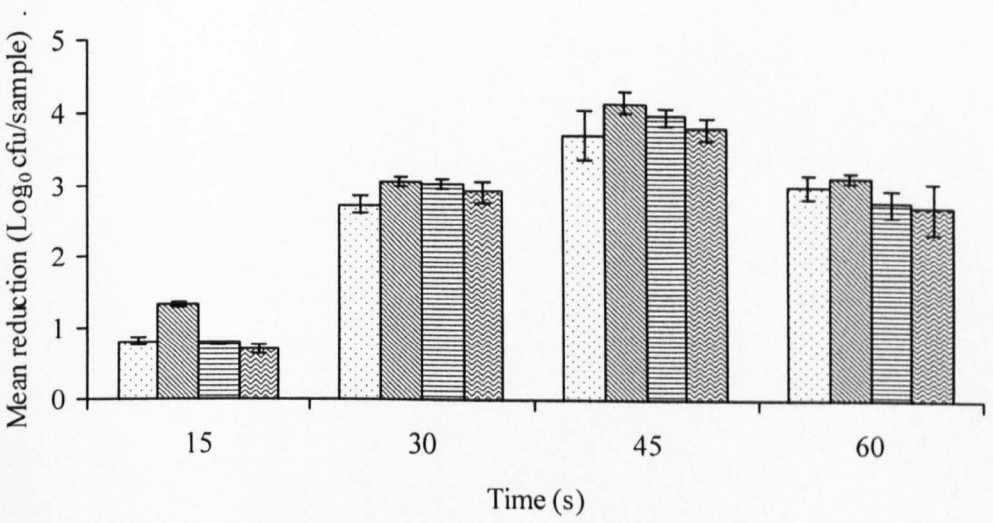


Figure 5.2: Growth of vancomycin resistant *E. faecalis* and *E. faecium* in the presence of orange/bergamot vapour (15mg/L air) at a) 37°C and b) 25°C (± SD, n = 6). *E. faecalis* (12203) in the presence of vapour (·····), *E. faecium* (12202) in the presence of vapour (✳), *E. faecalis* (12202) no vapour (□) and *E. faecium* (12203) no vapour (△)

On–food tests were carried out at 25°C as this was deemed to be a temperature that had greater relevance to the food sector generally. The greatest reduction of growth at both 5 and 8 $\log_{10}$ initial inocula (after 15 minutes accumulation of vapour) occurred at 45 seconds showing a significant difference to the other time points with an ANOVA statistical value of $F(23) = 1745.815$, $p \leq 0.001$ for 15 seconds $t(19) = -40.697$, $p \leq 0.001$, 30 seconds $t(19) = -60.227$, $p \leq 0.001$ and 60 seconds $t(19) = -75.531$, $p \leq 0.001$, with an overall effect size of r = 0.979 and r = 0.977 on cucumber skin and lettuce leaves respectively. A reduction of microbial load on cucumber skin ranged from 3.69 – 4.14 $\log_{10}$ and 1.05 – 2.02 $\log_{10}$ and on lettuce from 3.80 – 4.40 $\log_{10}$ and 1.40 – 2.37, from $\log_{10}$ 8 and 5 $\log_{10}$ initial inocula respectively (Fig. 5.3 & 5.4). There was a 99% reduction when the initial inoculum was 5 $\log_{10}$ and > 99.9% for 8 $\log_{10}$ on lettuce and cucumber after 45 seconds exposure to the blend vapour. There was no significant difference in reduction of microbial load between cucumber skin and lettuce leaf after being exposed to the orange/bergamot blend vapour at an initial innocula of either $\log_{10}$ 8 or 5 $\log_{10}$, $t(19) = 0.5183$, $p = 0.02$, or between the effect on the vancomycin resistant and susceptible strains $t(19) = -0.0967$, $p = 0.997$ for *E. faecalis* and $t(19) = -0.1033$, $p = 0.996$ for *E. faecium*.

a)



b)

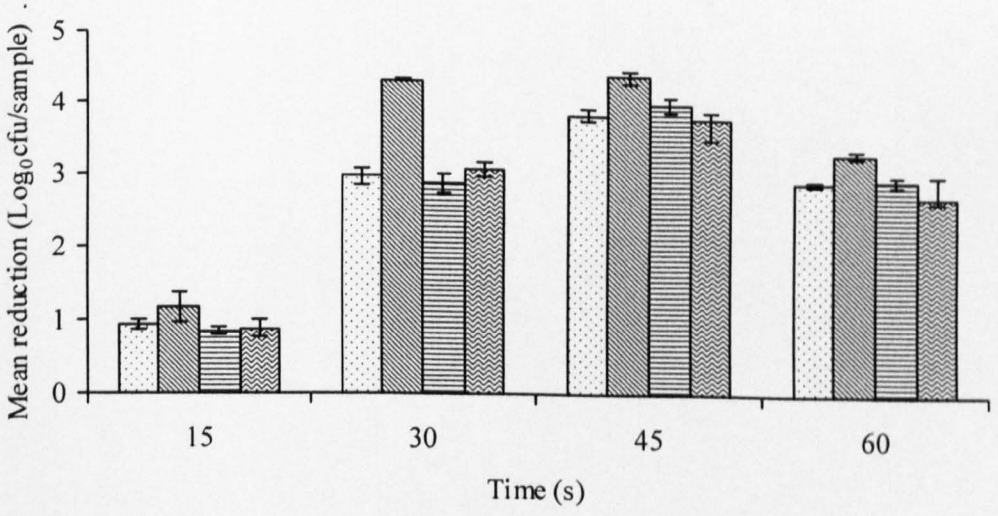
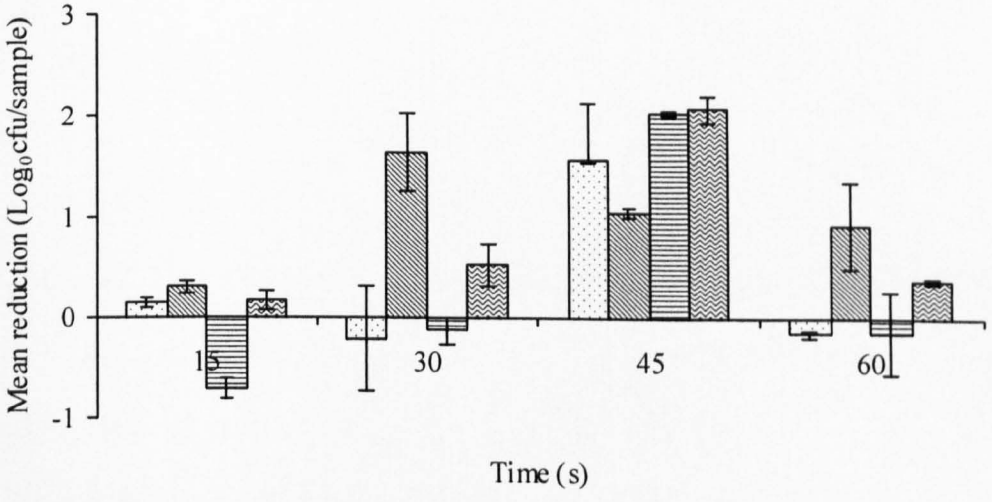


Figure 5.3: Reduction in cell numbers from 8 $Log_{(10)}$ cfu/2cm^2 sample of *Enterococcus* sp. in the presence of 15 mg/L air orange/bergamot vapour on a) cucumber skin and b) lettuce leaf (± SD, n = 6). *E. faecalis* ☐ , *E. faecium* ▨ , *E. faecalis* (12203) ▤ and *E. faecium* (12202) ▨

a)



b)

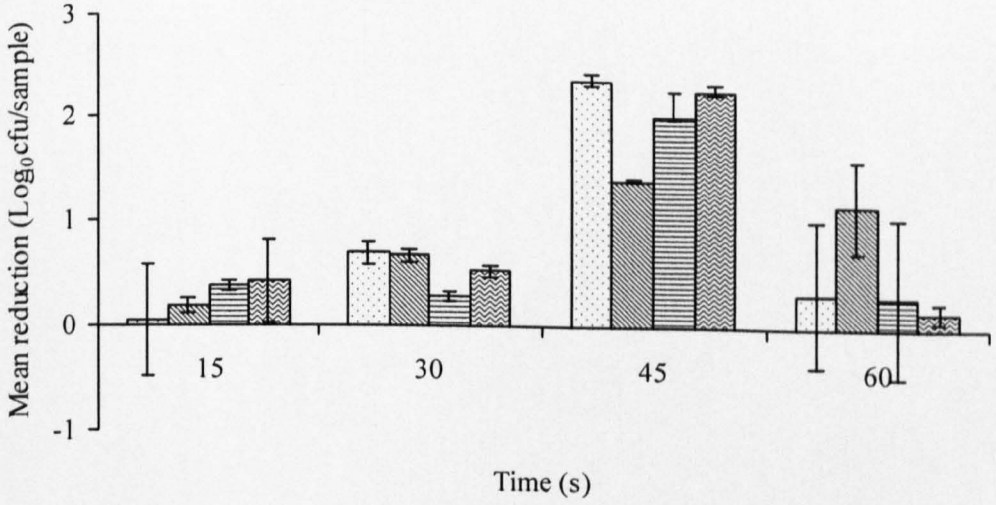


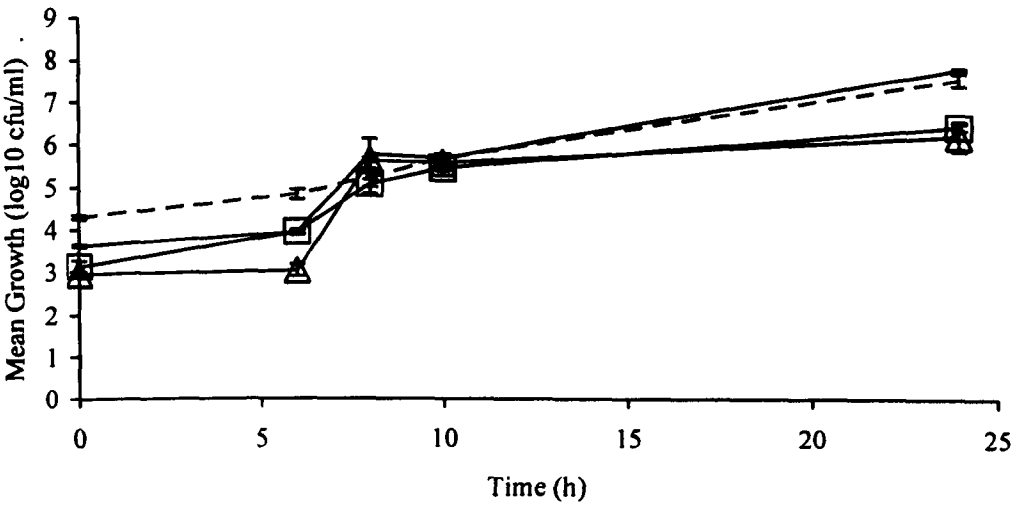
Figure 5.4: Reduction in cell numbers from 5 cfu/2cm^2 sample of *Enterococcus* sp. in the presence of 15 mg/L air of orange/bergamot vapour on a) cucumber skin and b) lettuce leaf (± SD, n = 6). *E. faecalis* ⬚ , *E. faecium* ▨ , *E. faecium* (12203) ▤ and *E. faecalis* (12202) ▨ .

The cells of both the vancomycin resistant and susceptible strains on lettuce leaves and cucumber skin begin to recover from the effect of the blend vapour after 6 hours after which there is no significant difference between those cells that have been exposed to the vapour and the controls with a ANOVA statistical value of $F(22) = 125.084$, $p = 0.001$, and post hoc analysis $t(19) = 0.1992$, $p = 0.753$. Generations per hour calculated from Figure 5.5 show that the growth rate of *E. faecium* and *E. faecalis* has slowed by 81.5% and 79.1% respectively between 45 seconds and 6 hours after exposure and by 57% and 97% for vancomycin resistant *E. faecium* (12202) and *E. faecalis* (12203) respectively. In the case of vancomycin susceptible *E. faecalis* the growth rate between 6-8 hours after exposure actually accelerates to above that of the cultures that have not been exposed to the vapour by 18% whereas the vancomycin susceptible *E. faecium* and vancomycin resistant *E. faecalis* (12203) cells return to approximately the same generations per hours as the control cells. For vancomycin resistant *E. faecium* (12202) the numbers of generations per hour is still 35% less than that of the control untreated cells.

Table 5.1: Mean Generations per hour of vancomycin susceptible *E. faecalis* and *E. faecium* and vancomycin resistant *E. faecalis* (12203) and *E. faecium* (12202) on lettuce and cucumber after being exposed to 15 mg/L air of vapour for 45 seconds (n = 6).

	Control	Test	Control	Test
Time after exposure	45 sec -6 (hrs)		6-8 (hrs)	
E. faecalis	0.97	0.18	0.86	1.01
E. faecium	1.41	0.29	1.47	1.45
12202	1.45	0.44	0.97	0.63
12203	1.7	0.05	1.45	1.43

a)



b)

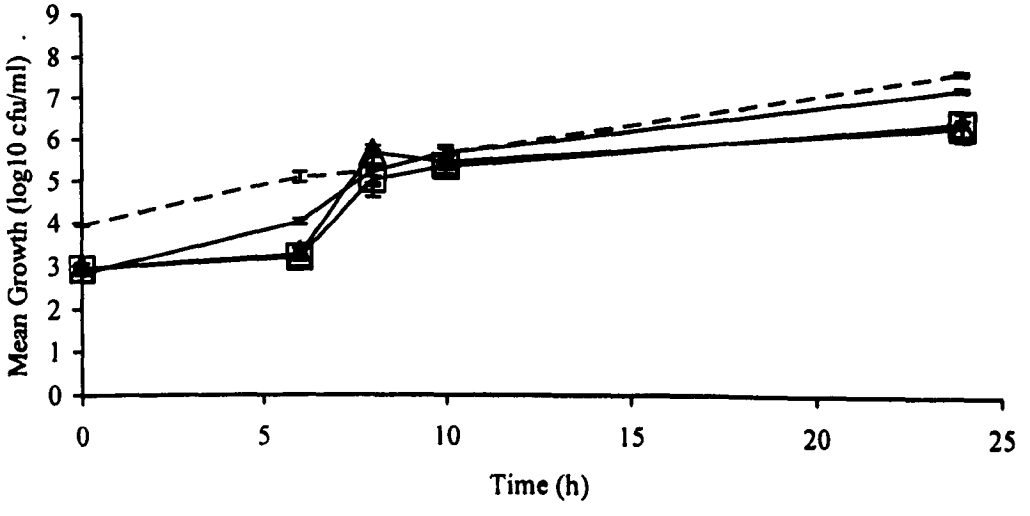


Figure 5.5: Growth of *Enterococcus* sp. on a) cucumber and b) lettuce over 24 hours after being exposed to a 15 minute accumulation of 15 mg/L air orange/bergamot vapours for 45 seconds (± SD, n = 6). *E. faecium* (.....), *E. faecalis* (-□-) , *E. faecium* (12202) (—) and *E. faecalis* (12203) (-▲).

Analysis of changes in the volatile components of the blend vapour headspace showed that, of the chemicals identified by GC-MS (Table 5.1, Appendix IIII) over a 25-minute period, there were no changes in the counts per minute of the components except for methanol, which showed a drop of between 12 – 35% from 14 to 18 minutes. The GC-MS showed that the highest counts per minute consisted of the alcohol based components such as methanol, ethanol and acetone and there was also a large peak at 136 & 154 m/z where a combination of some of the more specific components of citrus oils were observed, such as limonene, citral and linalool (Table 5.1, Appendix IIII).

Table 5.2: GC-MS head space analysis of the major components in the air after a 15-minute accumulation of the orange/bergamot vapour.

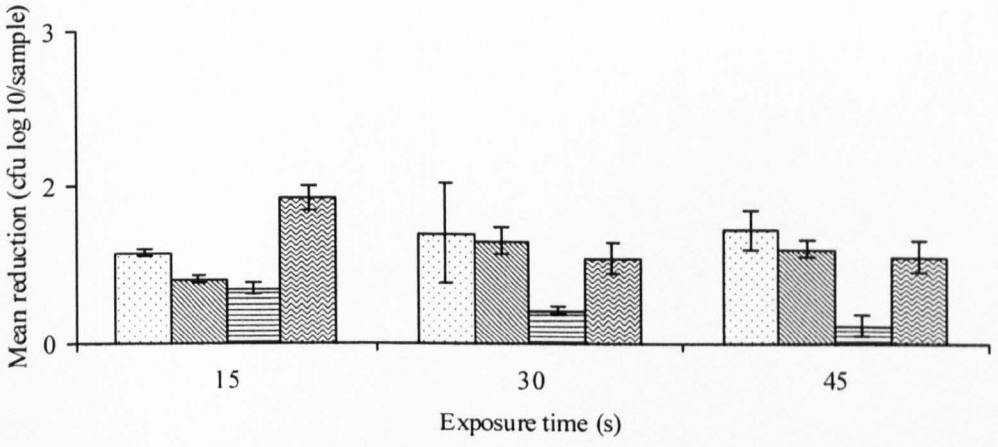
Components	m/z	Counts/min
Methanol	33	12790
Ethanol	47	3910
Acetone	59	716820
Isopropanol	60	22360
Fluoroacetic acid	77	45050
amine		
Trimethylsilyl fluoride	92	580*
Dimethylsilanediol	92	580*
3-heptanone	114	430
Butylacetate	116	1100
n-octanal	128	570
p-cymene	134	3420
Limonene	136	544400*
Alpha-pinene	136	544400*
Alpha-phellandrene	136	544400*
Camphene	136	544400*
Thujene	136	544400*
Beta pinene	136	544400*
Myrcene	136	544400*
Carene	136	544400*
Nonanal	142	360
Nonanol	144	360
Citral	154	1860*
Linalool	154	1860*
1-Fluorododecane	188	270*
Bergamol	196	260
Linalyl isobutyrate	224	250

*Combined peak for all components of the same molecular weight.

There was no significant difference in the taste for either lettuce leaf or cucumber skin after exposure to 45 seconds of the blend vapour with an alpha risk value of $N(35)$, $p = 0.5157$ and $N(28)$, $p = 0.6228$. Due to there being no significant difference in taste between the test and control samples, higher dosages of the vapours were investigated to assess whether this would reduce the microbial load of the foodstuff further, or reduce the length of exposure needed.

Overall 30 mg/L air of vapour was less effective than the 15 mg/L air dosage at all time intervals (Fig.5.6). Compared with the 15 mg/L air (Fig. 5.4) with an initial inoculum of $\log_{10}$ 5 reductions of vancomycin resistant *E. faecalis* (12203) 2.02, 0.37 and 2.03, 0.52 $\log_{10}$ *E. faecium* (12202) 2.06, 0.66 and 2.03, 0.52 $\log_{10}$ and vancomycin susceptible *E. faecalis* 1.58, 0.87 and 2.37, 0.86 $\log_{10}$ and *E. faecium* 1.05, 0.69 and 2.37, 0.86 $\log_{10}$ for 15 and 30 mg/L air on cucumber skin and lettuce leaf respectively. Therefore doubling the dosage of the vapour does not have an exponential relationship with reduction in growth of *Enterococcus* sp. on lettuce leaf and cucumber skin.

a)



b)

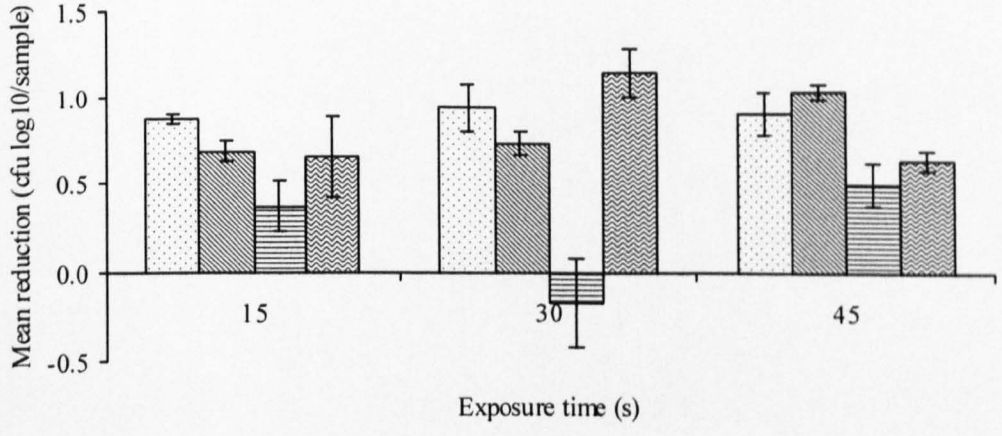


Figure 5.6: Reduction in cell numbers from an initial inoculum of 5 $Log_{(10)}$ cfu/2cm^2 sample of *Enterococcus* sp. in the presence of 30 mg/L air of orange/bergamot vapour on a) cucumber skin and b) lettuce leaf (± SD, n = 6). *E. faecalis*□ , *E. faecium* ▨ *E. faecalis* (12203) ▤ and *E. faecium* (12202) ▨.

5.4 Discussion

The use of a heat diffuser allowed the use of the blend EO vapour at lower temperatures (25°C). The use of nonthermal processing of foodstuff has recently been encouraged due to the adverse effects that heat can have on the organoleptic properties and nutritional value of the food as well as consumer trends changing from that of long life products to that of fresh, natural, minimally processed foods (Ross *et al.*, 2003). Only in recent years has the association of foods from origins other than animal been associated with foodborne illness, demonstrating that foodborne illness can arise from consumption of contaminated salads, fruits and vegetables (Little and Gillespie, 2008). Between 1992 and 2006, 2274 outbreaks of food poisoning were reported in England and Wales, 4% of which were associated with the consumption of prepared salads. Fresh salad, vegetables, or fruit may become contaminated from environmental sources, This is of concern because the organic sector of the food market has a retail value of £223M and is one of the fastest growing food sectors with an increase of 30% per annum from the 1990s to 2000. Although this has now began to slow to 15%, thought to be due to downward price pressures from the supermarket supply chain, if the Organic Action Plan Target is to be met with 70% of the indigenous organic products to be sourced from the UK by 2010, progress within the sector is required including the use of natural antimicrobials that can go some way in reducing the contamination and post-harvest decay of salads, fruits and vegetables (DEFRA, 2006).

The effectiveness of the EO blend in vapour form at decontaminating cucumber skin and lettuce leaf reducing microbial load by >99.9% with a large effect size of r = 0.97 (Fig. 5.3 & 5.4), meets DEFRA's requirements for a natural antimicrobial that could increase the output of the organic salad/vegetable sector due to possible

application as an antimicrobial against post harvest and spoilage organisms, as well as food poisoning pathogens. To date the safety of these products is based on correct-chill chain and hygienic practices which are both hard to implement and control and are not sufficient for the expanding market (Lanciotti *et al.*, 2004). Current research shows that the use of washing and sanitising methods are only capable of reducing microbial load by 90 – 99% (Allende *et al.*, 2006). Until recently the use of EOs as antimicrobials on-food has mainly been evaluated in oil form, despite EO vapours being shown to have antimicrobial properties against a range of pathogens (Inouye *et al.*, 2003, Inouye, 2003, Inouye *et al.*, 2001, Nedorostova *et al.*, 2008, Gaunt *et al.*, 2005) and their potential to overcome the concerns of the use of EOs in food because of their potent aromas and possible effects on the organoleptic properties of the food stuff.

The assessment of the vapours of eucalyptus and cinnamon EOs at concentrations of between 50–500 ppm against postharvest pathogens on fresh produce (strawberries, tomatoes) demonstrated that after exposure to the vapours for eight hours and transfer to ambient temperatures the fruits maintained a low severity of decay ($p < 0.05$) compared with the controls. However the vapours had an effect on the organoleptic properties of the foodstuff. Eucalyptus treated tomatoes had a 19% decline in firmness of the fruit, and there was a 1.1% weight loss in tomatoes exposed to both eucalyptus and cinnamon, although there was no change in fruit colour or aroma (Tzortzakis, 2007). The results of this present investigation demonstrate there to be no significant differences in the taste of the lettuce leaf and cucumber skin after being exposed to the blend vapour and citrus essential oils are also recognised as GRAS (Food and Drug Administration, 2005). Citrus EOs of mandarin, cider, lemon and lime have been shown to increase the shelf life of minimally processed fruit salads by reducing *E.*

coli levels at 13°C by 10^6 cfu/ml without changing any of the sensory properties (Lanciotti *et al.*, 2004).

In previous studies the use of citrus EOs (orange, lemon, bergamot, linalool and citral) in food was shown not to be as effective on chicken skin as on fresh produce (cabbage leaf), hence the use of fresh vegetable/salad produce in this investigation. The oils became effective against the pathogens on cabbage leaf after 15 seconds exposure and this inhibitory effect continued for 60 seconds, with a 5–7.5 $\log_{10}$ reduction (Fisher and Phillips, 2006). This is unlike the effect observed with the blend of EOs vapours in this investigation where a specific time point (45 seconds) was effective and within 15 seconds of further exposure the inhibition of *Enterococcus* sp. was reduced, the cells appeared to be recovering from the effect of the vapour (Fig. 5.3 & 5.4).

In-vitro experiments at 37°C show significant inhibition of growth of *E. faecalis* and *E. faecium* for the first eight hours of exposure to the vapour of the orange/bergamot blend only (Fig. 5.1 & 5.2). However when the temperature was reduced to 25°C this time period was reduced to one hour, which may be due to the increase in kinetic energy at higher temperatures. This may partially explain why the heating element was more effective than the fan diffuser at reducing the temperature needed for the blend vapour to be effective. An assessment of evaporating EOs (lavender, eucalyptus and tea tree) on the indoor air quality over 180 minutes, found that within the first 20 minute of evaporation the emissions of the volatile organic compounds occurred, with lowest levels of bacteria in air at 30 minutes after evaporation began. After this time period the levels of bacteria began to increase and

this phenomena also occurred with airborne fungi (Su *et al.*, 2007). The results of this present study agree with the findings of the Su *et al.* (2007) investigation in that the blend of EO vapour was most effective against *Enterococcus* sp. up to 15 minutes of exposure (Fig. 5.1 & 5.2). This may be due to the emission of all the antimicrobial volatile organic compounds and thus the effect diminishes after this time period. The increase of airborne bacteria/fungi after the 30 minute period as demonstrated by Su *et al.* (2007) is similar to the 45 second specific time period observed against *Enterococcus* sp. on lettuce leaf and cabbage skin in that the EO vapours have specific time periods for which they have an antimicrobial effect (Fig. 5.3 & 5.4).

The evaluation of the changes in emission of the volatile components within the air over 25 minute showed that of the components identified there was no change in counts/min other than a decrease in methanol between 14 to18 minutes, although an exact breakdown/separation of the components at 136 & 154 m/z would be needed to analyse this fully. The components identified (Table 5.1) were similar to previous studies on the composition of citrus EOs (Caccioni *et al.*, 1998, Satake *et al.*, 2004, Moufida and Marzouk, 2003).

One drawback of using the blend vapour as a food antimicrobial is that in this study the inhibitory effect only lasted for 6 hours after exposure (Fig. 5.5) during which time the growth of the *Enterococcus* sp. is significantly slowed by approximately 80%. However, between six and eight hours growth rates returned to normal or were even accelerated, which results suggests that the application of the vapours should be as close to consumption as possible. Other studies have shown by increasing the MIC of the oils the bactericidal effect was greater thus limiting the amount of recovery of the cells. The

use of tea tree oil against *E. coli* and *Staph. aureus* had MICs of 0.25% and 0.125% but a minimum bactericidal concentration (MBC) of 0.5% and 0.25% respectively. Reduction in viability in *Staph aureus* between the MIC and MBC was approximately 3 $\log_{10}$ over 120 minutes and cell respiration in *E. coli* was slowed at the MIC but completely inhibited at the MBC (Cox *et al.*, 2000). The main concern with increasing the MID was the effect of the blend on the organoleptic properties of the foodstuff, which after sensory evaluation showed not to be the case. When the dosage of the vapours was doubled (Fig. 5.6) there was no corresponding increase in the effect and the higher dosage was actually less effective by approximately 0.5 $\log_{10}$ with an initial load of 10^5. One reason might be that the vapours had reached a saturation point at which they were active against the cells and so the maximum effect was seen at 15 mg/L air.

The results of this present investigation show promise for the use of the citrus blend vapours as antimicrobials on fresh fruit, vegetable and salad produce but considerations must be made for the safety of the product. Although citrus EOs *per se* are GRAS, studies have shown that the volatile monoterpenes of the EOs, which have at least one unsaturated carbon-carbon bond can react with oxidants such as ozone, hydroxyl and nitrate radicals. As well as terpenes such as limonene and linalool containing high molecular weight oxidation products (aldehydes, ketones and organic acids) the formation of chemicals like formaldehyde can occur when these terpenes and oxidants react, thus creating secondary pollutants (Su *et al.*, 2007).

Due to the orange/ bergamot EO blend vapour being effective at 25 °C, it might have application during processing/storage/transit or modified atmosphere packaging

(MAP) against an array of pathogens on food. There was no significant difference in the decontamination levels of the foodstuff between vancomycin susceptible and resistant *E. faecalis* and *E. faecium* and therefore the citrus vapours may also have application within the clinical arena. The evaluation of the recovery of the cells and the possibility of secondary pollutant formation needs to be assessed before the citrus blend vapour as a product could be a viable option for industry. However, with changes in consumer trends and government legislation this investigation goes some way in addressing the constant hurdle of the food industry against microbial contamination of fresh foodstuff.

Chapter 6

Surface Decontamination

6.1 Introduction

6.1.1 Clinical VRE Control

Vancomycin resistant *Enterococcus* is able to survive in the environment and in humans with the length of human colonisation ranging from seven weeks to three years. Transmission of VRE can occur through direct contact with infected individuals, indirect contact with health care workers (HCW) or relative's hands or via contaminated patient care equipment or environmental surfaces The most common mechanism of transmission is thought to be from the hands of the HCW, but the control of cross contamination from environmental surfaces is paramount. It has been shown that strains isolated from patients during monoclonal outbreaks are genetically identical to those isolated from surfaces (Tacconelli and Cataldo, 2008). Enhanced control measures have been shown to eradicate the outbreaks of *vanB* phenotype VRE (*E. faecium*) in a 1600 bed tertiary care institution where the main factor of the control regime was cleaning contact areas such as walls, furniture, mattresses, sinks and doorknobs. A two-step procedure was used where the environments were disinfected twice a day with quaternary ammonium detergent then a phenolic based disinfectant (Kurup *et al.*, 2008).

The spread of VRE is a priority for Hospital Infection Control Practices Advisory Committee (HICPAC), as the vancomycin resistant genes can be transferred to other Gram-positive organisms such as *Staph. aureus* via the *vanA* gene which is often plasmid-borne. The other major concern is the ease with which VRE spreads

through a variety of transmission routes. The recommendations by the HICPAC are prudent use of vancomycin, educational programmes for HCW, the role of laboratories in detection and reporting of VRE and prevention and control of nosocomial transmission. The latter includes ensuring that hospitals have adequate procedures in place for the cleaning and disinfecting of environmental surfaces, with a Environmental Protection Agency (EPA) registered detergent-disinfectant (The Hospital Infection Control Practices Advisory Committe, 1995). These recommendations were used in a French university hospital and the results demonstrated that this aggressive multifaceted strategy resulted in reductions in outbreaks of VRE over a four-week period. The positive VRE cultures isolated within the hospital reduced from 70% to 20% in week one, to 18.8% and 1.6% in weeks two and three respectively. The results of this study shows that early intervention influences effective control of hospital outbreaks of VRE (Lucet *et al.*, 2007). Studies have also shown that aggressive routine environmental cleaning can actually contribute to the prevention of VRE outbreaks in the first place. This is vital as most studies show that once VRE are introduced to an environment they usually quickly become endemic (Boyce *et al.*, 1994, Montecalvo *et al.*, 1999). One hospital requested feedback from their housekeeping staff on new aggressive cleaning practices and their constructive comments was associated with significant reduction in the environmental contamination of VRE (Tacconelli and Cataldo, 2008). The rapid emergence of VRE in 1997 in the USA lead to a yearly screening between 1997-1998 of 32 healthcare facilities in the Siouxland district health department to assess the rates, control and transmission of VRE. The assessment of the infection control practices was carried out via a questionnaire and the results showed that in 1997 the responses were mainly anecdotal but by 1998 - 1999 through increased awareness the majority of the care units were following the Centre for Disease Control (CDC) guidelines and cleaning

policies were being followed which correlated with a decrease in colonisation of 2% in 1997 to 0.5% in 1999 (Ostrowsky *et al.*, 2001).

The application of disinfectants to surfaces can occur in a variety of ways including direct application via wipes or in the form of sprays. The use of wipes and sprays has been developed to provide fast efficient cleaning practices, which do not require rinsing after use. Due to the ease of use and speed of alternative cleaning products such as wipes and sprays it must be established that their use is more effective than that of the standard method of application of detergent following a rinse. The use of two antimicrobial wipes and sprays has been assessed against *E. coli* and *Staph. aureus* on wooden, plastic and glass surfaces. All applications showed a reduction of cell number from the surfaces used of 80% in the case of *Staph. aureus* and > 99% for *E. coli.* Overall one of the wipes tested was the least effective at disinfecting every surface, suggesting that the antimicrobial present and the way the carrier i.e. wipe is manufactured is vital to its effectiveness. Sprays were more effective than the wipes. This was thought to be due to the amount of antimicrobial applied to the surface being greater in the case of the sprays. Due to the antimicrobial being contained within the structure of the wipes it is essential that the bacterial contamination of the surface comes into direct contact with the wipe (DeVere and Purchase, 2007).

Research into the use of EOs within wipes as a disinfectant procedure is limited. A three-step protocol for the assessment of the efficiency of textile wipes within the hospital environment has been developed. This protocol was established with the use of wipes impregnated with grapefruit extracts against meticillin-resistant and susceptible strains of *Staph. aureus.* The protocol includes the assessment of ability to remove

contamination from surfaces, their ability to prevent cross contamination and the bactericidal effect of the wipe. Overall the EO wipes tested showed a significant reduction of contamination load compared with the controls of between 1.28 – 2.1 $\log_{10}$. However the grapefruit extracts within the wipes did not show large reduction in bacterial load when directly inoculated (0.58-0.68 $\log_{10}$), suggesting the reduction in surface contamination was mainly due to the action of wiping itself. The contaminated wipe did not retain the microbial load as subsequent contact with clean surfaces showed viable counts of bacterial strains i.e. the bacteria removed from the first contact surface was deposited on the next surface that the wipe came into contact with. Overall the use of grapefruit extracts within wipes is not that effective, but one outcome of the study has been the development of a protocol that allows for an evidence-based analysis of the effectiveness of antimicrobial wipes, thus allowing for more extensive research into the use of EOs as disinfectant wipes to be carried out (Williams *et al.*, 2007).

Although HICPAC have EPA registered detergent-disinfectant for use within hospitals, medical isolates of *Enterococcus* sp. have many virulence factors including aggregation substances, extracellular surface proteins and antibiotic resistant genes that allow successful resistance to antimicrobials that they encounter hence the rapid increase in multi-drug resistance strains. Therefore the need for novel ways in which to combat the cross-contamination of VRE to surfaces is required and the use of a citrus EO blend incorporated into a wipe may be one such way. Not only could this aid the control of VRE in hospitals, but also their fragrance may be superior in terms of acceptability to that of chemical disinfectants that are associated with the hospital environment.

6.1.2 Surgical Theatre Surface Decontamination

In English hospitals between 1997 and 2002, 4351 infections resulted from 107492 operations. Of these cases 10% were due to *Enterococcus* sp. whilst 49% were cased by *Staphylococcus* sp. of which 81% were *Staph. aureus* and 61% of these were resistant to meticillin (Health Protection Agency, 2008).

The importance of bacterial surface contamination in theatres has long been recognised as important in the epidemiology of infectious disease. The guideline of hospital cleaning of the environment is recommended to be "visual cleanliness" but just because a surface appears to be clean it does not mean that is microbiologically acceptable. It has been found that even after cleaning of operating theatre trolleys and door panels that the levels of aerobic colonies are still approximately 2.5 cfu/cm^2 and improvements in visual cleanliness results in no significant decrease in the number of colony forming units present (Griffith *et al.*, 2000). Antimicrobials often used for the decontamination of surgical sites include benzalkonium chloride, providone- iodine chlorhexidine and mercurial compounds, yet benzalkonium chloride is a poor disinfectant and allows for bacterial growth within it (Angelillo *et al.*, 1999). Infection as a result of surgery is commonplace and one study showed that when four intra-operative instruments were swabbed for contamination, between 11.1% and 19% showed bacterial contamination. However, once systematic procedures were put in place for cleaning regimes and behavioural changes a decrease in the postoperative infection was observed of up to 10% (Knobben *et al.*, 2006). General preventative measures in theatres to limit postoperative infection include the sterilisation of instruments and ultra clean air. In the Netherlands a theatre was built especially designed to take into consideration all of the postoperative infection measures and it

was found that the infection rate dropped by 2.1%, while a similar study in the USA found the same results, but in the UK infection rates dropped by up to 5.9% (van Griethuysen *et al.*, 1996). An epidemiological study of micro-organisms in a surgical ICU unit in 1999 and 2000 showed 7 and 28 isolates present to be *Enterococcus* sp. respectively. The results of this study also illustrated that inadequate sterilisation of equipment and linen may have lead to an outbreak of surgical site infection (Esel *et al.*, 2002).

The surface decontamination of surgical instruments is an ongoing dilemma. The UK Department of Health choose randomly selected estates offices of the NHS and asked them to collect surgical instruments after cleaning and before the point they would be returned to surgery. Visual analysis of the instruments showed them to be clean, but out of the 206 instruments tested 17% were found to have high levels of soiling demonstrating that the criteria of 99.9% of reduction in bacterial load was often not met, although this did vary between hospitals. No differentiation was made between the standard of cleaning and the type of instrument, therefore the lack of removal of substrate from the instruments was believed to be associated with the washer disinfectants not performing and meeting the required standards (Murdoch *et al.*, 2006).

The inanimate environment within a surgical setting is one contamination factor that can be more easily managed compared with the air and skin microflora from staff and patients. In the European Medical Devices Directive it is stated that surgical devices including drapes, linens and gowns should pose no risk of infection to the patient, although this is often not the case (Dharan and Pittet, 2002)

Although the contamination of operating theatre sites by bacteria is lower than in the wards, it has been suggested that this is only the case because the theatres run a "clean as you go" policy which means a cleaning regime is in place after the presence of each patient (Griffith *et al.*, 2000). The inadequate sterilisation of equipment and linen in theatres is also common place (Esel *et al.*, 2002), and the visually clean procedure adopted for surfaces does not remove all microbial contamination (Griffith *et al.*, 2000). Therefore the use of the citrus blend vapour as an antimicrobial within hospital surgical theatres may be an ideal addition to the procedures already in place by being a fail safe for the possible lack of efficiency of cleaning and sterilisation regimes.

6.1.3 Aims and objectives

The overall aim of the investigation was to assess the decontamination of surfaces by a citrus essential oil blend (orange: bergamot 1:1 (v/v)) and its vapours against vancomycin susceptible and resistant strains of *E. faecium* and *E. faecalis*

The objectives are:

- To determine the efficiency of wipes impregnated with the citrus essential oil blend in the removal of *Enterococcus* sp. from stainless steel surfaces.

- To establish the effectiveness of the citrus essential oils blend vapours in the removal of *Enterococcus* sp. from stainless steel surfaces

6.2 Methods

6.2.1 Wipes Investigation

The following investigations used the three-step protocol for testing the efficacy of disinfectant wipes as outlined by Williams, *et al* (2007).

6.2.1.2 Preparation of Neutraliser and Disinfectant Wipes

The neutraliser solution was prepared in tryptone soya chloride (TSC; 10g/L tryptone and 5g/L sodium chloride) (DM227, Mast, Merseyside, UK), 30g/L saponin (84510, Sigma-Aldrich, UK), 1g/L $_{L}$ – histidine (H8000, Sigma-Aldrich, UK), 30 g/L Polysorbate 80 (59924, Sigma-Aldrich, UK), 3g/L asolectin from soyabean (11145, Sigma-Aldrich, UK) and 5g/L sodium thiosulphate (S7026, Sigma-Aldrich, UK).

Wipes (10cm x 10cm non-woven swabs, ET9351, Econo-med, Peterborough, UK), were impregnated with 2ml of the blend. Control wipes were impregnated with 2 ml of sterile DH_2O and all wipes were left to dry for 5minutes.

6.2.1.3 Neutraliser Efficiency Tests

Test or control wipes were added to 50ml-covered beakers containing 5g of glass beads (Z143928, Sigma-Aldrich, UK) and 10ml of neutraliser and left in contact with the neutraliser for 5 minutes. Steel discs (2cm diameter with a Grade 2B finish,

Goodfellows Cambridge Ltd, Huntingdon, UK), inoculated with 20μL of bacterial culture (10^7 cfu/ml) of either vancomycin susceptible or vancomycin resistant *E. faecium* or *E. faecalis* were added to the beakers, with the inoculated side touching the glass beads. The beakers were shaken for one minute at 150 rpm. The neutraliser solution was then spiral plated, incubated at 37°C for 24 hours and viable counts determined. The neutraliser was deemed effective if there was no significant difference between the viable counts for the test and control wipes.

The neutralising ability of BHI agar containing 10% (v/v) neutraliser was assessed by placing test wipes on the surface of plates that had been spread plated with bacterial culture (10^7 cfu/ml) and incubated at 37°C for 24 hours, to ensure that growth could occur beneath and surrounding the wipes. Test wipes were also added to BHI broth containing 10% (v/v) neutraliser inoculated with culture (method 3.1.1) and incubated overnight at 37°C, The cultures were then spiral plated onto BHI and the plates incubated at 37°C. Bacterial viability was confirmed after 24 hours.

6.2.1.4 Removal of Microbial Load from Surfaces

The wipes were tested in clean conditions (0.3g/L BSA, Sigma-Aldrich, UK) and dirty conditions (3 g/L BSA). Steel discs were inoculated with 20μL of bacterial culture (10^7 cfu/ml) of either vancomycin susceptible or vancomycin resistant *E. faecium* or *E. faecalis*. The discs were fixed to the base of petri dishes. Either test or control wipes were attached to a steel rod of a drill (RW20, IKA, Staufen, Germany). The wipes were assessed by exerting 100 ± 10g pressure and a rotation speed of 60 rpm

for ten seconds on the discs. The discs were then placed into 50 ml covered beakers containing 5g of glass beads and 10ml of neutraliser and shaken horizontally for 1 minute at 150rpm and then left for 5 minutes. The solution was then spiral plated onto BHI, incubated at 37°C for 24 hours and viable counts determined. The controls were wipe impregnated with sterile water and a control surface that was not wiped with either an EO or water impregnated wipe.

6.2.1.5 Bacterial Transfer from Wipes: Adpression Test

The contaminated test and control wipes (section 6.2.1.4) were then pressed, exerting a weight of 100 ± 10g on eight consecutive BHI plates containing 10% (v/v) neutraliser. The wipes were placed in 100 ml of BHI broth containing 10% (v/v) neutraliser; both the broth and plates were incubated at 37°C for 24 hours. The plates were assessed for viable counts and 50µl of the broth spiral plated onto BHI agar, re-incubated at 37°C for 24 and viable counts determined.

6.2.1.6 Bactericidal Properties of the Wipes

Control and test wipes (4 cm^2) were directly inoculated with 20µL bacterial culture (10^7 cfu/ml) of either vancomycin susceptible or vancomycin resistant *E. faecium* or *E. faecalis* containing BSA at concentrations of either clean (0.3g/L BSA) or dirty conditions (3g/L BSA). After 10 second exposure the wipes were transferred to 50 ml covered beakers containing 5g of glass beads and 10ml of neutraliser and shaken horizontally for 1 minute at 150rpm and then left for 5 minutes. The neutraliser

solution was then spiral plated, incubated at 37°C for 24 hours, viable counts determined and bactericidal effect of the wipes calculated.

6.2.1.7 Statistical Analysis

See section 3.1.5

Efficiency of Neutraliser: Significant differences were calculated between the growth of the control and test samples subjected to neutraliser.

Removal of Microbial Load: Significant differences in removal of *Enterococcus* sp. from surfaces between antimicrobial wipes and control wipes were determined

Transfer of Microbial Load: Growth of *Enterococcus* sp. in the wipes was assessed for significant difference between that of the control and test wipes.

Bactericidal Properties: Any significant differences between the survival of *Enterococcus* sp. directly applied to control and test wipes were established.

6.2.2 Vapour Investigation

6.2.2.1 Assessment of Vapours on the Decontamination of Surfaces

Steel discs (2cm diameter) were inoculated with 20µl of overnight culture of either vancomycin susceptible or vancomycin resistant *E. faecium* or *E. faecalis* and allowed to dry for 20 minutes. Discs were then placed in a 600L vapour chamber and subjected to 15mg/L air of the citrus blend vapour via a heat diffuser for different time intervals (15 minutes, 1, 2, 4, 15 and 24 hours). The discs were then placed in 50ml-covered beakers containing 5g of glass beads (inoculated side of disc touching the beads) and 10ml of PBS and shaken for 1 minute at 150 rpm. The PBS solution was then spiral plated onto BHI agar, plates incubated at 37°C for 24 hours and viable counts determined. This (method 6.2.2.1) was repeated with a dose of 30mg/L air of the EO blend. The controls were surfaces that had not been exposed to the orange/bergamot EO blend vapour.

6.2.2.2 Statistical Analysis

Removal of Microbial Load: Significant differences between the removal of *Enterococcus* sp. contamination from surfaces between the controls and those subjected to vapour were assessed.

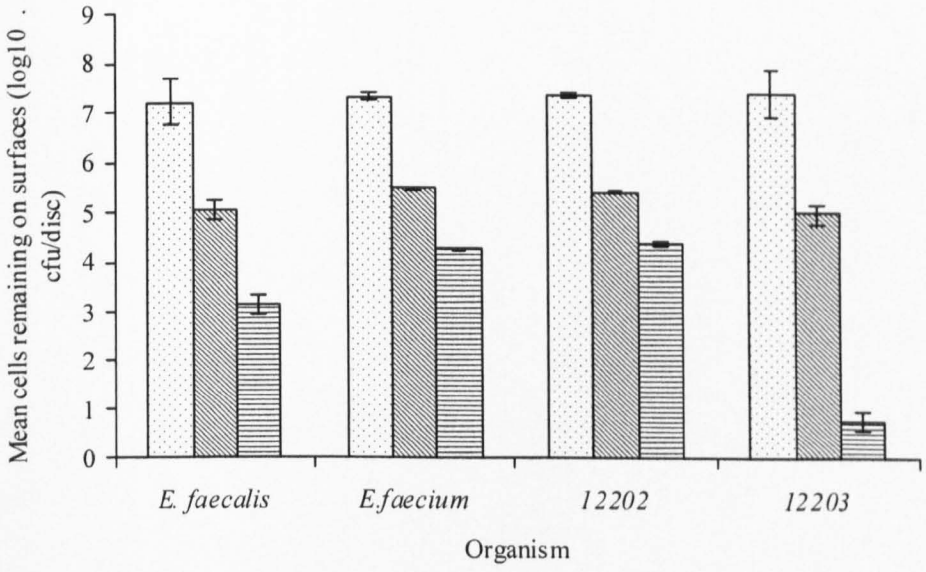
6.3 Results

6.3.1 Wipes Investigation

The neutraliser both in the BHI broth and in agar was effective at quenching the antimicrobial activity of the orange/bergamot blend with no significant difference between those cells subjected to the blend and then neutraliser and the controls (no blend) with a ANOVA statistical value of $F(35) = 6.286$, $p \leq 0.001$, post hoc analysis showed that in agar $t(6) = -0.27$, $p = 0.281$ and in broth $t(6) = 0.2150$, $p = 0.525$.

In clean conditions there was a significant increase in removal of cells from the stainless steel surface after being wiped for 10 seconds with test wipes compared with that of the control with a removal of > 99.9% on all strains of *Enterococcus* sp. with a ANOVA statistical value of $F(93) = 572.189$, $p \leq 0.001$ and post hoc analysis showed $t(15) = -2.0383$, $p \leq 0.001$, $r = 0.98$, with a reduction of $\log_{10}$ 4.05, 3.06, 3 and 6.6 for vancomycin susceptible *E. faecalis, E. faecium* and vancomycin resistant *E. faecium* (12202) and *E. faecalis* (12203) respectively (Figure 6.1a). Under dirty conditions the test wipes were not as effective with surface microbial load reductions of $\log_{10}$ 1.86, 2.14, 2.26 and 2.05 for vancomycin susceptible *E. faecalis, E. faecium* and vancomycin resistant *E. faecium* (12202) and *E. faecalis* (12203) respectively (Figure 6.2a) still demonstrating a removal of approximately 99%, and a significant increase in removal of microbial load from the surface between the test and control wipes with a ANOVA statistical value of $F(47) = 126.743$, $p \leq 0.001$, post hoc analysis $t(18) = -2.5067$, $p \leq 0.001$, $r = 0.97$. Under both clean and dirty conditions there was no significant difference between the removal of microbial load of vancomycin resistant and susceptible *Enterococcus* sp. $t(15) = -0.1200$, $p = 1$ and $t(15) = -0.2667$, $p = 0.976$ respectively.

a)



b)

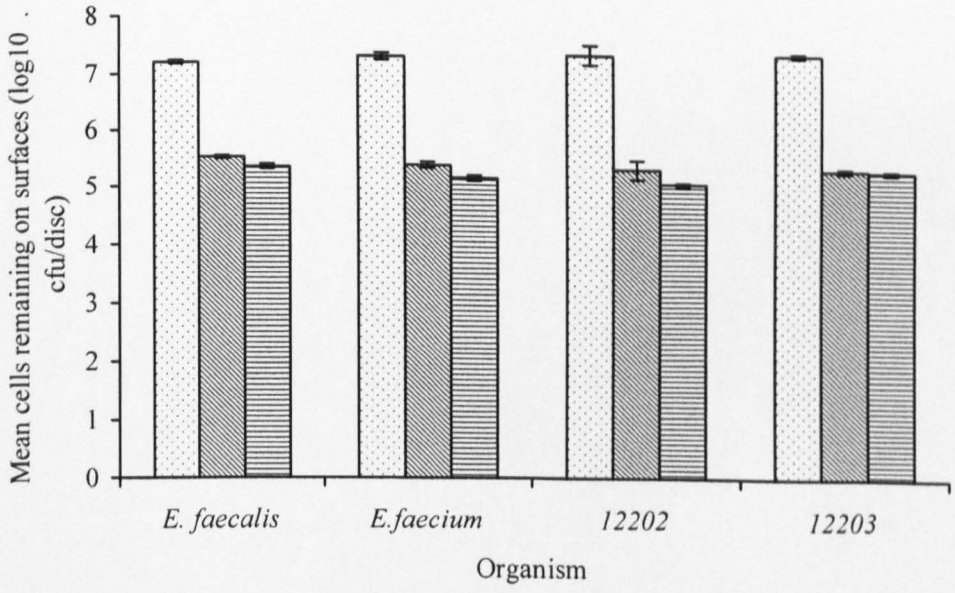


Figure 6.1: Remaining microbial load on surfaces of vancomycin susceptible *E. faecium, E. faecalis* and vancomycin resistant *E. faecium* (12202) and *E. faecalis* (12203) after being subjected to an orange/bergamot impregnated wipe on a) clean surface and b) dirty surface, (± SD, n= 6). Control surface⬚, control wipe ▨ and test wipe▤.

The adpression test showed that all the strains tested were transferred from the surface of both the test and control wipes onto the eight agar plates consecutively. The re-growth of *Enterococcus* sp. for the test and control wipes after the removal of microbial load from the surface was $Log_{(10)}$ 7.9, 7.17, 6.51 and 7.09 in clean conditions and 7.36, 8.73, 7.63 and 8.56 in dirty conditions for vancomycin susceptible *E. faecalis, E. faecium* and vancomycin resistant *E. faecium* (12202) and *E. faecalis* (12203) respectively (Figure 6.2). Overall the recovery from the test wipes was significantly different by approximately 2 log_{10} less than the control wipes with a ANOVA statistical value of $F(45) = 14.773$, $p = 0.001$ and a post hoc analysis for clean conditions of $t(7) = -0.815$, $p \leq 0.001$ and dirty conditions of $t(7) = 1.135$, $p \leq 0.001$, with the overall r value of 0.98.

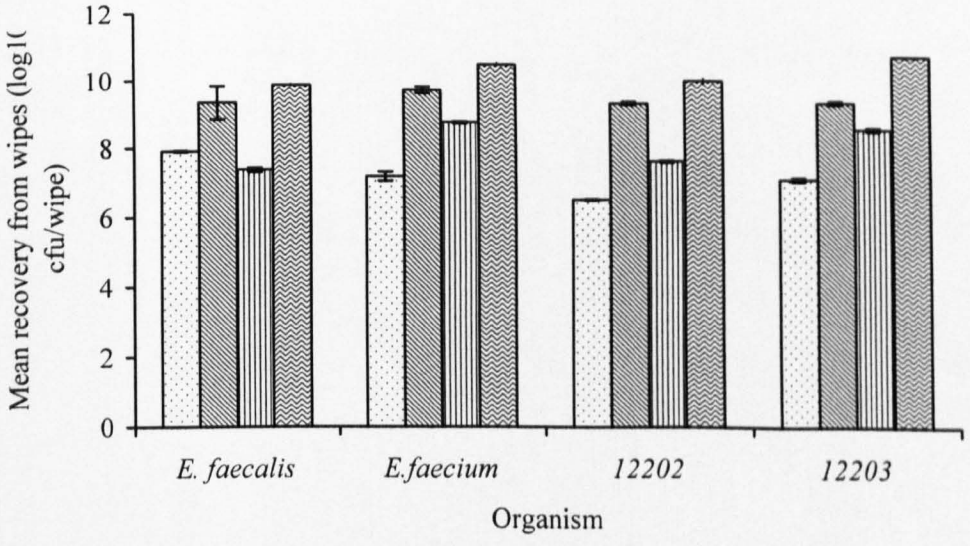


Figure 6.2: Recovery of vancomycin susceptible *E. faecium, E. faecalis* and vancomycin resistant *E. faecium* (12202) and *E. faecalis* (12203) from the wipes after removal from the surfaces, (± SD, n = 6). Clean test wipe clean control wipe, dirty test wipe and dirty control wipe.

The bactericidal effect of the wipes after 10 seconds exposure was assessed, the test wipes were significantly better than the control wipes with a ANOVA statistical value of $F(96) = 36.240$, $p \leq 0.001$, post hoc analysis of $t(15) = -1.183$, $p \leq 0.001$ and $t(15) = -1.1433$, $p \leq 0.001$, overall r value = 0.93 under both clean and dirty conditions respectively with a mean difference in cell growth of 1.18 – 1.31, 1.01- 1.39, 1.2 – 1.21 and 1.26 – 1.12 in clean and dirty conditions for vancomycin susceptible *E. faecalis, E. faecium* and vancomycin resistant *E. faecium* (12202) and *E. faecalis* (12203) respectively (Fig. 6.3). The bactericidal effect was greater under dirty conditions than clean for the vancomycin resistant *Enterococcus* sp.

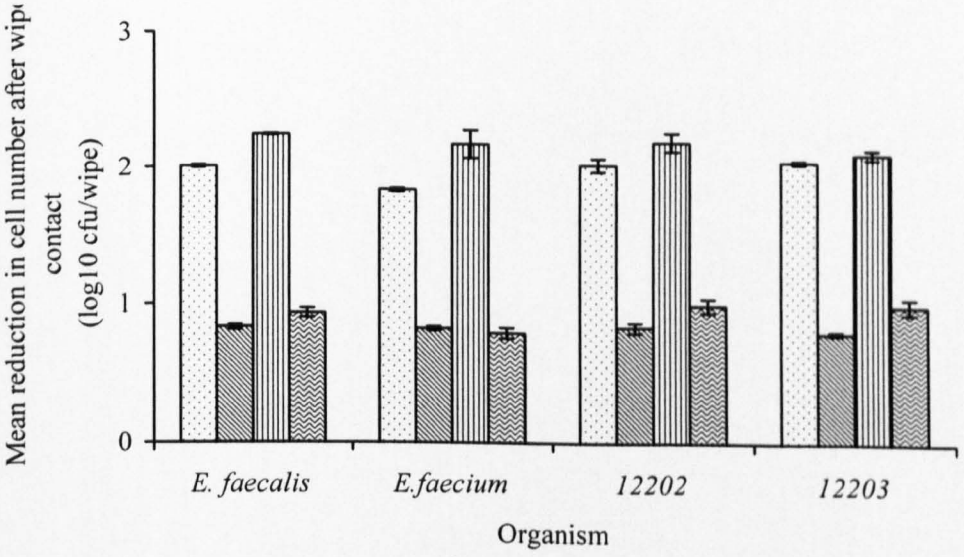
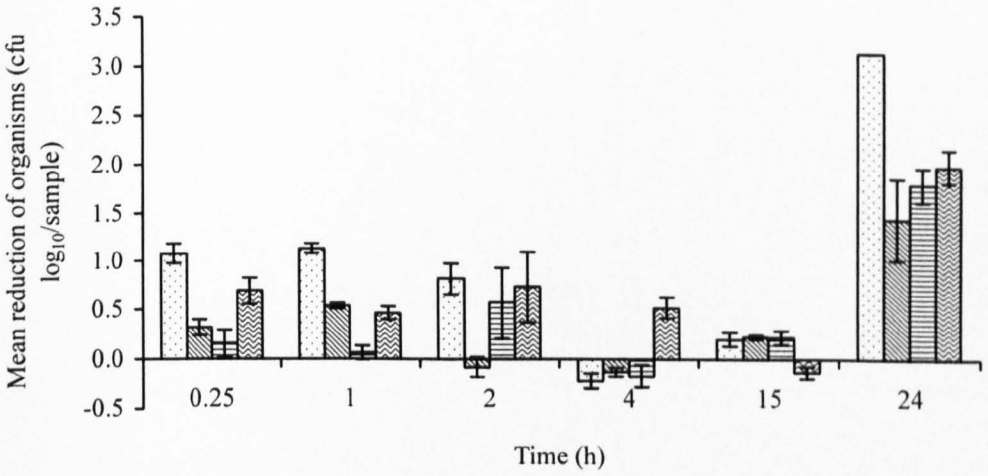


Figure 6.3 Reduction of vancomycin susceptible *E. faecium, E. faecalis* and vancomycin resistant *E. faecium* (12202) and *E. faecalis* (12203) after exposure to orange/bergamot impregnated wipes (± SD, n = 6). Clean test wipe clean control wipe dirty test wipe and dirty control wipe.

6.3.2 Vapour Investigation

Overall the vapours were more effective at reducing microbial load on a stainless steel surface than the controls (Fig. 6.4). There was no significant difference in the effectives of the EO vapours between the antibiotic resistant and susceptible strains. The dose of 15mg/L air had a significant difference to that of the controls at 24 hrs only with a ANOVA statistical value of $F(41) = 6.347, p \leq 0.001$, post hoc analysis of $t(6) = 58.549, p \leq 0.001, r = 0.95$. Whereas at the use of 30 mg/ml shows a significant difference at both 15 and 24 hours $F(47) = 4.905, p \leq 0.001$, post hoc analysis of $t(7) = 22.736, p \leq 0.001, r = 0.94$ for 15 hours and $t(7) = 58.714, p \leq 0.001, r = 0.95$ for 24 hours. The 15mg/L air reached the 99.9% reduction required for disinfectants at 24 hours across all species, whilst the 30mg/L air do not meet this threshold requirement. Overall in the first 2 hours the 15 mg/L air was more effective than the 30 mg/L air with reductions of approximately 0.5 – 1.2 $\log_{10}$ compared with 0.2 – 0.5 $\log_{10}$ reductions for 30 mg/L air with the exception of vancomycin susceptible *E. faecalis* at 1 hour.

a)



b)

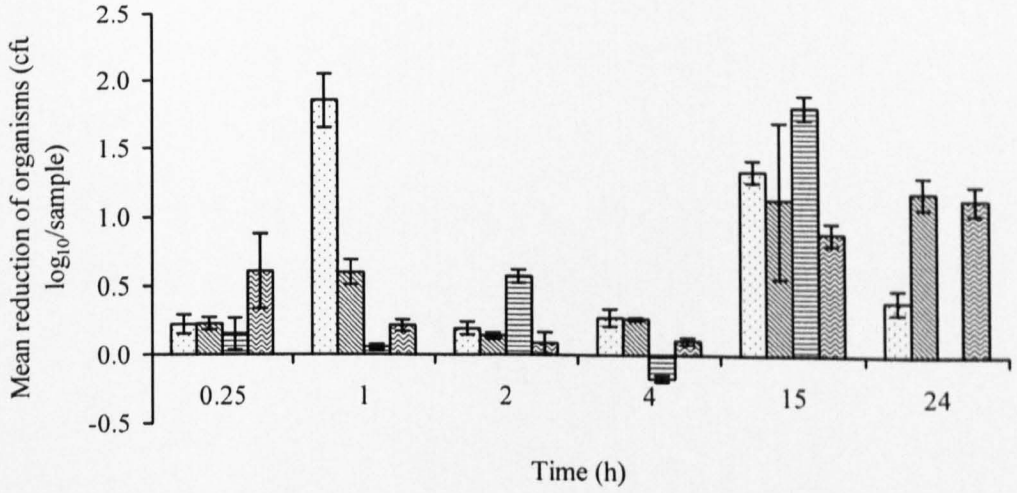


Figure 6.4: Reduction of vancomycin susceptible *E. faecium*, *E. faecalis* and vancomycin resistant *E. faecium* (12202) and *E. faecalis* (12203) on a stainless steel surface to orange/bergamot blend vapour compared with the control samples a) 15 mg/L air and b) 30 mg/ml. Vancomycin susceptible *E. faecalis* □ and *E. faecium* ▨, vancomycin resistant *E. faecalis* (12203) ▤ and *E. faecium* (12203) ▨.

6.4 Discussion

6.4.1 Wipes Investigation

Following the three step protocol to determine the efficacy of disinfectant wipes outlined by Williams *et al.*, (2007), the incorporation of the citrus EO blend into non-woven swabs showed some promise as a potential disinfectant wipe that could be use against VRE within the clinical arena. Overall the wipes were more effective on clean surfaces with approximately 2 $\log_{10}$ of *Enterococcus* sp. less remaining under clean conditions than dirty conditions (Fig. 6.1). When assessing the efficacy of disinfectants it is important to stimulate the environmental parameters they are to be used in, thus the use of protein loads to simulate clean and dirty conditions in this study. The procedure depends on the application of the disinfectant to be tested. For example the protein loads used to simulate a veterinary arena would be greater than those used in this investigation. A study into the use of a halogen containing disinfectant against *P. aeruginosa* showed that under dirty conditions (0.3% BSA) the concentration of the disinfectant and the contact time had to be twice as high to that of the use of the disinfectant in clean conditions (0.03% BSA), the dirty conditions were thought to have a conflicting effect on the efficacy of the disinfectant to act on the cell membrane (Bessems, 1998). The results of a study carried out in 2002 on aldehyde and alcohol based disinfectants tested against VRE and VSE showed no difference in effect between clean and dirty conditions, with the array of aldehyde based disinfectants tested killing the organisms at concentrations of 0.5 – 3% in 1 – 5 minutes, whilst the pure alcohol based disinfectants were needed in higher concentrations and contact times of 50% for 2.5-30 minutes or 70% for 15 seconds to 1 minute. Disinfectants containing some

alcohol rather than a pure alcohol disinfectant were deemed the most effective, being able to kill VRE and VSE in 15 – 30 seconds at low concentrations (Sakagami and Kajimura, 2002). Quanternary ammonium compounds and hypochlorite were required in concentrations of 37% and 67% less in clean compared with dirty conditions for inactivation of Gram-negative and Gram-positive bacteria tested respectively (Bessems, 1998), indicating that protein load has a greater protective effect on Gram- positive bacteria.

The results of the study using grapefruit EO impregnated wipes against Meticillin resistant and susceptible *Staph. aureus* (Williams *et al.*, 2007) agree with the findings of this present investigation i.e. removal of microbial load of 1.86 – 2.26 and 3 – 6.6 $\log_{10}$ on dirty and clean surfaces respectively from that of the control surface (Fig. 6.1). Overall the effect of grapefruit EO impregnated wipes on *Staph. aureus* resulted in approximately the same level of reduction in microbial load as the citrus EO blend wipes on *Enterococcus* sp. this may be due to the EOs being from the same citrus family or that the strains tested were both Gram-positive. Both studies found no significant difference in the removal of antibiotic resistant and susceptible strains by the EO impregnated wipes. The results of these studies suggest therefore that the adhesion mechanisms by which antibiotic resistant and their equivalent susceptible strains adhere to surfaces is similar. In the removal of bacteria from different surface types it has been shown to be key to the efficacy of disinfectants, the use of two types of antimicrobial wipes against *Staph. aureus* and *E. coli* on two types of plastic surfaces (polyethylene and Microban®), and glass were assessed, the glass surface allowed for undetectable levels of decontamination. This was thought to be due to lack of adhesion the smooth surface. On polyethylene the wipes had greater difficulty at reducing the microbial loads

with the use of one wipe allowing approximately 2.5 log_{10} cells to remain on the surface. On Microban®, dependent on the wipe, the reduction in microbial load was up to 100% for one wipe tested, whereas the other wipes reduction was between 72 –97% (DeVere and Purchase, 2007), illustrating not only that the organisms ability to adhere to a surface is important in decontamination but the actual disinfectant used within the wipe.

Prevention of cross contamination of *Enterococcus* sp. has been highlighted as key in controlling the spread of VRE within the hospital environment. Routes of transmission are thought mainly to be due to patient to patient, patient to staff contact and from the environment surrounding the patients (Tacconelli and Cataldo, 2008). Within the domestic environment cloths and sponges have been shown as vehicles for cross contamination of bacteria. When 35 households were assessed for *Listeria* sp. six out of seven dish cloths were positive, resulting in the cross-contamination by the organism in routine hygiene practices (Cox *et al.*, 1989). The transfer rates of *Staph. aureus, Camp. jejuni* and *S. enteritidis* from kitchen sponges to stainless steel surfaces showed that after inoculation of a sponge with 10 ml of bacterial suspension, the transfer rate to surfaces was 21 – 43% which was not dependent on organism. The number of cells remaining on the sponge ranged from 10^5 to 10^6 after contact with the initial surface, indicating use of the sponge in subsequent cleaning practices would lead to further cross-contamination (Kusumaningrum *et al.*, 2003). Reservoirs for dissemination of microrganisms such as cloths, sponges and rags with a high frequency of contamination, are a constant transfer risk with a requirement for use of disinfectant via targeted chemicals, heat or disposable usage to prevent further contamination (Scott, 1999). The cross contamination from the use of the citrus blend impregnated wipes is

high, transfer was observed when adpression test were carried out on eight consecutive agar plates, with the recovery of *Enterococcus* sp. from the wipes being 7 –8 $\log_{10}$ from both clean and dirty surfaces (Fig. 6.2). Should these wipes be developed for use in the clinical arena they would only have application if they were disposed of after one surface clean. However, this may not be cost effective, especially due to the NHS limited funds and that adequate surveillance practices that would need to be implemented to ensure this procedure was adhered to, otherwise the wipes could lead to further cross contamination of wards. The grapefruit EO impregnated wipes against *Staph. aureus* showed similar results (Williams *et al.*, 2007).

The activity of the citrus blend wipes was approximately 1.5 $\log_{10}$ better than the control wipes in both clean and dirty conditions (Fig. 6.3), a bacteriostatic effect is observed due to recovery of 0.5 – 1 $\log_{10}$ bacteria directly from the wipes (Fig. 6.3). The antimicrobial effect of EOs is well documented (Deans and Ritchie, 1987, Burt, 2004, Fisher and Phillips, 2006, Fisher *et al.*, 2007, Bakkali *et al.*, 2007). Overall the results from this present investigation showed the citrus EO blend had a greater bactericical effect of 1.8 –2.2 $\log_{10}$ reduction (Fig. 6.3) compared with that of the grapefruit wipes where $\log_{10}$ reductions were only 0.68 and 0.58 in clean and dirty conditions respectively (Williams *et al.*, 2007). In this investigation the antibiotic resistant strains were found to be less susceptible to the citrus blend wipes under dirty conditions, whereas in the grapefruit wipes the antibiotic susceptible strains were less susceptible in dirty conditions (Williams *et al.*, 2007). In both cases the increase levels in protein is thought to aid in the resistance of the organisms to the EOs. Although the orange/beragmot wipes showed a greater bactericidal activity than the grapefruit wipes, overall the removal of the microbial load from the stainless steel surfaces was

approximately the same suggesting that the antimicrobial action of the wipes may be due to the physical action of wiping rather than the disinfectant content.

In conclusion under clean conditions the orange/bergamot blend EO wipes are more effective than under dirty conditions at the removal of *Enterococcus* sp. from surfaces. Increased concentrations of the EO or longer application time could improve the effectiveness of the wipes in dirty conditions, but the parameters used in this investigation were based on observations of ITU staff using wipes and thus recreating the hospital environment (Williams *et al.*, 2007). The wipes tested have a high potential for cross contamination and would be best used as disposable product but did show an approximate reduction in cells of 2 $\log_{10}$. The wipes were just as effective against vancomycin resistant *Enterococcus* sp. as susceptible strains, indicating potential use within the clinical arena to combat the ever-growing problem of emerging antibiotic resistant organisms.

6.4.2 Vapour Decontamination

The use of the citrus blend vapour as a disinfectant for stainless steel surfaces is only effective over a 15 to 24 hours dependant on dosage (Fig.6.4), thus limiting the potential applications of its use. The threshold for a disinfectant to be classed as effective is 99.9% (Murdoch *et al.*, 2006), the 30 mg/L air of EO vapour does not fulfil this criteria but has optimum inhibition on *Enterococcus* sp. at 15 hrs compared to the 15 mg/L air of EO vapour having a 99.9% inhibition at 24 hrs (Fig. 6.4). The proposed application of the vapours would be the decontamination of surgical theatres when they are not in use (overnight). Therefore the 15 hrs would be a more appropriate time

period, making the 30 mg/ml the preferred choice, but this dosage does not meet the required reduction in microbial load for a disinfectant, thus causing a dilemma of cost of time over percentage reduction of bacteria. The use of the vapours would not be an alternative to the cleaning regimes already in place in hospitals but an additional cleaning practice, especially given the failings of the current cleaning infrastructure and postoperative infections rates (van Griethuysen *et al.*, 1996, Knobben *et al.*, 2006). Seventeen percent of surgical instruments after cleaning have shown to be above the threshold of 200 μg of protein contamination, making the potential for bacterial colonisation high, as well as the final rise water of the washers for such equipment also presenting a microbiological hazard (Murdoch *et al.*, 2006). The key element of a disinfectant being effective is that it is able to reach the target site in high enough concentrations to achieve a lethal effect (Russell, 1999). In the case of vapours they are able to reach any area that air comes into contact with, thus reaching areas that may be missed during routine cleaning.

The decontamination of theatres by the citrus blend vapour after cleaning may also prevent the emergence of resistant strains especially due to the increasing cases of multi drug resistance in bacteria, as well as those to disinfectant. The use of a double cleaning regime may eliminate any bacteria that may have survived the first disinfectant, much like the use of multi-drug therapy on antibiotic resistant strains. In this study there was no significant difference between the inhibition of the vancomycin susceptible and resistant strains (Fig. 6.4). The use of Pine oil as a disinfectant has also shown similar results against *E. coli* that had multiple antibiotic resistant mutants and showed resistance to multiple antibiotics. Due to this strain of *E. coli* having a deletion of the *acrAB* locus that encodes for a protonmotive force-dependent efflux pump it

increased its susceptibility to the pine oil (Russell, 1999). Tolerance can also emerge form the lack of destabilisation of biofilms by disinfectants allowing for the development of resistance within this habitat (Latasa *et al.*, 2006). For the vapour of the citrus blend to be a viable option for clinical cleaning it must be able to penetrate biofilms. The use of the vapour as a secondary cleaning practice may prevent the adhesion of bacteria to surfaces, as the initial adhesion of bacteria depends on physicochemical surface properties including electrokinetic characteristics and surface hydrophobicity of both the bacterium and substratum (Van Merode *et al.*, 2008). Should the components of the vapours leave a residue on the surfaces it may act as an antimicrobial barrier to prevent colonisation of subsequent bacteria.

The main cause of postoperative infection is *Staphylococcus* sp. (Health Protection Agency, 2008). It has been demonstrated that *Enterococcus* sp. are more resistant to disinfectants than *Staphylococcus* sp., this indicates that the 15 hours of 30 mg/L air of vapour (Fig. 6.4) may reach the 99.9% inhibition threshold required against *Staphylococcus* sp. that are more problematic in the surgical environment (Russell, 1999). Although not assessed in this investigation the dual role for the vapours for air contamination control must also be considered. Contaminated air in operating theatres has been suggested as the main contributor to infection, primarily caused by contaminated skin scales shed from the surgical staff, within the theatre environment, 10 cfu/m^2 is the estimated safe level (Pasquarella *et al.*, 2007). Ultra clean air in surgeries is achieved by the use of ventilation systems and maintaining this air flow during operating, most systems are not used whilst the theatres are vacant (Verkkala *et al.*, 1998). The potential residue components remaining in the air after a 15 hour air exposure to the vapours could possibly be utilised by the ventilation systems to add an

antimicrobial element to the systems. The use of EO vapours to reduce bacterial load in the air has been discussed in chapter 6 (Su *et al.*, 2007, Gaunt *et al.*, 2005, Nedorostova *et al.*, 2008).

New disinfectants are needed to combat the resistant stains that are emerging within the hospital environment, as well as addressing the problem of postoperative infection from surgery. The use of orange/bergamot EO blend vapour have shown promise as having a potential application in the decontamination of stainless steel surfaces, but further investigations needs to be carried out to assess there use against a range of different pathogens and surfaces and also its potential to decontaminate the theatre air which is the main cause for concern in the surgical environment.

Chapter 7

Mechanisms of Action

7.1 Introduction

7.1.1 Mechanisms of Antimicrobial Action of EO's

The mechanisms by which EOs bring about their antibacterial effect is incompletely understood but there are a number of proposed mechanisms (Holley and Patel, 2005). Certainly there are morphological changes that are apparent. The outer membrane of both *E. coli* and *S. typhimurium* disintegrates following exposure to carvacrol and thymol (Helander *et al.*, 1998) and major thickening and disruption of the cell wall, together with increased roughness and lack of cytoplasm has recently been reported in *L. monocytogenes* on treatment with thyme essential oil (Rasooli *et al.*, 2006b). Similar observations have been made with *E. coli* O157:H7 and *L. monocytogenes,* in the presence of oregano and cinnamon respectively (Oussalah *et al.*, 2006).

Carvacrol has been shown to increase membrane fluidity and cause the leakage of protons and potassium ions, resulting in a collapse of membrane potential and inhibition of ATP synthesis (Ultee *et al.*, 2002, Ultee *et al.*, 1999). This has also shown to be the case with tea tree oil against *Staph. aureus*, which affects the cell wall and produces a loss of 260-nm nuclear material, K^+ ions and salt tolerance. The presence of mesosome-like structures is observed and inhibition of glucose-dependent respiration occurs (Halcon and Milkus, 2004). Terpenes have the ability to disrupt and penetrate the lipid structure of the cell wall of bacteria, leading to denaturing of proteins and

destruction of cell membrane leading to cytoplasmic leakage, cell lysis and eventually cell death. The total amount of K^+ ions lost in *Staph. aureus* increased with increasing concentrations of tea tree oil. The decrease in pH that occurs due to this cell membrane disruption means that control of cellular processes such as DNA transcription, protein synthesis and enzyme activity is lost (Oussalah *et al.*, 2006). Not only do EOs penetrate the cell membrane but also the mitochondrial leading to greater permeability of the organelle and the same ion leakage process (Raybaudi-Massilis *et al.*, 2006).

Some studies have found Gram-positive bacteria to be more sensitive to EOs than Gram-negative bacteria, which, it has been suggested, may be due to the relatively impermeable outer membrane that surrounds Gram-negative bacteria (Smith-Palmer *et al.*, 2001). Although this explanation has been generally well accepted (Delaquis *et al.*, 2002, Burt, 2004), there have also been studies that suggest there is only a time delay in the growth of Gram-negative bacteria. Therefore, over a longer time period the essential oils would have the same effect on both Gram-negative and Gram-positive bacteria (Deans and Ritchie, 1987, Tassou *et al.*, 2000). This differential sensitivity has been observed when using citrus oils/components *in vitro*, but not in on food studies (Fisher and Phillips, 2006).

7.1.2 Stress Proteins

The synthesis of Heat Shock Proteins (HSP) is increased in the presence of extremes of temperature or other environmental stresses. They are mainly chaperones and proteases that are involved in protein re-folding or degradation to protect the cell against possible dangers (Laport *et al.*, 2004).

The effect of subjecting *E. faecalis* to sub-lethal stress conditions as a pre-treatment, cause it to adapt, and enhances its survival against further stresses i.e. adaptation to exposure at 50°C induces a 6000 fold increase in heat tolerance at 62°C and adaptation to 0.08% bile salts increases survival by 1500 fold when the cells were subjected to 0.3% bile salts (Rince *et al.*, 2000). Bacteria have also been shown to adapt to both domestic and industrial detergents, with as little as five seconds exposure with cross protection being a feature of the cells that have previously been exposed to bile salts show a strong cross resistance to sodium dodecyl sulfate (Flahaut *et al.*, 1996a). When both stationary and exponential cells of *L. plantarum* were subjected to 42 °C for 1 hour they adapted in that their heat resistance to 72 °C for 90 seconds increased by 2 and 3 $Log_{(10)}$ cycles. The proteins identified as being involved in this response were DnaK and GroEL (Koch *et al.*, 1998).

The ability for *Enterococcus* sp. to survive such adverse conditions i.e. its growth over large ranges of temperature and pH suggests that it has proteins that are able to function at extreme parameters and also able to adapt (alteration in gene expression) to a range of conditions. HSPs have been shown to be increased in alteration of the pH environment in as little as a change from pH 7 to 8.8 (Flahaut *et al.*, 1996b). It has been noted that unlike the cross-response of alkaline to acid stresses in *E. coli*, *E. faecalis* does not have this ability, therefore acid adaptation does not then result in resistance to for example bile salts, acid and alkaline the tolerance to which involves different resistance pathways (Flahaut *et al.*, 1996b). This is not always the case as starvation stress responses in *E. faecalis* show enhance resistance to subsequent

exposure to heat, acid, ethanol and NaOCl, showing that starvation in the cells leads to the development of a generally resistant phenotype (Hartke *et al.*, 1998).

Of 167 general stress proteins that have been identified by 2-D electrophoresis in *E. faecalis*, 67 are induced by two or more stresses and only six stress proteins were induced when six or more stresses were encountered (Rince *et al.*, 2000). Using western blot, the major chaperones identified were DnaK and GroEL (Rince *et al.*, 2000). In bacteria with low G + C content, GroEL and DnaK operons have been demonstrated to be controlled by HrcA and CtsR repressors (Laport *et al.*, 2004). Thirty-seven proteins are induced twofold under alkaline stress, nine of which are amplified more than fivefold. The treatment of *E. faecalis* at pH 10.5 induced two HSPs, DnaK and GroEL, by 2.9 and 8.5 fold respectively (Flahaut *et al.*, 1996b). DnaK belongs to the family of chaperone proteins Hsp70 which control folding of proteins. They bind non-native substrates regulated by ATPase activity. The ATPase of DnaK is stimulated by DnaJ, which in turn stimulates GrpE to increase the rate of ATP hydrolysis (Motohashi *et al.*, 1997). In *E. coli* DnaK has been shown to have many functions including prevention of protein aggregation, acting as a protein stabiliser factor and adaptation to stress factors, with both DnaJ and GrpE stimulating DnaK activity, in the presence of these two HSPs the ATPase activity of DnaK increases by up to 50 times (Fukuda *et al.*, 2002).

As well as DnaK being involved in refolding of proteins it also plays a role in ATP-dependent proteolysis, as it contains an amino-terminal ATP binding domain (Koch *et al.*, 1998). Mutations in the DnaK causes increased expression of heat shock genes, even when optimum conditions are maintained (Koch *et al.*, 1998).

The differences in the genomes of *E. faecalis* and *E. faecium* have been assessed using competitive DNA hybridization. *E. faecalis* specific sequences are mainly those encoding for surface-exposed proteins. Overall 6.4% of the *Enterococcus* sp. genome is associated with cell surface proteins and 22.6% of the differences between the two species are found in these genes. Thus one of the major differences between *E. faecalis* and *E. faecium* is the proteins associated with the cell membrane. This variation is thought to have implications in avoiding different host immune responses (Shanks *et al.* 2006).

7.1.3 Effect of EOs on DNA, Enzymes and Proteins

Geraniol, a component of essential oils, is an inhibitor of mevalonate (MVA) metabolites, thus inhibiting 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase activity. When applied to colon cancer cells the usual cell membrane disruption was observed and after 6 hours exposure a 60% reduction in protein kinase C activity occurred and after 16 hours a 50% decrease in active forms of p44/ p42 was observed (Carnesecchi *et al.*, 2002). Caco-2 cells are inhibited by geraniol, by reducing DNA synthesis, after a 48 and 72hr exposure to geraniol the amount of thymidylate synthase (TS) mRNA was reduced by at least 2-fold. There are two different targets involved in the resistance of the cancer cells to the current treatment of 5-fluorouracil (5-FU). Firstly, changes in membrane permeability resulting in changes in membrane potential, triggering modifications in membrane bound protein activity and intracellular signalling pathway and, secondly, the intracellular metabolism of drugs (Carnesecchi *et al.*, 2004). Garlic

organosulfur compounds bring about regulation of cyclin and Cdk7 proteins in human liver tumour cells (Wu *et al.*, 2004).

The EOs of *Origanum compactum, Artemisia herba alba* and *Cinnamomum camphora* can induce oxidative stress, cytotoxic effects and expression of DNA-responsive genes in *Saccharomyces cerevisiae.* They cause cytoplasmic petite mutations but do not lead to nuclear genetic effects. The mitochondria damage caused by these cytoplasmic petite mutations leads to respiratory-competent cells without mitochondrial damage being altered to respiratory-deficient cytoplasmic petites with damaged mitochondria. Therefore EOs not only affect cell survival but the expression of nuclear genetic events (Bakkali *et al.*, 2006). Two DNA damage-responsive genes were studied. RNR3, induced in the presence of DNA damage and RAD51 a DNA damage-inducible gene as these it was thought could be induced by the cytotoxicity of the EOs. Mitochondrial damage was noted but no nuclear DNA damage, suggesting either membrane damage or damage to the organelles resulting from oxidative stress. Both genes in this study were thought to be induced by EOs in the same way that H_2O_2 triggers the pathway (Bakkali *et al.*, 2005).

Linalool has an inhibitory effect on inducible nitric oxide synthetase (iNOS) although western blotting showed no reduction of protein expression for this enzyme (Peana *et al.*, 2006). Anethole occurs naturally in EOs and has been shown to increase intracellular levels of glutathine and glutathione-S-transferase. It also inhibits TNF-induced cellular responses (Yea *et al.*, 2006). Chamomile exhibited inhibition of cytochrome P450 enzymes involved in the oxidative metabolism of drugs, which might explain some of the observations noted by Carnesecchi (2004). Results from *in vitro*

experiments demonstrated that CYP1A2 was particularly sensitive with k_i values of between 0.82 and 0.19 μm. CYP3A4 is the most relevant in this group of enzymes from a clinical perspective as it is effective against the largest range of substrates and found in the largest quantities in the body and chamomile was found to have moderate inhibition of all isoforms (Ganzera *et al.*, 2006).

Little is known about the mechanism of action of citrus EOs and especially their vapours despite the potential ability for them to be used as alternative antimicrobials against those bacterial strains developing antibiotic resistance. Due to the bacteriostaic nature of many of the EOs the findings of research in this area only go part of the way in understanding the mechanisms by which EOs produce their antimicrobial effect and further investigations need to be carried out on the effect of citrus EOs on specific enzymes, proteins and metabolic processes and the mechanisms by which the cells recover.

7.1.4 Aim and Objectives

The overall aim of the project was to investigate the mechanisms of action of a blend of citrus essential oil orange: bergamot 1:1 (v/v) against *E. faecium* and *E. faecalis.*

The objectives are:

- To assess the effect of the blend and its vapours on the cell membrane permeability and how this affects cell ATP concentrations, membrane potential and intracellular pH.
- To establish any morphological changes to the cells after being exposed to the citrus blend and its vapours.
- To determine whether DnaK stress protein is observed after cells have been subjected to the citrus blend and its vapours.

7.2 Methods

Cells of an overnight culture grown at 37 °C at pH 7.5 of vancomycin susceptible strains of *E. faecalis* and *E. faecium* only were used in the following experiments. When assessing the effect of the vapour, cell cultures were exposed to 15mg EO/L air vaporised using a heat diffuser at 37 °C in a 600L sealed vapour chamber. In the case of the EO *per se* the oil was added at a concentration of 2% (v/v) during the investigations. Controls were not subjected to either the oil or vapour.

7.2.1 Membrane Permeability- NPN Assay

A solution of N-Phenyl-1-napthylamine (NPN) 0.5mmol l^{-1} (Sigma, 104043-100G) was prepared in acetone and diluted in 5mmol l^{-1} of *n*-heptadecanoic acid methyl ester (HEPES buffer, Sigma H3375-25G), pH 7.2 to give a final concentration of 40μmol l^{-1}

Samples (1.5ml) of overnight cultures were taken and cells concentrated by centrifugation for 20 minutes at 25°C at 8000 rpm (Rota, F 45-30-11, Eppendorf 5864R, Hamburg, Germany). The pellet was washed twice with 10 ml of DH_2O for 10 minutes, diluted with DH_2O to an OD_{660} of 1.0 and then re-suspended in a ratio of 1:1 (v/v) of HEPES buffer with NPN. The uptake of NPN was measured immediately using a spectrofluorometer (Fluorimeter 6200, Jenway Essex England) at excitation 355 nm and emission 405 nm. The EO blend was then added to the solution and the change in NPN uptake monitored. In the case of the vapour NPN uptake was measured before and after the cells had been exposed to the vapour.

7.2.2 Determination of Intra- and Extracellular ATP Concentrations

Intra and extra-cellular ATP concentrations were measured using a method adapted from Ultree *et al.* (1999). Cells from an overnight culture were washed three times with sterile DH_2O and re-suspended in 25mM potassium phosphate buffer (pH 7). The cell suspensions were adjusted to an OD_{660} of 1.0 and glucose added at a concentration of 0.5% (w/v). Aliquots (200 µl) were removed every two minutes for 14 minutes, with the EO being added after six minutes. Samples were added to eppendorf tubes containing 200 µl of silicon oil on top of 100 µl of trichloroacetic acid (10%) (T9159, Sigma UK) -EDTA buffer (2 mM). The samples were then centrifuged for five minutes at 12 000 rpm (Rota, F 45-30-11, Eppendorf 5864R, Hamburg, Germany). The extracellular ATP (upper layer) and intracellular ATP (lower layer) were measured using an FLAA- 1KT assay kit (Sigma, UK). Lumininescence was measured with an Optocomp 1 luminometer (MGM instruments, USA). In the case of the vapour, ATP concentrations were assayed before and after the cells had been subjected to the vapour.

7.2.3 Effect of Citrus Blend on Membrane Potential ($\Delta\Psi$)

Cells of an overnight culture were washed twice in a solution of 1mM $MgSO_4$ and 50 mM potassium HEPES buffer pH 7 (H0527, Sigma, UK) and diluted to an OD_{660} of 1.0. Membrane potential was measured using a method adapted from that of Ultree *et al.* (1999). In brief, 30 µl of the cell suspension was added to 2ml of buffer containing 3,3-dipropylthiacarbocyanine ($DiSC_3(5)$) (318434, Sigma, UK). The membrane $\Delta\Psi$ was monitored using a spectrofluorometer (Fluorimeter 6200, Jenway Essex England) at excitation wavelength 643 nm: emission wavelength 666nm. Once a steady reading

was obtained the cells were energised with 15 mM of glucose and when equilibrium occurred 1nM of nigericin was added to neutralise the pH gradient across the membrane before the EO was added and fluorescence continued to be measured for a further 120-150s. In the case of the vapour-exposed cells the experiment was carried out on untreated cells and treated cells. Valinomycin (1nM) was used as a control to dissolve the membrane potential.

7.2.4 Intracellular pH Determination

A calibration curve was carried out using buffers containing 50mM glycine, 50mM citric acid, 50mM $NaHPO_4$ $2H_2O$ and 50mM KCL. The pH of the buffer was adjusted with NaOH or HCl to pHs from 3 to 10 in intervals of 1.0. The intracellular pH (pH_{in}) and extracellular pH (pH_{out}) were equilibrated by the addition of 1 μM of valinomycin (Sigma V0627) and nigericin (Sigma, 72445), as described by Ultee *et al.* (1999).

Cells from an overnight culture were washed three times in 50mM HEPES buffer (pH 7) and diluted to an OD_{660} of 1.0. Cell suspensions were incubated for 10 min at 30°C in the presence of 1.5 μM of carboxyflurescein diacetate succinimidyl ester (Molecular Probes, C1311). To remove any nonconjugated carboxyflurescein diacetate succinimidyl ester, the cells were washed twice in 50mM potassium phosphate buffer pH 5.81 and incubated for 30 minutes at 30°C in the presence of 10mM of glucose. Cells were then washed twice in 50mM potassium phosphate (pH 5.81) with 1 μM of valinomycin (rule out any other gradients apart from proton gradients, due to the membrane potential being dissolved). The cells were kept on ice until required.

A 30 μl of the cell suspension was added to 3 ml of phosphate buffer (pH 5.81) and mixed. Fluorescence was measured at emission 525nm; excitation at 490nm (pH sensitive) and 440nm (pH insensitive). The EO was then added and changes in fluorescence monitored. For vapour exposed cells fluorescence was measured before and after treatment to determine any differences. Intracellular pH was calculated as the ratio of the 490nm and 440nm excitation, Δ pH is pH in – pH out.

7.2.5 Transmission Electron Microscopy (TEM)

Overnight cultures were subjected to either 15 mg/L air of vapour or 2% v/v of oil for one hour. Samples of 15ml were taken and the cells concentrated by centrifugation for 20 minutes, at 25°C at 8000 rpm (Rota, F 45-30-11, Eppendorf 5864R, Hamburg, Germany). The pellet were washed three times with 10 ml of DH_2O for 10 minutes.

Spurr Resin Embedding:

The samples were placed in sodium cacodylate buffer pH 7.2 with 2% (w/v) of paraformaldehyde and 2% (v/v) of glutaraldehyde for 2 hours then rinsed for ten minutes in cacodylate buffer pH 7.2. The second fixing was 1 hour in osmium tetroxide. The samples were then rinsed four times for 10 minutes in ultra-pure water. Samples were block stained in 0.5% (v/v) aqueous uranyl acetate overnight and again rinsed in ultra-pure water for 10 minutes. Dehydration was carried out for 30 minutes in a water/ethanol series of :20; 30; 50; 70; 90; 100% (v/v) and then dried in 100%

ethanol this was repeated twice. Infiltration in agar 100 resin was carried out gradually using resin diluted in 100% dried ethanol at the following ratios 25%, 50%, 75% and 100% v/v resin for 1 hour, 100% v/v resin overnight, 8 hours, overnight and then 8 hours. Polymerisation occurred by placing the samples in an oven for 12 hours at 333°K.

Microscopy:

The polymerised blocks were sectioned at 70-80 nm thickness using a Reichart Ultracut E ultramicrotome and samples were picked up on formvar coated grids. The samples were then stained with Reynold's lead citrate for 10 minutes. Samples were viewed on a Jeol 1200EX, operating at 120 kV

7.2.6 UV Spectrophotometry of EO Blend Remaining in the Cells

The remaining EO within the cell was determined using method 4.2.2.4

7.2.7 Western Blot

Overnight cultures were subjected to either 15 mg/L air of vapour or 2% (v/v) of oil for one hour. Samples of 15ml were taken and the cells concentrated by centrifugation for 20 minutes, at 25°C at 8000 rpm (Rota, F 45-30-11, Eppendorf 5864R, Hamburg, Germany). The pellet were washed three times with 10 ml of DH_2O for 10 minutes.

Both membrane and cytoplasmic proteins were extracted from the samples using a ProteoPrep Universial extraction kit (Sigma Aldrich, UK). The pellet was suspended in 10 ml of soluble cytoplasmic reagent and sonicated on ice for 2 minutes. The suspension was then centrifuged (Jovan GR2002) at 14 000 g for 45 minutes at 4°C and the supernatant decanted. This was repeated twice and the supernatants combined (SUP1, cytoplasmic proteins). The supernatant was then lyophilised for 36 hours in a Christ freeze drier (Sciquip, Shropshire) at -40 °C. The lyophilised protein was then placed in 2 ml of protein resuspension reagent. The pellets were placed in 2 ml of protein extraction reagent and sonicated for 2 minutes on ice and centrifuged at 14 000g for 45 minutes for at 4°C, the pellet was discarded and the supernatant decanted (SUP2, membrane proteins). Tributylophospine (TBP) was added to SUP1 and SUP2 at a final concentration on 5mM and incubated at room temperature for one hour. The supernatants were then alkylated by adding iodoacetamide at a final concentration of 15 mM and incubated at room temperature for 1.5 hours. TBP (5mM) was then added to each of the supernatants and incubated at room temperature for 15 minutes. The samples were centrifuged at 20 000g for 5 minutes at room temperature, the pellets discarded and the supernatants used to run the Western blot. Protein concentrations were determined using a Bradford assay (B6916, Sigma UK).

A ProteoQwest™ colorimetric western blotting kit (Sigma- Aldrich, UK, PQ0101) was used for the identification of DnaK. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% Tris HCL gels (161-1173, Bio-Rad, UK). Aliquots of 10 µl protein samples (100 µg) were suspended in 10 µl of 10% Tris/glycine SDS buffer (161-0732 Bio-Rad UK) and heated for four minutes at 95°C, before being subjected to SDS-PAGE. Also loaded in the gel was a chemichrome

Western control and 50ng of DnaK active recombinant protein (Spp-630, Sigma, UK). Proteins were transferred to a nitrocellulose membrane (162-0145, Bio-Rad, UK), using a semi-dry blotting system at 64mA (V10, Scie-Pas, UK). For the detection of the DnaK protein, the samples were firstly probed with a monospecfic mouse anti-DnaK monoclonal antibody (2μl/ml) (SPA-880, Cambridge Bioscience, UK) and then a anti-mouse IgG horse radish peroxidase conjugated antibody (A5225, Sigma, UK) at a constant 1:10 000 dilution, 3,3',5,5'-Tetramethylbenzidine (TMB) was used to detect immobilised protein, this was visualised using a gel-doc system (Gel Doc 1000, Bio-Rad, UK). Limits of detection were established using purified DnaK and serial dilutions from 50ng to 3.125ng.

7.3 Results

The addition of the bergamot:orange EO blend to *E. faecium* and *E. faecalis* cells increased the permeability of cell membrane by twofold while exposure to the bergamot:orange vapour increased cell permeability by 32- 40 times (Table 7.1).

Table 7.1: NPN uptake induced by citrus essential oil blend and increase in permeability (as a multiple of the untreated cells) of cells ± SE (n = 6).

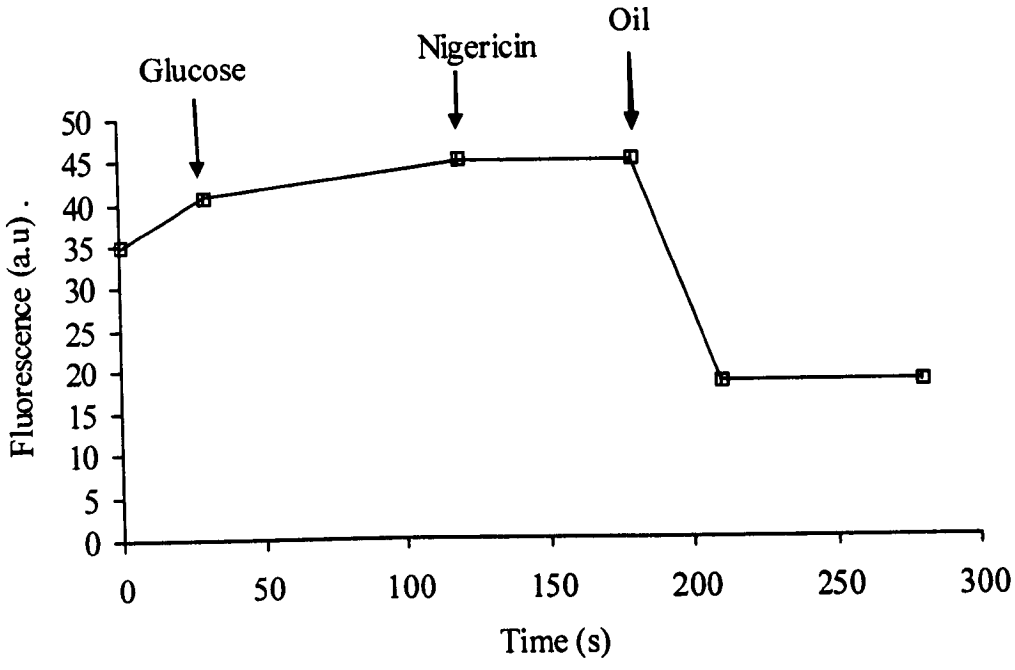
	NPN & EO*	Control	Oil		Vapour	
	Fluorescence value	Fluorescence value**	Fluorescence value**	Increase	Fluorescence value**	Increase
E. faecalis	42 ± 0.8	128.46 ± 24	308.3 ± 8	2.4 ± 0.1	4191.65 ± 9	32.63 ± 0.2
E. faecium	42 ± 0.8	130.66 ± 18	309.96 ± 5	2.37 ± 0.2	5243.39 ± 10	40.13 ± 0.3

*EO fluorescence value = 0

** Fluorescence values after background subtracted

The membrane potential of the cells is reduced from 35 to 20 a.u. in *E. faecium* (Fig. 7.1a) and 32 to 12 a.u. in *E. faecalis* (Fig. 7.1b) when subjected to the EO and from 35 -12.61 a.u. and 45 -14.97 a.u. in *E. faecium* and *E. faecalis* respectively after exposure to the vapours (Fig. 7.2). Increased permeability of the cells (Table 7.1) and loss of membrane potential leads to loss of intracellular ATP in both *E. faecalis* and *E. faecium* (Fig. 7.3) from approximately 18 pmol/mg protein to undetectable levels when being subjected to both the EO and the vapour (Table 7.2). This is coupled with a decrease in intracellular pH in *E. faecium* from pH 6.34 to 5.46 and 5.32 and in *E. faecalis* from pH 6.51 to 4.98 and 4.25 by oil and vapour respectively (Table 7.3).

a)



b)

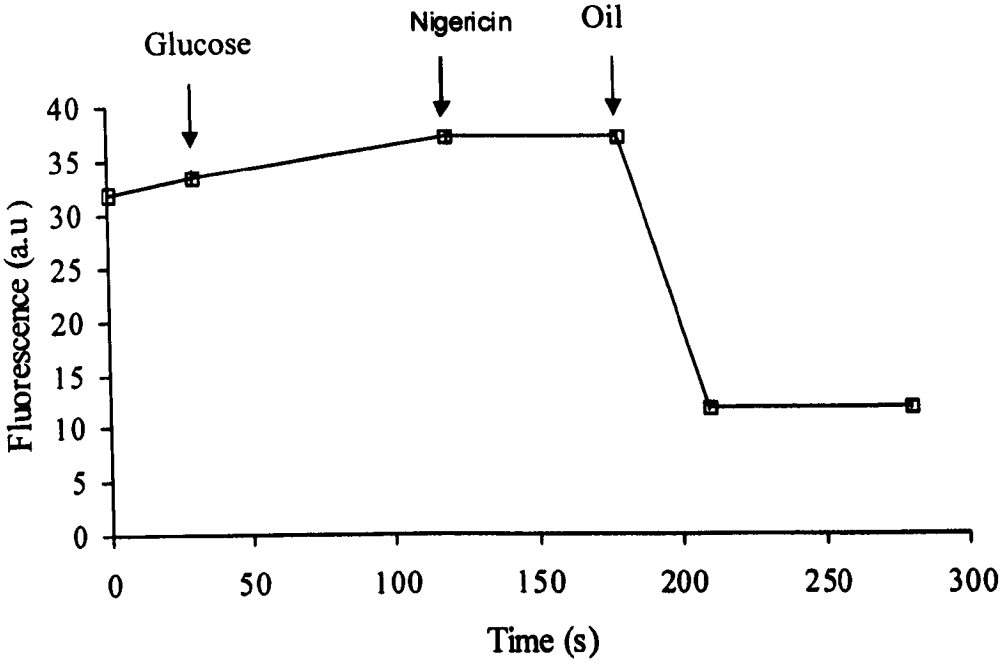


Figure 7.1: Changes in membrane potential after the addition of bergamot:orange (1:1 v/v) EO blend to vancomycin susceptible a) *E. faecium* and b) *E. faecalis* (± SD, n = 6).

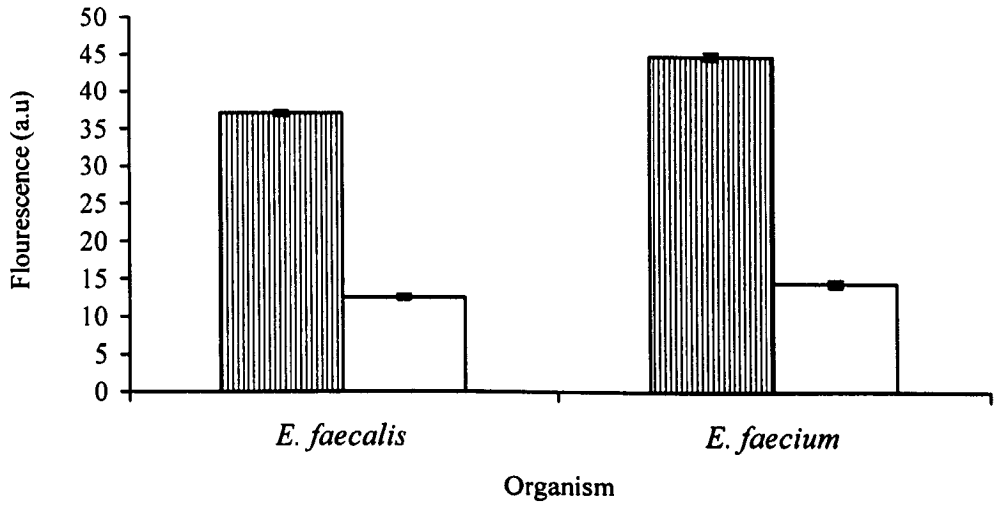
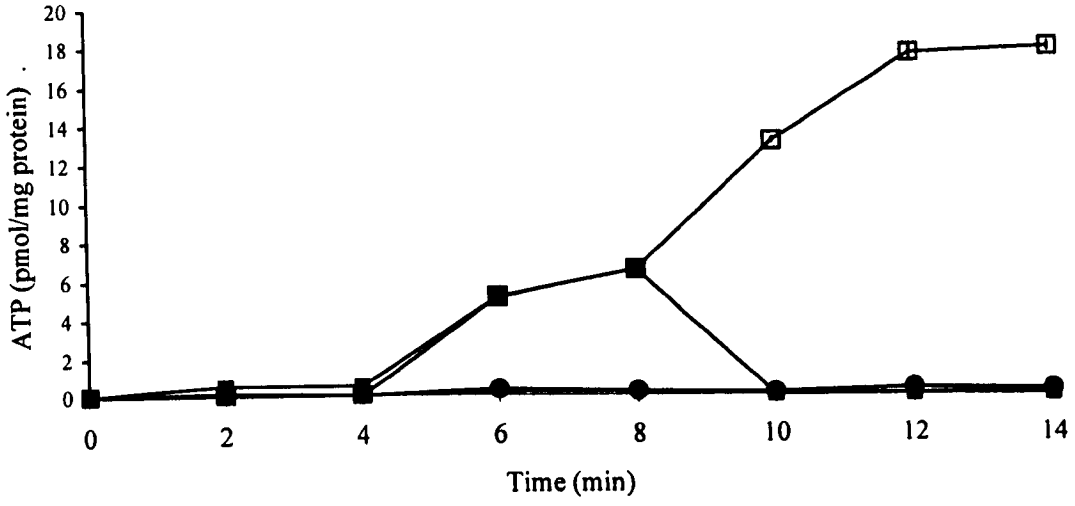


Figure 7.2 Changes in membrane potential of vancomycin susceptible *Enterococcus* sp. before ▥ and after □ the addition of bergamot:orange (1:1 v/v) EO vapours (± SD, n = 6)

a)



b)

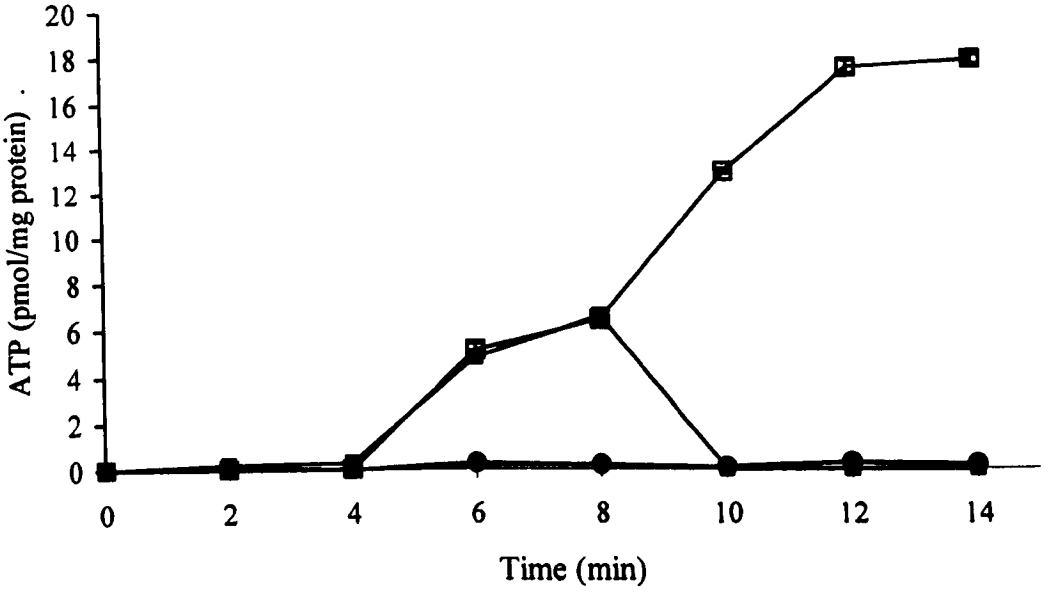


Figure 7.3: Changes in intra/extra-cellular ATP concentrations in vancomycin susceptible a) *E. faecalis* and b) *E. faecium* after being subjected to bergamot:orange (1:1 v/v) EO blend (± SD,n = 6). Intra- cellular ATP in test sample (—■—), Extra-cellular ATP in test sample (—◊—), Intra-cellular ATP control (—⊟—) and Extra-cellular ATP control
(—●—).

Table 7.2: Changes in intra and extra cellular ATP (pmol/mg protein) of vancomycin susceptible *Enterococcus* sp. (± SE), before and after the addition of orange:bergamot (1:1 v/v) EO vapours (n = 4)

	Before		After	
	Intra	Extra	Intra	Extra
E. faecalis	17.53 ± 0.3	0 ± 0	0 ± 0	0 ± 0
E. faecium	17.8 ± 0.2	0 ± 0	0 ± 0	0 ± 0

Table 7.3: Intracellular and Δ pH (± SE) of vancomycin susceptible *Enterococcus* sp., before and after cells were subjected to a citrus blend oil or vapour (n = 6).

		E. faecium		*E. faecalis*	
		Before	After	Before	After
Oil	pH intracellular	6.34 ± 0.24	5.46 ± 0.18	6.51 ± 0.38	4.98 ± 0.27
	Δ pH (pH_{in}-pH_{out})	1.35 ± 0.21	0 ± 0.2	1.63 ± 0.08	0 ± 0.11
Vapour	pH intracellular	6.34 ± 0.24	5.32 ± 0.16	6.51 ± 0.38	4.25 ± 0.14
	Δ pH (pH_{in}-pH_{out})	1.35 ± 0.21	0 ± 0.26	1.63 ± 0.08	0 ± 0.12

Morphological changes occur in the cells after being in contact with either the EO or its vapour including a loss of distinction of the membrane. Cells that have been subjected to the vapour are elongated in shape whilst those cells that have been in direct contact with the EO appear to have vacuoles containing the EO within them. Cell lysis also occurs (Fig. 7.4) and this appears to be occurring at the point where EO is trapped within the cells.

After the removal of externally attached EO and subsequent cell lysis, the remaining supernantants did not absorb at 321nm (the peak of absorbance of the EO blend) suggesting that control cells and those subjected to the vapour did not contain EO. Whereas, those cells that had been in direct contact with the EO blend had 9.19 and 8.5 µl/ml of oil remaining in the supernatant of *E. faecium* and *E. faecalis* respectively.

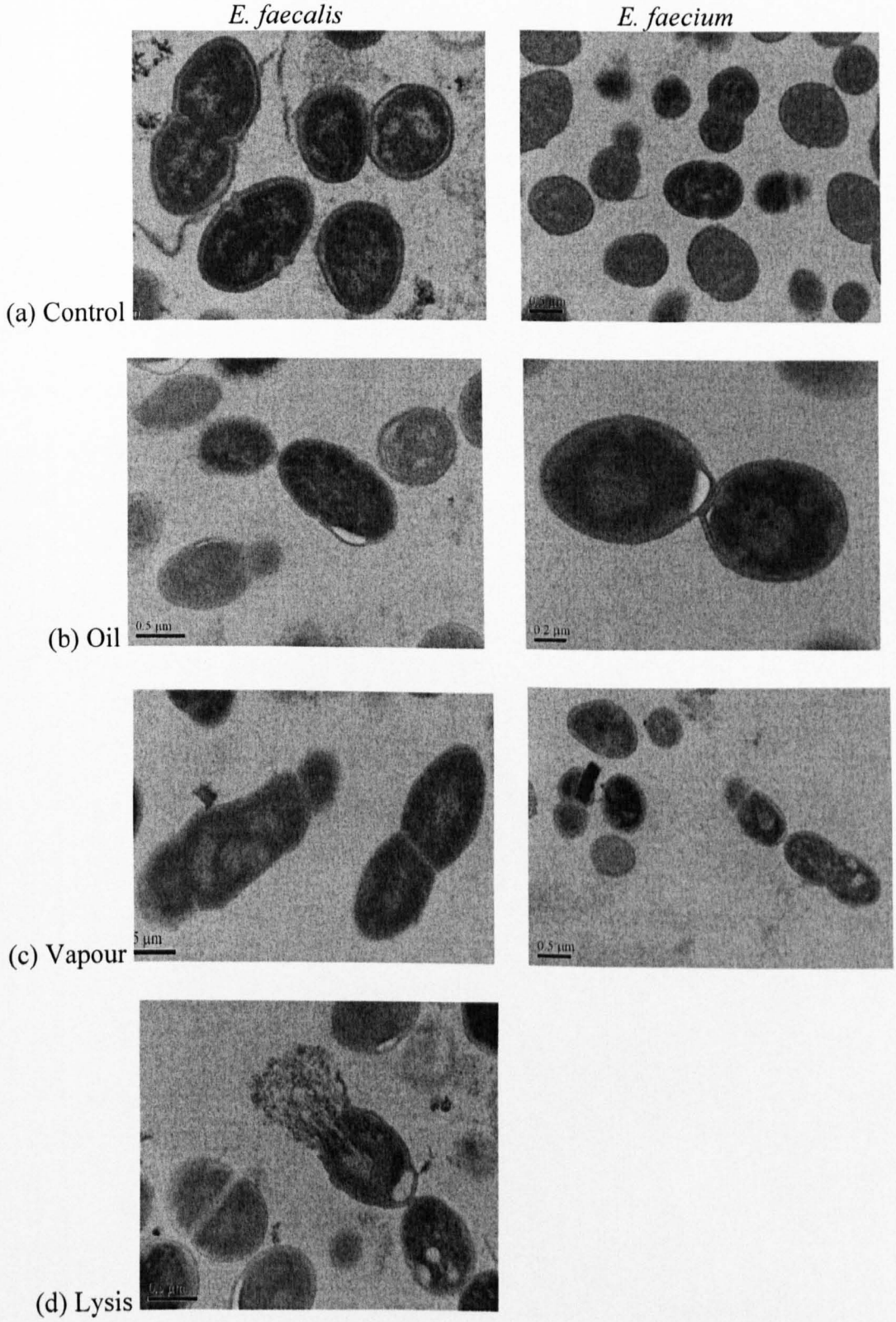


Figure 7.4: TEM images of vancomycin susceptible *E. faecalis* and *E. faecium*: a) cells that had not been exposed to either oil or vapour, b) cells that had been exposed to the blends oil c) cells that had been exposed to the vapour of the blend and d) lysis of a *E. faecalis* cell after being subjected to the oil blend.

Western Blot analysis showed that the heat shock protein (HSP) DnaK was undetectable in the cytoplasmic protein fraction (Fig 7.5b), but a faint band with a molecular mass of approximately 70 KDa, corresponding to that of DnaK, was apparent in the membrane protein fraction (Fig 7.5b) of the control cells. DnaK was not detected in either the cytoplamic or membrane proteins of the cells after treatment with either the citrus blends oil or vapours (Fig 7.5 a & b) in *E. faecalis* or *E. faecium* (Results not shown, Fig 7.5 is representative of both strains). The minimum detection concentration of DnaK active recombinant protein was 6.25 ng.

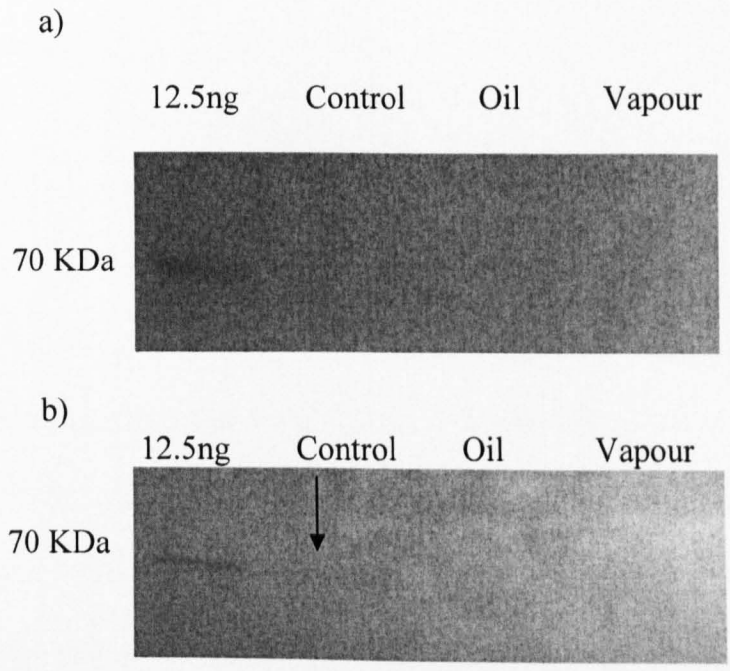


Figure 7.5. Western blot analysis of the presence of DnaK protein in vancomycin susceptible *E. faecalis* before and after being exposed to a to a citrus blend oil or vapour a) DnaK present in the membrane proteins of the cells and b) DnaK present in the cytoplasmic proteins of the cells.

7.4 Discussion

The findings of this investigation suggest that the antimicrobial action of a bergamot:orange (1:1 v/v) EO on *Enterococcus* sp., is effected by the uptake of the EO into the cell (Fig. 7.4). Vacuoles are observed within the cells (believed to contain the EO), which were not present in the control cells. The lipophilic characteristic of the EOs will allow the preferential partitioning from the aqueous phase into the membrane structure of the cell (Cox *et al.*, 2000), and thus could result in the uptake of the EO into the cell. However, the mechanism of action of the vapour is not clear and, although it may work via the same mechanism, no residue of oil was noted when being examined by UV spectrophotometry unlike when the cells were treated with EO. Figure 7.3 shows complete lysis of some cells. Although the anti-microbial effect of citrus oils has been shown to be bacteriostatic (Fisher and Phillips, 2008) other studies have suggested that the only partial damage to the cell wall may occur after being subjected to EOs, causing increased permeability to the cell contents (Helander *et al.*, 1998, Ultee *et al.*, 1999, Oussalah *et al.*, 2006). The images in Fig. 7.4 also show deformities in the cells, with the membranes being less distinct after exposure and those cells having been subjected to the vapour being elongated. Previous studies have shown that when *L. monocytogenes* and *E. coli* are treated with Spanish oregano EO, similar morphological changes occur with deformed shape to the cells and incomplete cell membranes and a depleted cell content was observed suggesting damage to the cell membrane and thus permeability. *E. coli* showed holes in the cell membrane which appeared as white dots along the cell wall. The use of Chinese cinnamon against *L. monocytogenes* did not alter the morphological shape of the cell although it had been shown to have an inhibitory effect against the bacteria (Oussalah *et al.*, 2006). Rasooli *et al* (2006) also

showed *L. monocytogenes* to have degenerative changes to the cell wall including thickening, disruption and increased roughness, a reduction in the amount of cytoplasm thought to be due to the cell membrane being able to function as a barrier, a decrease in overall cell size and clumping of cells was also noted after being exposed to *Thymus x-porlock.* A study of the inhibitory effect of chitosan against *E. coli* showed the outer membrane after exposed to be covered in vesicular structures (Helander *et al.*, 2001).The following studies when using TEM to assess cell damage from EOs have suggested the vacuoles observed when cells are treated with EOs may be due to co-agulation of the membrane, when using cinnamon and oregano in combination with packaging against *E. coli* and *Staph. aureus* (Becerril *et al.*, 2007, Ultee *et al.*, 2002), oregano, cinnamon and savory against *E. coli* and *L. monocytogenes* (Oussalah *et al.*, 2006). The use of thyme against *L. monocytogenes* also showed splitting of the cell wall layers (Rasooli *et al.*, 2006b). The uptake of the EOs into the cell to form vacuoles and thus having an antimicrobial effect from the inside is a novel theory to mechanisms of action of EOs.

The barrier of the cell membrane is essential for normal cellular function including such processes as energy transduction, solute transport, metabolic regulation and control of energy status and turgor pressure (Cox *et al.*, 2000). After exposure of *E. faecalis* and *E. faecium* to the citrus blend there is an increase in cell membrane permeability with a twofold increase in the case of the EO and a 32-40 times increase in the case of exposure to vapour (Table 7.1). This might suggest that the vapour may be bringing about its effect via a different mechanism or because of the smaller molecular size or of specific particles evaporating from the oil, the vapour may be more effective at penetrating the membrane and thus causing damage to the cell. Damaged membranes

occur in *E. coli* when exposed to vanillin with a 29% increase in the number of cells with a damaged membrane after one hour exposure, but after 24hrs recovery appeared to occur with only 13% of the cells being damaged (Fitzgerald *et al.*, 2004). Using flow cytometry the permeability of *L. innocua* after exposure to EOs *Thymus vulgaris*, *Cymbopogon citratus* and *Ocimum gratissimum* for 30 seconds showed that the fluorescence index decreased from approximately 0.73 to undetectable levels (Nguefack *et al.*, 2004). The uptake of fluorescent ethidium bromide (EB) is limited by the cell membrane after the addition of 0.1% (v/v) of thymol or carvacrol to *P. aeruginosa* and *Staph. aureus* resulted in an uptake in 90% of the cells (Lambert *et al.*, 2001).

Increased permeability of bacterial cells after being exposed to EOs has been shown in many studies to result in a loss of cell content. The addition of 0.25% (v/v) of tea tree oil to *Staph. aureus* and *E. coli* cells showed that leakage of potassium ions occurred immediately from *E. coli* and after 30 minutes the cells had nearly lost 100% of the cellular free potassium. In comparison five minutes exposure was required before the *Staph. aureus* became permeable to potassium ions and after 30 minutes only a loss 20% was observed (Cox *et al.*, 2000). A significant increase ($p \leq 0.05$) of cell content release was observed when *E. coli* cells were subjected to Spanish oregano (0.013% v/v) using the measurement of UV absorbance at 260nm compared to that of control cells that had not been treated with the oil (Oussalah *et al.*, 2006). This loss of cell constituents was suggested to mean that an irreversible damage to the cell membrane had occurred, although other studies have demonstrated the action of EOs to bacteriostatic indicating cell recovery (Fisher and Phillips, 2006, Burt, 2004, Lambert *et al.*, 2001).

Although an increased permeability of the cell membrane is observed the loss of ATP that occurs within the enterococcal cells does not leak into the surrounding fluid (Fig. 7.1). This suggests the loss of intracellular ATP may be due to the lack of synthesis or increased hydrolysis of ATP, the uptake of the oil/vapour into the cell thus may be interfering with normal cellular function rather than increased permeability of the cell membrane to ATP. Similar results have been found using carvacrol against *B. cereus* where intracellular ATP was reduced to near zero after 14 minutes exposure, yet there was no increase in the extracellular ATP pool (Ultee *et al.*, 1999), whilst Helander *et al* (1998) observed a leakage of ATP from the cells to the surrounding environment when using carvacrol. Ultee *et al.* (1999) suggested this might be due to the differences in the Gram-negative cell membrane studied by Helander *et al.* Ossalah *et al.* (2005) found that the use of EO of Chinese cinnamon oil against *E. coli* and *L. monocytogenes* had the same effect on both Gram-positive and Gram-negative membranes with a loss of intracellular ATP and an increase of extracellular ATP, agreeing with Helander *et al* (1998). However the use of the Chinese cinnamon oil (0.025% v/v) causes a gradual decrease in intracellular ATP against *L. monocytogenes* (6.26 to 4.09 ng/ml), whereas only a slight increase was seen in the extracellular ATP. Increasing concentrations of EO resulted in greater loss of intracellular ATP 4.09 ng/ml, 1.60 ng/ml and 0.22 ng/ml with 0.025, 0.05, and 0.1% (v/v) respectively of EO. In *E. coli* when subjected to 0.025% (v/v) of the EO the intracellular ATP significantly decreased (1.84 ng/ml to 1.09 ng/ml), as did the increase in extracellular ATP (0.03 ng/ml to 0.05 ng/ml), but the decreases in ATP levels remained stable with increasing concentrations of the EO (Oussalah *et al.*, 2006). Although the use of vanillin (50 mmol l^{-1}) against *E. coli* only leaked small quantities of ATP into the external environment, yet when carvacrol 3.3

(50 mmol l^{-1}) was added there was a rapid decrease of internal ATP to undetectable levels within 10 minutes and a greatly increased external ATP pool (Fitzgerald *et al.*, 2004). The conflicting data presented by these studies suggests the way in which the use of EOs affects intracellular ATP is dependent on the EO itself and the concentration and the bacterial strain that it is acting upon. The most relevant data to this present study is the probably the use of EOs against *Lactobacillus* sp., a closely related species to *Enterococcus* sp. The addition of carvacrol (50 mmol l^{-1}) against *L. plantarum* showed deceases in internal ATP and only small increases in extracellular ATP and *L. innocua* treated with vanillin (50 mmol l^{-1}) showed reduced levels of intracellular ATP and no extracellular ATP was detected agreeing with the findings of this present study (Fitzgerald *et al.*, 2004).

The membrane potential is the driving force of ATP synthesis thus the reduction of internal ATP is coupled with the loss of membrane potential observed in these experiments (Fig. 7.2). Studies on carvacrol have shown that the loss of membrane potential is proportional to the concentration of the acting substance present with reductions of 0.01 and 0.25 U/s at 0.01 and 0.5 mM respectively in *B. cereus* cells (Ultee *et al.*, 1999). Should the MIC/MID be increased from the values used in this present study then complete loss of membrane potential may have been observed. However, this would have implications in the application of this citrus EO blend, within the food industry since increasing concentration may affect organoleptic properties of the food and, in the clinical arena, the increased concentrations could bring about allergic reactions.

Normal homeostasis of the cell would allow for the internal pH to be maintained via ion channels, in the presence of the citrus EO blend this delicate balance is lost and due to the disruption of the membrane integrity there is a loss of control of the H^+ ion gradients, with a drop of approximately pH 1.5 after being subjected to both direct oil and vapour (Table 7.3). Most studies into the mechanism of action of EOs observed the loss of pH homostatsis when being subjected to tea tree, vanillin, thyme, oregano and carvacrol (Cox *et al.*, 2000, Fitzgerald *et al.*, 2004, Lambert *et al.*, 2001). The internal pH of *E. coli* was reduced from pH 7.25 to 6.68 and pH 5.16 to 5.94 in the presence of 0.025% Spanish oregano and Chinese cinnamon respectively, this loss of pH correlated with the accumulation of the compounds in the cytoplasmic membrane, as the hydrophobic nature of EOs allows them to dissolve in the membrane (Oussalah *et al.*, 2006).

Homeostasis of internal pH is essential for cellular processes such as DNA transcription, protein synthesis and enzyme activity (Oussalah *et al.*, 2006). Therefore change in internal pH of the cell could also limit ATP synthesis with the denaturing of some of the enzymes involved in the process and the loss of specific ions involved in the proton motive force driving the proton pumping H^+-ATPase, regardless of glucose present needed as an energy source for this process (Cox *et al.*, 2000). The presence of glucose does have some protective role against the loss of internal pH in cells subjected to EOs. The results of study by Lambert *et al.* (2001) showed that when the external pH of *E. coli* was adjusted to 5.05 it took two hours for the internal pH to reach 5.05, whereas in the presence of 0.05% oregano it only took 30 minutes. When the same experiment was carried out in the presence of glucose where external pH was adjusted to 4.05, the control had an internal pH of 4.6 after two hours but the cells subjected to

0.05% of oregano reached pH 4.05 in the same time period, thus the presence of glucose slows the effect of the EO on changes in pH.

The bacteriostatic effect of the citrus blend suggests that the cells have a mechanism in place to recover from the damage incurred after being exposed to the blend oil/vapour. DnaK is involved in the general stress response of *Enterococcus* sp. and *E. faecalis* has been shown to rapidly up regulate the synthesis of DnaK when exposed to environmental stresses, such as heat, acid, H_2O_2, NaCl and UV irradiation (Laport *et al.*, 2004). Therefore DnaK up-regulation was investigated as a possible mechanism by which the cells may be recovering from the EO blend. A faint band with a molecular mass of around 70 KDa corresponding to DnaK was present in the cytoplasmic proteins of both *E. faecalis* and *E. faecium* (Fig. 7.5b), but was undetected in the membrane protein fractions (Fig. 7.5a) of the control cells. In a study on *Cyanobacterium synechococcus* investigating the distribution of DnaK within the cell, it was found that it was mainly located in the cytosolic fraction as opposed to the membrane fractions where only a small amount of a subpopulation of DnaK was detected (Nimura. K *et al.*, 1996). It has also been demonstrated in *E. coli* that exponentially growing cells under control conditions have DnaK levels of 6.72 ng/μg of protein and that these levels rise to 14.93 ng/μg of protein after four hours of glucose starvation (Rackabrand *et al.*, 1995). The use of stationary cells in this investigation may be why only low levels of DnaK were detected. The synthesis of DnaK has been demonstrated to correlate with the growth rate of the cells and therefore is at its lowest levels when in stationary phase of growth (Koch *et al.*, 1998). However, when 56 strains of *Streptococcus* in exponential and stationary phase were exposed to acid, osmotic, oxidative and heat stresses and monitored for their adaptive responses and HSP

present, it was found there was a significant changes in the protein range of 70-55 kDa and 25-10 kDa which included the chaperones DnaK, GprE, GroEL and GroES. The increase in intensity of these bands correlated with the growth phase of the cells as no significant correlation was made between the band intensity and stress response (Zotta *et al.*, 2008).

The absence of DnaK in the cytoplasmic proteins of *E. faecalis* and *E. faecium* which have been exposed to the citrus blend, compared to the presence of DnaK in the control cytoplasmic protein fraction, indicates that the citrus blend reduces DnaK protein expression (Fig. 7.5). DnaK expression has been shown to be dependent on the bacterial species and the type of stress. In *E. coli* DnaK is a general stress protein and is induced by a range of stresses whereas in *B. subtilis* DnaK is induced by heat but not salt or glucose deprivation, thus having a more limited role in the stress response of the cells (Koch *et al.*, 1998). The EO blend may not induce the synthesis of DnaK in a stress response in *Enterococcus* sp., but another HSP such as GroEL could be involved. HSPs are induced in *L. plantarum* when subjected to 72°C, but when a bacteriostatic concentration of chloramphenicol (1 mg ml^{-1}) was applied to the cells the synthesis of HSPs and tolerance of the strain to this temperature were greatly reduced, demonstrating that the overall environment i.e. pH, water activity, salt content and preservatives has an affect on the expression on HSPs (De Angelis *et al.*, 2003). Therefore the expression of DnaK may be induced by the citrus oil blend if combined with other stresses. Another reason for the apparent reduction of expression of DnaK in the test cells may be associated with the loss of ATP activity (Fig. 7.3 & Table 7.2) DnaK controls protein folding after thermal damage in a ATP-dependent process involving the co-chaperones DnaJ and GrpE (Rackabrand *et al.*, 1995) and binds and

dissociates from its substrate in an ATP-dependent manner (Nimura. K *et al.*, 1996). Therefore, the depletion of ATP within the cells caused by the addition of the EO blend may also result in ATP-dependent processes, such as the recovery of cells by the re-folding of proteins induced by DnaK, to lapse, leading to further cell damage and limitations on the "choice" of recovery pathway of the cell. Eukaryotic cytosolic Hsp70 proteins require K^+ for ATPase activity and have two K^+ bound in the catalytic site. One study on *Thermus thermophilus* found that ATPase activity was dependent on K^+ and T.GrpE and that the system did not work with either alone. K^+ could not be replaced by NaCl, indicating the specificity of the system (Motohashi *et al.*, 1997). Thus the permeability of the cell (Fig. 7.1) may have resulted in the loss of K^+ ions and contributed to DnaK being unable to play a protective role against the EO blend. The faint band visualised in the cytoplasmic protein fraction of the control cells may suggest that low levels of DnaK are present in *E. faecium* and *E. faecalis* under non-stress growth conditions, similar results have been found in *E. coli* with DnaK being present at a level of 6.72 ng/μg of protein under non-stress conditions (Rackabrand *et al.*, 1995). Due to the colorimetric system of detection used in this investigation not being quantitative, the levels visualised in Fig 7.5b would be between 6.25 ng/μg and 3.125 ng/μg of protein, at which point the system used can no longer detect the DnaK protein. Further investigations would need to be carried out to quantify the faint band detected in the control cells of the cytoplasmic protein fractions of *E. faecium* and *E. faecalis* using a more sensitive and quantitative detection system such as luminescence.

In conclusion this study has demonstrated that the uptake of the citrus EO blend into enterococcal cells changes permeability of the cell membrane and reducing ATP synthesis and internal pH. Although the vapours of the orange/bergamot blend result in

the same damage, they are probably acting via different mechanisms to those of the EO because of the increased permeability of the cell membrane and particularly the different morphological changes that they induce. The HSP DnaK does not aid recovery of the cells from exposure to the citrus blend; this may be related to the ATP-dependent nature of the protein. Due to the bacteriostaic nature of EO blend the findings of this investigation only go part of the way in understanding the mechanisms by which EOs produce their antimicrobials effect and further investigations need to be carried out on the effect of the blend on specific enzymes, proteins and metabolic processes and the mechanisms by which the cells recover.

Chapter 8

Discussion

8.1 The inhibitory effect of a range of citrus essential oils and their vapours on the growth and survival of E. faecium and E. faecalis at a range of temperatures and pHs.

Enterococcus sp. are adapted to withstand a large range of adverse environmental conditions including temperatures from 5 °C to 50°C, pH (4.5-10.5), up to 40% (w/v) bile salts and 6.5% NaCl, as well as having a antibiotic resistance to a large range of antibiotic glycopeptides within both the clinical arena and food industry (Klein, 2003). Thus when EOs are being tested as antimicrobials, overall *Enterococcus* sp. is found to be one of the most resistant bacterial species. When the EO *Ziziphora persica* and its methanol extract was tested against 98 laboratory strains of bacteria *E. faecalis* was one of eleven that were resistant to the EO (Ozturk and Ercisli, 2006). Only three out of seven species from the Brazilian EOs genus *Cunila* had a strong inhibitatory effect against *E. faecalis* compared with *E. coli. L. monocytogenes, P. aeuruginosa* and *S. enteritidis* where all seven species showed a strong inhibition of with MICs > 5 mg/ml (Sandri *et al.*, 2007). Black thyme and wild savory EOs at a 1/50 dilution showed the smallest zones of inhibition 34.5 and 33 (mm) respectively against *E. faecalis* when compared with 14 other bacteria with the exception of *L. monocytogenes* (Baydar *et al.*, 2004). EO components borneol, carvacrol, limonene, methone and nerol showed no inhibition against *E. faecalis* although, all the components had an antimicrobial effect against other species of bacteria (Dorman and Deans, 2000). The results of a study investigating the effect of vanillin against a range of Gram- positive and Gram-negative bacteria showed that the lactic acid producing strains were most resistant to the EO (Fitzgerald *et al.*, 2004). What these studies

demonstrate is that the strong antimicrobial effect observed in this investigation by the citrus EO blend against both vancomycin susceptible and resistant *Enterococcus* sp. (Table 4.3, Figs. 4.7, 4.8, 5.4, 5.3 & 5.3) means that in the wider context the effect of the citrus blend may be effective against a large array of Gram-negative and Gram-positive bacteria, thus making the applications of wipes and vapours explored plausible general antimicrobial products.

The bacteriostatic effect of the citrus blend was observed throughout this study. When the cells were subject to the most inhibitory combination of temperature, pH and MIC of the citrus oil/vapour blend, the cells recovered from between 1-3 and 4.5 $\log_{10}$ from oil and vapour respectively. This bacteriostatic effect was further demonstrated when cells were subjected to the combination of temperature, pH and MIC/MID of the orange/bergamot blend or vapours over 24 hours, the live:dead ratio gradually increased to that of the control cells of approximately 23:0.5 (Table 4.6 and Fig. 4.9). The application of the vapours and oil on surfaces showed a recovery of cells from the use of impregnated citrus blend wipes of up to 10 $\log_{10}$ (Fig. 6.2) even though direct contact with the wipe destroyed up to 2 $\log_{10}$ (Fig 6.3). On-food investigations showed that after *Enterococcus* sp. were exposed to the orange/bergamot blend vapour on cucumber and lettuce cell recovery occurred after six hours (Fig. 5.5). The nature by which the citrus EO blend brings about its effect on *Enterococcus* sp. has implications on the potential application of the blend, due to the recovery of the cells.

The unique mixture of components observed within any given EO or blend of EO is thought to give it its antimicrobial effect. When the most inhibitory combination of citrus EO components were assessed (citral and linalool) an antagonistic effect was

observed (Table 4.5) with FICs of >4. Due to these components being the most effective against *Enterococcus* sp. individually (Table 4.3), it was thought that in combination this effect would increase, but this was not the case. When the components of the vapours were assessed for changes in composition at the most inhibitory time point of 45 seconds (Table 5.2) a drop in methanol was observed, yet this component is known to be inhibitory against microrganisms (Bajpai *et al.*, 2007, Al-Bayati, 2008), again suggesting that an individual component is not responsible for the antimicrobial effect of the EOs. Previous studies have suggested that overall the aldehydes, phenolics, terpenes and alcohols are responsible for the inhibition of growth of microrganisms (Su *et al.*, 2007, Burt, 2004, Fitzgerald *et al.*, 2004). Yet these major components only make up approximately 85% (Burt, 2004) of the EO, the other 15% may play some key role in the inhibition of bacteria. Otherwise blends of EOs would not be more effective than the oils alone (Table 4.3), considering citrus EOs have approximately the same major components (Borgmann *et al.*, 2004, Banes-Marshall *et al.*, 2001, Smith *et al.*, 2001, Flamini *et al.*, 2007) and also the major components are antagonistic when assessed alone. Therefore the results suggest that the unique blend of all the components and their interactions is what is vital for the antimicrobial effect of the citrus EO blend.

8.2 Potential applications of the citrus essential oils in food systems and on surfaces.

Overall the application of either the orange/bergamot EO in wipes or as a vapour on the decontamination of surfaces or on food showed no significant difference in inhibition against vancomycin susceptible or vancomycin resistant *E. faecium* and *E. faecalis* (Fig 6.1, 6.4, 5.4 & 5.5). Other studies have shown that alcohol, iodine and aldehyde based disinfectants also have no difference in effect against vancomycin resistant or

vancomycin susceptible *Enterococcus* sp. (Sakagami and Kajimura, 2002). This was also observed when the oil of EO blend was used in combination with temperature and pH with a $\log_{10}$ reduction of up to 10 for both susceptible and resistant strains (Figs. 4.6 & 4.7). Yet the vapours were less effective against the vancomycin resistant strains in combination with temperature and pH, suggesting that the virulence factors that lead to antibiotic resistance also create resistance to the vapours of the citrus blend, but when tested on-food and surfaces this seemed not to be the case. When the EOs were tested as an application in true-life situations the inhibitory action of the EO blend whether that be in wipe or vapour format is not as effective as when in combination with temperature and pH in culture. Reduction ranged from 2 $\log_{10}$ in EO impregnated wipes, 2.5 $\log_{10}$ for vapours on stainless steel surfaces and up to 4 $\log_{10}$ for vapours on food compared with a 5.5-10 $\log_{10}$ reduction in culture (Figs. 6.1, 5.4, 5.5, 4.6 & 4.7). Similar results were found when individual citrus EOs and their vapours were tested *in vitro* and on cabbage leaf and chicken skin (Fisher and Phillips, 2006). The use of the orange/bergamot blend vapours both on-food and on surfaces showed that by doubling the dosage from 15mg/L air to 30mg/L air a greater inhibitory effect was not observed (Figs. 5.6 & 6.4b), although in the case of the investigations into the use of EO vapour as a decontaminant, the greatest inhibitory effect did occur in the shorter time period of 15 hrs compared with 24 hrs, but the reduction in growth of *Enterococcus* sp. was not as great as 1.5 $\log_{10}$ compared to 2.5 $\log_{10}$ for 30mg/L air and 15mg/L air respectively.

8.3 To investigate the mechanisms of action of citrus essential oils.

When cells were subjected to the orange/bergamot EO blend oil and vapour the homeostasis of the cell was disrupted with a loss of membrane integrity and potential, pH and ATP (Table 7.1, 7.2 & 7.3 and Figs. 7.1, 7.2 & 7.3) which lead to sublethal damage, complete lysis of the cells was not observed. In the case of blend EO the cell was only twice as permeable as the control cells. However in the presence of the vapours there was a 30 times increase in the permeability of the cell (Table 7.1). This may be due to the differential separation of the molecules and increased kinetic energy resulting in the molecules having a greater ability to penetrate the cell. If this were the case it might be expected that there would be a greater bactericidal effect, but when assessing the results from Table 4.6 the cells recover in the same period of time to approximately the same number as when being subjected to the citrus blend oil. The membrane potential of the cells is approximately halved (Figs. 7.1 & 7.2) and, although the internal pH is greatly reduced, it is not completely lost (Table 7.3). The fact that internal ATP is not being leaked into the external environment (Fig 7.3) indicates that the membrane remains partially intact thus further indicating the bacteriostatic effect of the blend of citrus EOs. Other studies have shown when assessing the modes of action of EOs both bactericidal and bacteriostatic effects have occurred. The use of vanillin against both Gram-positive and Gram-negative bacteria showed that after the bacteria had been subjected to the EO only partial loss of viability was observed, whereas when cells were subjected to carvacrol complete loss of viability occurred. An investigation into the mechanism of action of vanillin showed ATP activity was inhibited but was dependent on the dosage at 20 – 100μmol l^{-1} and the loss of pH varied from complete loss of pH to no effect (Fitzgerald *et al.*, 2004). The use of the carvacrol (0.12mM)

against *B. cereus* showed a depletion of ATP, complete dissipation of pH gradient with leakage of K^+ ions into the external environment (Ultee *et al.*, 1999). The bacteriostatic, bactericidal differences of effect are thought to be due to the amount of phenolic compounds within the EO.

The cell size of *Enterococcus* sp. did not significantly alter after being subjected to the most inhibitory combination of temperature, pH and MIC/MID of the orange/bergamot blend (Fig 4.6), yet studies have shown a loss of cytoplasmic contents of cells after being subjected to EO (Rasooli *et al.*, 2006a, Helander *et al.*, 1998). This may be due to the uptake of the citrus blend into the cells as suggested in the TEM images (Fig. 7.4), the exchange of cytoplasmic contents for EO may occur, resulting in no change in cell size. Although there is no significant difference in size, *E. faecalis* after being exposed to the vapours is slightly smaller in size at 1.1μm compared with the control and oil-exposed cells (Fig 4.6) (1.6 and 1.3μm respectively). The other possibility is that, due to the bacteriostatic nature of the blend of EOs and no complete loss of membrane integrity, the loss of cell cytoplasmic content does not occur to such an extent that the cell size is altered significantly.

Overall the differences in the combinations of the temperature/pH and MIC/MID required to inhibit the growth of *E. faecium* and *E. faecalis* cells may be in some way explained by the nature of the mechanisms by which the citrus blend brings about its effect. Acid resistance of *E. faecalis* requires H^+ -ATPase activity (Nakajo *et al.*, 2005). The loss of ATP synthesis/hydrolysis (Fig. 7.3 & Table 7.2) could have lead to the lack of resistance to acid conditions with the combination of the blend and pH 5.5 in both the vancomycin resistant and vancomycin susceptible strains (Figs. 4.7, 4.8 & 4.9) having

the greatest inhibitory effect. The phospholipid concentration of the cell membrane alters dependent on the growth temperature and the more fluidity there is, the easier it is for EOs to cross the membrane (Karatzas *et al.*, 2000). The TEM images (Fig. 7.4) illustrate that at 37°C the citrus blend oil is able to cross the cell membrane thus suggesting that the cells have enough phospholipid fluidity to allow the EO to do so. Figure 4.6 showed that reduction of growth of vancomycin susceptible strains by the vapours was greater than the oil, but the vancomycin resistant strains were more susceptible to the oil than the vapours (Fig 4.6). This suggests that the membrane of the resistant strains may have greater phospholipid fluidity than the susceptible strains, thus allowing more EO blend to cross the membrane and act upon the cell. In the case of the susceptible strains the vapours possibly due to the smaller particle size or the lack of fluidity of the phospholipid in the membrane was able to enter the cell and have a greater inhibitory effect than the oil.

Due to the recovery of the cells observed during the hurdle experiments (Table 4.6 and Fig. 4.9) and the on-food and surface decontamination investigations (Fig. 6.2 & 5.5), the presence of HSP DnaK was assessed as a means of cell recovery (Fig. 7.5). The oils and vapours alone (temperature and pH were kept at optimal conditions i.e. 37°C and pH 7.5) did not induce DnaK this was thought to be partly due to the lack of ATP in the cells (Fig. 7.3 & Table 7.2). DnaK has been shown to aid in the recovery in cells under other stresses such as temperature. Increasing the temperature by 8°C from that of optimum conditions for *Bacillus* sp. showed 15 HSPs to be expressed one of which was DnaK (Periago *et al.*, 2002) and increased quantaties of DnaK is also expressed in heat stressed *S. theromphilus* (Zotta *et al.*, 2008). It was observed when *Enterococcus* sp. were subjected to combinations of hurdles at 25°C and 37°C, that the cells were more

resistant to the blend of EOs in combination with a temperature of 25°C (Figs. 4.6, 4.7 & 4.8), this could mean that the temperature stress could have increased levels of DnaK that then had a cross protective role against the blend of EOs. Due to the loss of ATP taking approximately six minutes (Fig. 7.3) to occur, increased expression of DnaK in response to temperature stress (25°C) may have already taken place and thus given the cells some protection to the blend at this temperature, however due to the lack of temperature stress at 37°C, DnaK expression may not have occurred and therefore no cross protection to the EO blend occurred.

8.4 Future Studies

The breadth of further research resulting from this project includes the mechanism of recovery of the cells from the EO blend and other possible stress proteins involved in this process for example the possible role of GroEL. However, for the orange/bergamot blend to be a viable commercial product it firstly needs to be tested against a range of pathogens including post harvest organisms such as *Penicillium* sp. (Caccioni *et al.*, 1998) and clinical pathogens including *Staphylococcus* sp. and its antibiotic resistant strain MRSA. Further applications of the vapours need to be investigated including possible uses within the greenhouse environment as an antimicrobial. Especially due to its inhibition of growth at high temperatures such as 50°C (Figs. 4.1, 4.2, 4.3, 4.4 & 4.5 and Table 4.3) and that pathogenic fungi alone reduce the yield of major foods and cash crops by 20% (Tzortzakis, 2007). The use of EOs for the decontamination of air has only recently been explored (Su *et al.*, 2007, Gaunt *et al.*, 2005, Nedorostova *et al.*, 2008) and the use of the EO orange/bergamot

blend vapours for this application in the clinical, domestic and food arena needs to be further investigated. Other studies may include the incorporation of the EO into food packaging as EOs *per se* have been shown to be effective in modified atmosphere packaging (MAP) with oregano reducing *S. typhimurium* by 1-2 $\log_{10}$ cfu/g^{-1} in meat when combined with MAP (Skandamis *et al.*, 2002). Due to the citrus blend being a natural product it may also have applications as an antimicrobial within the organic food sector.

Conclusions

In conclusion this study has shown that the combination of an orange/bergamot blend is more effective than the individual oils alone against both vancomycin susceptible and vancomycin resistant *Enterococcus* sp. In combination with pH and temperature, nonthermal stress conditions are more effective at inhibition, whilst the pH needed is dependent on species. The overall effect of the blend is bacteriostatic, with a mode of action where the uptake of the blend leads to the cell membrane being permeable, resulting in dissipation of the membrane potential and loss of pH and ATP. The blend has been shown to have potential application in both an impregnated wipe on surfaces and as a vapour against contamination of foodstuff and surfaces. With increasing antibiotic resistant bacteria and the organic sector of the food market being the fastest growing, at the same time as increasing incidents of food poisoning occurring from fresh salad and vegetable, the use of the citrus EO blend in both oil and vapour form could be the answer to the problems of both the clinical arena and food industry.

References

Al-Bayati, F. A. (2008) Synergistic antibacterial activity between *Thymus vulgaris* and *Pimpinella anisum* essential oils and methanol extracts. *Journal of Ethnopharmacology,* **116,** 403-406.

Al-Shuneigat, J., Cox, S. D. & Markham, J. L. (2005) Effects of a topical essential oil-containing formulation on biofilm-forming coagulase-negative *Staphylococci*. *Letters in Applied Microbiology,* **41,** 52-55.

Allende, A., Tomas-Barberan, F. A. & Gil, M. I. (2006) Minimal processing for healthy traditional foods. *Trends in Food Science & Technology,* **17,** 513-519.

American Chemical Society (2008). CAS Registry. Accessed 11 May 2008. Available from http://www.cas.org/expertise/cascontent/registry/regsys.html

Ananou, S., Garriga, M., Hugas, M., Maqueda, M., Martinez-Bueno, M., Galvez, A. & Valdivia, E. (2005) Control of *Listeria monocytogenes* in model sausages by enterocin AS-48. *International Journal of Food Microbiology,* **103,** 179-190.

Angelillo, I. F., Mazziotta, A. & Nicotera, G. (1999) Nurses and hospital infection control: knowledge, attitudes and behaviour of Italian operating theatre staff. *Journal of Hospital Infection,* **42,** 105-112.

Arce, A., Pobudkowska, A., Rodriguez, O. & Soto, A. (2007) Citrus essential oil terpenless by extraction using 1-ethyl-3-methylimidazolium ethylsulphate ionic liquid: Effect of the temperature. *Chemical Engineering Journal,* **133,** 213-218.

Arias, B. A. & Ramon-Laca, L. (2005) Pharmacological properties of citrus and their ancient and medieval uses in the Mediterranean region. *Journal of Ethnopharmacology,* **97,** 89-95.

Aumo, J., Warna, J., Salmi, T. & Murzin, D. Y. (2006) Interaction of kinetics and internal diffusion in complex catalytic three-phase reactions: Activity and selectivity in citral hydrogenation. *Chemical Engineering Science,* **61,** 814-822.

Baharak, S., Hallgren, A., Jonasson, J., Nilsson, L., Hanberger, H. & Isaksson, B. (2002) Modified pulsed-field gel electophoresis protocol for typing of enterococci. *APMIS,* **110,** 869-74.

Bajpai, V. K., Rahman, A., Choi, U. K., Youn, S. J. & Kang, S. C. (2007) Inhibitory parameters of the essential oil and various extracts of *Metasequoia glyptostroboides* Miki ex Hu to reduce food spoilage and food-borne pathogens. *Food Chemistry,* **105,** 1061-1066.

Bakkali, F., Averbeck, D., Averbeck, S. & Idaomar, M. (2007) Biological effects of essential oils - a review. *Food and Chemical Toxicology,* **46,** 446-475.

Bakkali, F., Averbeck, S., Averbeck, D., Zhiri, A., Baudoux, D. & Idaomar, M. (2006) Antigenotoxic effects of three essential oils in diploid yeast (*Saccharomyces cerevisiae*) after treatments with UVC radiation, 8-MOP plus UVA and MMS. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* **606,** 27-38.

Bakkali, F., Averbeck, S., Averbeck, D., Zhiri, A. & Idaomar, M. (2005) Cytotoxicity and gene induction by some essential oils in the yeast *Saccharomyces cerevisiae*. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* **585,** 1-13.

Banes-Marshall, L., Cawley, P. & Phillips, C. A. (2001) *In vitro* activity of *Melaleuca alternifolia* (tea tree) oil against bacterial and *Candida* spp. isolates from clinical specimens. *British Journal of Biomedical Science,* **58,** 001-007.

Bauer, W. A., Kirby, W. M., Sherris, J. C. & Turck, C. (1966) Antibiotic susceptability testing by a standardised single disc method. *American Journal of Clinical Pathology,* **45,** 493-496.

Baydar, H., Sagdic, O., Ozkan, G. & Karadogan, T. (2004) Antibacterial activity and composition of essential oils from Origanum, Thymbra and Satureja species with commercial importance in Turkey. *Food Control,* **15,** 169-172.

Bearman, G. M. L. & Wenzel, R. P. (2005) Bacteraemias: A leading cause of death. *Archives of Medical Research,* **36,** 646-659.

Becerril. R, Gómez-Lus. R, Goñi. P, López. P & Nerín. C (2007) Combination of analytical and microbiological techniques to study the antimicrobial activity of a new active food packaging containing cinnamon or oregano against *E. coli* and *S. aureus, Analytical and Bioanalytical Chemistry.* **388,** 1003-1011.

Belletti, N., Nadagijimana, M., Sisto, C., Guerzoni, M. E., Lanciotti, R. & Gardini, F. (2004) Evaluation of the antimicrobial activity of citrus essences on *Saccharomyces cerevisiae. Journal of Agricultural and Food Chemistry,* **17;52,** 6932-8.

Benvenuti, F., Gironi, F. & Lamberti, L. (2001) Supercritical deterpenation of lemon essential oil, experimental data and simulation of the semicontinuous extraction process. *The Journal of Supercritical Fluids,* **20,** 29-44.

Bessems, E. (1998) The effect of practical conditions on the efficacy of disinfectants. *International Biodeterioration & Biodegradation,* **41,** 177-183.

Borgmann, S., Niklas, D. M., Klare, I., Zabel, L. T., Buchenau, P., Autenrieth, I. B. & Heeg, P. (2004) Two episodes of vancomycin-resistant *Enterococcus faecium* outbreaks caused by two genetically different clones in a newborn intensive care unit. *International Journal of Hygiene and Environmental Health,* **207,** 386-389.

Boyce, J. M., Opal, S. M., Chow, J. W., Zervos, M. J., Potter-Bynoe, G., Sherman, C. B., Romulo, R. L., Fortna, S. & Medeiros, A. A. (1994) Outbreak of multidrug-resistant *Enterococcus faecium* with transferable *vanB* class vancomycin resistance. *Journal of Clinical Microbiology,* **32,** 1148-1153.

Brown, D. F. J., Brown, N. M., Cookson, B. D., Duckworth, G., Farrington, M., French, G. L., King, L., Lewis, D., Livermore, D. M. & Macrae, B. (2006) National glycopeptide-resistant enterococcal bacteraemia surveillance working group report to the department of health -- August 2004. *Journal of Hospital Infection,* **62,** 1-27.

Bruni, R., Medici, A., Andreotti, E., Fantin, C., Muzzoli, M., Dehesa, M., Romagnoli, C. & Sacchetti, G. (2004) Chemical composition and biological activities of Ishpingo essential oil, a traditional Ecuadorian spice from Ocotea quixos (Lam.) Kosterm. (Lauraceae) flower calices. *Food Chemistry,* **85,** 415-421.

Buiarelli, F., Cartoni, G. P., Coccioli, F. & Leone, T. (1996) Analysis of bitter essential oils from orange and grapefruit by high-performance liquid chromatography with microbore columns. *Journal of Chromatography A,* **730,** 9-16.

Burt, S. (2004) Essential oils: their antibacterial properties and potential applications in foods--a review. *International Journal of Food Microbiology,* **94,** 223-253.

Busani, L., Del Grosso, M., Paladini, C., Graziani, C., Pantosti, A., Biavasco, F. & Caprioli, A. (2004) Antimicrobial susceptibility of vancomycin-susceptible and -resistant *Enterococci* isolated in Italy from raw meat products, farm animals, and human infections. *International Journal of Food Microbiology,* **97,** 17-22.

Busatta, C., Vidal, R. S., Popiolski, A. S., Mossi, A. J., Dariva, C., Rodrigues, M. R. A., Corazza, F. C., Corazza, M. L., Vladimir Oliveira, J. & Cansian, R. L. (2008)

Application of *Origanum majorana L.* essential oil as an antimicrobial agent in sausage. *Food Microbiology,* **25,** 207-211.

Caccioni, D. R. L., Guizzardi, M., Biondi, D. M., Agatino, R. & Ruberto, G. (1998) Relationship between volatile components of citrus fruit essential oils and antimicrobial action on *Penicillium digitatum* and *Penicillium italicum. International Journal of Food Microbiology,* **43,** 73-79.

Campos, C. A., Rodriguez, O., Calo-Mata, P., Prado, M. & Barros-Velazquez, J. (2006) Preliminary characterization of bacteriocins from Lactococcus lactis, *Enterococcus faecium* and *Enterococcus mundtii* strains isolated from turbot (Psetta maxima). *Food Research International,* **39,** 356-364.

Canzek Majhenic, A., Rogelj, I. & Perko, B. (2005) *Enterococci* from Tolminc cheese: Population structure, antibiotic susceptibility and incidence of virulence determinants. *International Journal of Food Microbiology,* **102,** 239-244.

Carnesecchi, S., Bradaia, A., Fischer, B., Coelho, D., Scholler-Guinard, M., Gosse, F. & Raul, F. (2002) Perturbation by geraniol of cell membrane permeability and signal transduction pathways in human colon cancer cells. *Journal Pharmacological Experimental Ther,* **303,** 711-5.

Carnesecchi, S., Bras-Goncalves, R., Bradaia, A., Zeisel, M., Gosse, F., Poupon, M.-F. & Raul, F. (2004) Geraniol, a component of plant essential oils, modulates DNA synthesis and potentiates 5-fluorouracil efficacy on human colon tumor xenografts. *Cancer Letters,* **215,** 53-59.

Carr, F. J., Chill, D. & Maida, N. (2002) The lactic acid bacteria: A literature survey. *Critical Reviews in Microbiology,* **28,** 281-370.

Celiktas, O. Y., Kocabas, E. E. H., Bedir, E., Sukan, F. V., Ozek, T. & Baser, K. H. C. (2007) Antimicrobial activities of methanol extracts and essential oils of *Rosmarinus officinalis,* depending on location and seasonal variations. *Food Chemistry,* **100,** 553-559.

Chang, S.-T., Chen, P.-F. & Chang, S.-C. (2001) Antibacterial activity of leaf essential oils and their constituents from *Cinnamomum osmophloeum. Journal of Ethnopharmacology,* **77,** 123-127.

Chenn, H. & Hoover, D. G. (2003) Bacteriocins and their food applications. *Comprehensive Reviews in Food Science and Food Safety,* **2,** 82-100.

Chida, M., Tyamashita, K., Izumyia, Y., Wantanabe, K. & Tamura, H. (2006) Aroma impact compunds in three Citrus oils: cross-matching test and correspondence analysis approach. *Journal of Food Science,* **71,** 56-58.

Chun, S.-S., Vattem, D. A., Lin, Y.-T. & Shetty, K. (2005) Phenolic antioxidants from clonal oregano (*Origanum vulgare*) with antimicrobial activity against *Helicobacter pylori. Process Biochemistry,* **40,** 809-816.

Clarys, P., Alewaeters, K., Jadoul, A., Barel, A., Manadas, R. O. & Preat, V. (1998) *In vitro* percutaneous penetration through hairless rat skin: influence of temperature, vehicle and penetration enhancers. *European Journal of Pharmaceutics and Biopharmaceutics,* **46,** 279-283.

Cleveland, J., Montville, T. J., Nes, I. F. & Chikindas, M. L. (2001) Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology,* **71,** 1-20.

Courvalin, P. (2005) Genetics of glycopeptide resistance in Gram-positive pathogens. *International Journal of Medical Microbiology,* **294,** 479-489.

Cox, L. J., Kleiss, T., Cordier, J. L., Cordellana, C., Konkel, P., Pedrazzini, C., Beumer, R. & Siebenga, A. (1989) *Listeria* spp. in food processing, non-food and domestic environments. *Food Microbiology,* **6,** 49-61.

Cox, S. D., Mann, C. M., Markham, J. L., Bell, H. C., Gustafson, J. E., Warmington, J. R. & Wyllie, S. G. (2000) The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *Journal of Applied Microbiology,* **88,** 170-175.

Dabbah, R., Edwards, V. M. & Moats, W. A. (1970) Antimicrobial action of some citrus fruit oils on selected food-borne bacteria. *Applied Microbiology,* **19,** 27-31.

De Angelis, M., Di Cagno, R., Huet, C., Crecchio, C., Fox, P. & Gobbetti, M. (2003) Heat Shock Responce in *Lactobacillus plantarum. Applied and Environmental Microbiology,* **0099-2240,** 1336-1346.

De Castro, A., Montano, A., Casado, F. J., Sanchez, A. H. & Rejano, L. (2002) Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starter cultures for Spanish-style green olive fermentation. *Food Microbiology,* **19,** 637-644.

De Fatima Silva Lopes, M., Ribeiro, T., Abrantes, M., Figueiredo Marques, J. J., Tenreiro, R. & Crespo, M. T. B. (2005) Antimicrobial resistance profiles of dairy and clinical isolates and type strains of *Enterococci. International Journal of Food Microbiology,* **103,** 191-198.

De Kwaadsteniet, M., Todorov, S. D., Knoetze, H. & Dicks, L. M. T. (2005) Characterization of a 3944 Da bacteriocin, produced by *Enterococcus mundtii* ST15, with activity against Gram-positive and Gram-negative bacteria. *International Journal of Food Microbiology,* **105,** 433-444.

De Vuyst, L., Foulquie Moreno, M. R. & Revets, H. (2003) Screening for enterocins and detection of hemolysin and vancomycin resistance in *Enterococci* of different origins. *International Journal of Food Microbiology,* **84,** 299-318.

Deans, S. G. & Ritchie, G. (1987) Antibacterial properties of plant essential oils. *International Journal of Food Microbiology,* **5,** 165-180.

Deegan, L. H., Cotter, P. D., Hill, C. & Ross, P. (2006) Bacteriocins: Biological tools for bio-preservation and shelf-life extension. *International Dairy Journal,* **16,** 1058-1071.

DEFRA (2006) The UK organic vegetable market (2004-2005 season).

Delaquis, P. J., Stanich, K., Girard, B. & Mazza, G. (2002) Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *International Journal of Food Microbiology,* **74,** 101-109.

DeVere, E. & Purchase, D. (2007) Effectiveness of domestic antibacterial products in decontaminating food contact surfaces. *Food Microbiology,* **24,** 425-431.

Dharan, S. & Pittet, D. (2002) Environmental controls in operating theatres. *Journal of Hospital Infection,* **51,** 79-84.

Domig, K. J., Mayer, H. K. & Kneifel, W. (2003) Methods used for the isolation, enumeration, characterisation and identification of *Enterococcus spp.*: 2. Pheno- and genotypic criteria. *International Journal of Food Microbiology,* **88,** 165-188.

Dordevic, S., Petrovic, S., Dobric, S., Milenkovic, M., Vucicevic, D., Zizic, S. & Kukic, J. (2007) Antimicrobial, anti-inflammatory, anti-ulcer and antioxidant activities of *Carlina acanthifolia* root essential oil. *Journal of Ethnopharmacology,* **109,** 458.

Dorman, H. J. D. & Deans, S. G. (2000) Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology,* **88,** 308-316.

Dufour, M., Simmonds, R. S. & Bremer, P. J. (2003) Development of a method to quantify in vitro the synergistic activity of "natural" antimicrobials. *International Journal of Food Microbiology,* **85,** 249-258.

Dugo, P., Mondello, L., Dugo, L., Stancanelli, R. & Dugo, G. (2000) LC-MS for the identification of oxygen heterocyclic compounds in citrus essential oils. *Journal of Pharmaceutical and Biomedical Analysis,* **24,** 147-154.

Dupont, S., Caffin, N., Bhandari, B. & Dykes, G. A. (2006) *In vitro* antibacterial activity of Australian native herb extracts against food-related bacteria. *Food Control,* **17,** 929-932.

Dusan, F., Marian, S., Katarina, D. & Dobroslava, B. (2006) Essential oils - their antimicrobial activity against *Escherichia coli* and effect on intestinal cell viability. *Toxicology in Vitro,* **20,** 1435-1445.

Eaton, T. J. & Gasson, M. J. (2002) A variant Enterococcal surface protein Espfm in *Enterococcus faecium*; distribution among food, commensal, medical, and environmental isolates. *FEMS Microbiology Letters,* **216,** 269-275.

Edwards-Jones, V., Buck, R., Shawcross, S. G., Dawson, M. M. & Dunn, K. (2004) The effect of essential oils on meticillin-resistant *Staphylococcus aureus* using a dressing model. *Burns,* **30,** 772-777.

El-Sharoud, W. M. (2005) Two-component signal transduction systems as key players in stress responses of lactic acid bacteria. *Science Progress,* **88,** 203-28.

Emborg, H. D., Andersen, J. S., Seyfarth, A. M., Andersen, S. R., Boel, J. & Wegener, H. C. (2003) Relations between the occurrence of resistance to antimicrobial growth promoters among *Enterococcus faecium* isolated from broilers and broiler meat. *International Journal of Food Microbiology,* **84,** 273-284.

Esel, D., Doganay, M., Bozdemir, N., Yildiz, O., Tezcaner, T., Sumerkan, B., Aygen, B. & Selcuklu, A. (2002) Polymicrobial ventriculitis and evaluation of an outbreak in a surgical intensive care unit due to inadequate sterilization. *Journal of Hospital Infection,* **50,** 170-174.

Fernandez-Lopez, J., Zhi, N., Aleson-Carbonell, L., Perez-Alvarez, J. A. & Kuri, V. (2005) Antioxidant and antibacterial activities of natural extracts: application in beef meatballs. *Meat Science,* **69,** 371-380.

Fisher, K. & Phillips, C. (2006) The effect of lemon, orange and bergamot essential oils and their components on the survival of *Campylobacter jejuni, Escherichia coli O157, Listeria monocytogenes, Bacillus cereus* and *Staphylococcus aureus in vitro* and in food systems. *Journal of Applied Microbiology,* **101,** 1232-1240.

Fisher, K. & Phillips, C. (2008) Potential antimicrobial uses of essential oils in food: is citrus the answer? *Trends in Food Science & Technology,* **19,** 156-164.

Fisher, K., Rowe, C. & Phillips, C. (2007) The survival of three strains of *Arcobacter butzleri* in the presence of lemon, orange and bergamot essential oils and their components *in vitro* and on food. *Letters in Applied Microbiology,* **44,** 495-499.

Fitzgerald, D. J., Stratford, M., Gasson, M. J., Ueckert, J., Bos, A. & Narbad, A. (2004) Mode of antimicrobial action of vanillin against *Escherichia coli, Lactobacillus plantarum* and *Listeria innocua. Journal of Applied Microbiology,* **97,** 104-113.

Flahaut, S., Frere, J., Boutibonnes, P. & Auffray, Y. (1996a) Comparison of the bile salts and sodium dodecyl sulfate stress responces in *Enterococcus faecalis. Applied Environmental Microbiology,* **62,** 2416-20.

Flahaut, S., Hartke, A., Giard, J. C. & Auffray, Y. (1996b) Alkaline Stress Respnce in *Enterococcus faecalis*: Adaptation, Cross-Protection and Changes in Protein Synthesis. *Applied and Environmental Microbiology,* **0099-2240,** 812-814.

Flamini, G., Tebano, M. & Cioni, P. (2007) Volatiles emission patterns of different plant oragns and pollen of *Citrus limon*. *Analytica Chimica Acta*, **589**, 120-124.

Food and Agriculture Organisation & World Health Organisation (2002) Guidelines for the evaluation of probiotics in food.

Food and Drug Administration (2005). GRAS notifications. Accessed 13 December 2005. Available from www.fda.gov

Foulquie Moreno, M. R., Sarantinopoulos, P., Tsakalidou, E. & De Vuyst, L. (2006) The role and application of *Enterococci* in food and health. *International Journal of Food Microbiology*, **106**, 1-24.

Franz, C. M. A. P., Holzapfel, W. H. & Stiles, M. E. (1999) *Enterococci* at the crossroads of food safety? *International Journal of Food Microbiology*, **47**, 1-24.

Fritsche, T. R., Sader, H. S., Stilwell, M. G., Dowzicky, M. J. & Jones, R. N. (2005) Potency and spectrum of tigecycline tested against an international collection of bacterial pathogens associated with skin and soft tissue infections (2000-2004). *Diagnostic Microbiology and Infectious Disease*, **52**, 195-201.

FSA (2006) An evaluation of probiotic effects in the human gut: Microbial aspects.

Fukuda, D., Watanabe, M., Sonezaki, S., Sugimoto, S., Sonomoto, K. & Ishizaki, A. (2002) Molecular characterization and regulatory analysis of *dnaK* operon of halophilic lactic acid bacterium *Tetragenococcus halophila*. *Journal of Bioscience and Bioengineering*, **93**, 388-394.

Ganzera, M., Schneider, P. & Stuppner, H. (2006) Inhibitory effects of the essential oil of chamomile (*Matricaria recutita L.*) and its major constituents on human cytochrome P450 enzymes. *Life Sciences*, **78**, 856-861.

Garcia-Galaz, A., Perez-Morales, R., Diaz-Cinco, M. & Acedo-Felix, E. (2004) Resistance of *Enterococcus* strains isolated from pigs to gastrointestinal tract and antagonistic effect against *Escherichia coli* K88. *Rev Latinoam Microbiolology*, **46**, 5-11.

Garcia, M. T., Martinez Canamero, M., Lucas, R., Ben Omar, N., Perez Pulido, R. & Galvez, A. (2004) Inhibition of *Listeria monocytogenes* by enterocin EJ97 produced by *Enterococcus faecalis* EJ97. *International Journal of Food Microbiology*, **90**, 161-170.

Gardini, F., Martuscelli, M., Caruso, M. C., Galgano, F., Crudele, M. A., Favati, F., Guerzoni, M. E. & Suzzi, G. (2001) Effects of pH, temperature and NaCl concentration on the growth kinetics, proteolytic activity and biogenic amine production of *Enterococcus faecalis*. *International Journal of Food Microbiology*, **64**, 105-117.

Gastmeier, P., Schwab, F., Barwolff, S., Ruden, H. & Grundmann, H. (2006) Correlation between the genetic diversity of nosocomial pathogens and their survival time in intensive care units. *Journal of Hospital Infection*, **62**, 181-186.

Gaunt, L. F., Higgins, S. C. & Hughes, J. F. (2005) Interaction of air ions and bactericidal vapours to control micro-organisms. *Journal of Applied Microbiology*, **99**, 1324-1329.

Giraffa, G. (2002) *Enterococci* from foods. *FEMS Microbiology Reviews*, **26**, 163-171.

Giraffa, G. (2003) Functionality of enterococci in dairy products. *International Journal of Food Microbiology*, **88**, 215-222.

Gomes, B. C., Esteves, C. T., Palazzo, I. C. V., Darini, A. L. C., Felis, G. E., Sechi, L. A., Franco, B. D. G. M. & De Martinis, E. C. P. (2008) Prevalence and characterization of Enterococcus spp. isolated from Brazilian foods. *Food Microbiology*, **25**, 668-675

Griffith, C. J., Cooper, R. A., Gilmore, J., Davies, C. & Lewis, M. (2000) An evaluation of hospital cleaning regimes and standards. *Journal of Hospital Infection,* **45,** 19-28.

Guerra, N. P., Bernardez, P. F., Mendez, J., Cachaldora, P. & Pastrana Castro, L. (2006) Production of four potentially probiotic lactic acid bacteria and their evaluation as feed additives for weaned piglets. *Animal Feed Science and Technology,* **134,** 89-107.

Gutierrez, J., Barry-Ryan, C. & Bourke, P. (2008) The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology,* **124,** 91-97.

Halcon, L. & Milkus, K. (2004) *Staphylococcus aureus* and wounds: A review of tea tree oil as a promising antimicrobial. *American Journal of Infection Control,* **32,** 402-408.

Hartke, A., Giard, J. C., Laplace, J. M. & Auffray, Y. (1998) Survival of *Enterococcus faecalis* in an oligotrophic microcosm: changes in morphology, development of general stress resistance, and analysis of protein synthesis. *Appl Environ Microbiol,* **64,** 4238-45.

Hayouni, E. A., Bouix, M., Abedrabba, M., Leveau, J.-Y. & Hamdi, M. (2008) Mechanism of action of *Melaleuca armillaris (Sol. Ex Gaertu) Sm.* essential oil on six LAB strains as assessed by multiparametric flow cytometry and automated microtiter-based assay. *Food Chemistry,* **In Press, Accepted Manuscript.**

Hazra, A., Dollimore, D. & Alexander, K. (2002) Thermal analysis of evaporation of compounds used in aromatherapy using thermogravimetry. *Thermochimica Acta,* **392-393,** 221-229.

Health Protection Agency (2005). *Enterococcus* spp and Glycopeptide-Resistant *Enterococci* (GRE)
Accessed 2 February 2006. Available from: http://www.hpa.org.uk/infections/topics_az/enterococci/default.htm

Health Protection Agency (2006). The Health Protection Agency has 12 tips for avoiding infection from food. Accessed 28 September 2006. Available from http://www.hpa.org.uk/northwest/press/060608_foodsafety.htm

Health Protection Agency (2007). Bacteraemia
Accessed 05 June 2007. Available from: www.hpa.org.uk/cdr/pages/bacteraemia.htm#entero

Health Protection Agency (2008). Surveillance of surgical site infection in English hospitals 1997 - 2002. Accessed 22.07.08. Available from: http://www.hpa.org.uk

Helander, I. M., Alakomi, H. L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E. J., Gorris, L. G. M. & von Wright, A. (1998) Characterization of the action of selected essential oil components on gram-negative bacteria. *Journal of Agricultural and Food Chemistry,* **46,** 3590-3595.

Helander, I. M., Nurmiaho-Lassila, E. L., Ahvenainen, R., Rhoades, J. & Roller, S. (2001) Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. *International Journal of Food Microbiology,* **71,** 235-244.

Hickey, D. K., Kilcawley, K. N., Beresford, T. P. & Wilkinson, M. G. (2006) Starter bacteria are the prime agents of lipolysis in cheddar cheese. *J Agric Food Chem,* **54,** 8229-35.

Hili, P., Evans, C. S. & Veness, R. G. (1997) Antimicrobial action of essential oils : the effect of dimethylsulphoxide on the activity of cinnamon oil. *Letters in Applied Microbiology,* **24,** 269-275.

Holley, R. A. & Patel, D. (2005) Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiology,* **22,** 273-292.

Hugas, M., Garriga, M. & Aymerich, M. T. (2003) Functionality of *Enterococci* in meat products. *International Journal of Food Microbiology,* **88,** 223-233

Inouye, S. (2003) Laboratory of evaluation of gaseous essential oils (Part 1). *The International Journal of Aromatherapy,* **13,** 95-107.

Inouye, S., Abe, S., Yamaguchi, H. & Asakura, M. (2003) Comparative study of antimicrobial and cytotoxic effects of selected essential oils by gaseous and solution contacts. *International Journal of Aromatherapy,* **13,** 33-41.

Inouye, S., Takizawa, T. & Yamaguchi, H. (2001) Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. *Journal of Antimicrobial Chemotherapy,* **47,** 565-573.

Inouye, S., Uchida, K., Maruyama, N., Yamaguchi, H. & Abe, S. (2006) A novel method to estimate the contribution of the vapour activity of essential oils in agar diffusion assay. *Japanese Journal of Medical Mycology,* **47,** 91-98.

Ivanov, I. T., Boytcheva, S. & Mihailova, G. (1999) Parallel study of thermal resistance and permeability barrier stability of *Enterococcus faecalis* as affected by salt composition, growth temperature and pre-incubation temperature. *Journal of Thermal Biology,* **24,** 217-227.

Jeannot, V., Chahboun, J., Russell, D. & Baret, P. (2005) Quantification and determination of chemical composition of the essential oil extracted from natural orange blossom water (*Citrus aurantium L. ssp. aurantium*). *International Journal of Aromatherapy,* **15,** 94-97.

Kacmaz, B. & Aksoy, A. (2005) Antimicrobial resistance of *Enterococci* in Turkey. *International Journal of Antimicrobial Agents,* **25,** 535-538.

Karapinar, M. (1985) The effects of citrus oils and some spices on growth and aflatoxin production by *Aspergillus parasiticus* NRRL 2999. *International Journal of Food Microbiology,* **2,** 239-245.

Karatzas, A. K., Bennik, M. H. J., Smid, E. J. & Kets, E. P. W. (2000) Combined action of S-carvone and mild heat treatment on *Listeria monocytogenes* Scott A. *Journal of Applied Microbiology,* **89,** 296-301.

Katla, A.-K., Kruse, H., Johnsen, G. & Herikstad, H. (2001) Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products. *International Journal of Food Microbiology,* **67,** 147-152.

Kim, J., Marshall, M. R. & Wei, C. (1995a) Antibacterial activity of some essential oil components against five foodborne pathogens. *Journal of Agricultural and Food Chemistry,* **43,** 2839-2845.

Kim, J. M., Marshall, M. R., Cornell, J. A., Preston, J. F. & Wei, C. I. (1995b) Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella typhimurium* in culture medium and on fish cubes. *Journal of Food Science,* **60,** 1364-1374.

Klare, I., Konstabel, C., Badstubner, D., Werner, G. & Witte, W. (2003) Occurrence and spread of antibiotic resistances in *Enterococcus faecium. International Journal of Food Microbiology,* **88,** 269-290.

Klein, G. (2003) Taxonomy, ecology and antibiotic resistance of *Enterococci* from food and the gastro-intestinal tract. *International Journal of Food Microbiology,* **88,** 123-131.

Klein, G., Pack, A., Bonaparte, C. & Reuter, G. (1998) Taxonomy and physiology of probiotic lactic acid bacteria. *International Journal of Food Microbiology,* **41,** 103-125.

Knobben, B. A. S., van Horn, J. R., van der Mei, H. C. & Busscher, H. J. (2006) Evaluation of measures to decrease intra-operative bacterial contamination in orthopaedic implant surgery. *Journal of Hospital Infection,* **62,** 174-180.

Koch, B., Kilstrup, M., Vogensen, F. K. & Hammer, K. (1998) Induced levels of heat shock proteins in a *dnaK* mutant of *Lactococcus lactis. Journal of Bacteriology,* **180,** 3873-81.

Kolodjieva, V., Yafaev, R., Yermolenko, E. & Suvorov, A. (2006) Incidence of virulence determinants in Enterococcal strains of probiotic and clinical origin. *International Congress Series,* **1289,** 367-369.

Kotzekidou, P., Giannakidis, P. & Boulamatsis, A. (2007) Antimicrobial activity of some plant extracts and essential oils against foodborne pathogens *in vitro* and on the fate of inoculated pathogens in chocolate. *LWT - Food Science and Technology,* **41,** 119 127.

Kuhn, I., Iversen, A., Burman, L. G., Olsson-Liljequist, B., Franklin, A., Finn, M., Aarestrup, F., Seyfarth, A. M., Blanch, A. R. & Vilanova, X. (2003) Comparison of Enterococcal populations in animals, humans, and the environment - a European study. *International Journal of Food Microbiology,* **88,** 133-145.

Kuipers, O. P., Buist, G. & Kok, J. (2000) Current strategies for improving food bacteria. *Research in Microbiology,* **151,** 815-822.

Kurup, A., Chlebicki, M. P., Ling, M. L., Koh, T. H., Tan, K. Y., Lee, L. C. & Howe, K. B. M. (2008) Control of a hospital-wide vancomycin-resistant Enterococci outbreak. *American Journal of Infection Control,* **36,** 206-211.

Kusumaningrum, H. D., Riboldi, G., Hazeleger, W. C. & Beumer, R. R. (2003) Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *International Journal of Food Microbiology,* **85,** 227-236.

Lambert, R. J. W., Skandamis, P. N., Coote, P. J. & Nychas, G. J. E. (2001) A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology,* **91,** 453-462.

Lanciotti, R., Gianotti, A., Patrignani, F., Belletti, N., Guerzoni, M. E. & Gardini, F. (2004) Use of natural aroma compounds to improve shelf-life and safety of minimally processed fruits. *Trends in Food Science & Technology,* **15,** 201-208.

Laport, M. S., Lemos, J. A., Bastos Md Mdo, C., Burne, R. A. & Giambiagi-De Marval, M. (2004) Transcriptional analysis of the *groE* and *dnaK* heat-shock operons of *Enterococcus faecalis. Research in Microbiology,* **155,** 252-8.

Latasa, C., Solano, C., Penadés, J. R. & Lasa, I. (2006) Biofilm-associated proteins. *Comptes Rendus Biologies,* **329,** 849-857.

Leistner, L. (2000) Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology,* **55,** 181-186.

Leroy, F., Foulquie Moreno, M. R. & De Vuyst, L. (2003) *Enterococcus faecium* RZS C5, an interesting bacteriocin producer to be used as a co-culture in food fermentation. *International Journal of Food Microbiology,* **88,** 235-240.

Little, C. L. & Gillespie, I. A. (2008) Prepared salads and public health. *Journal of Applied Microbiology,* **In Press.**

Lloyd, S., Zervos, M., Mahayni, R. & Lundstrom, T. (1998) Risk factors for Enterococcal urinary tract infection and colonization in a rehabilitation facility. *American Journal of Infection Control,* **26,** 35-39.

Lopez-Malo, A., Alzamora, S. M. & Palou, E. (2005) *Aspergillus flavus* growth in the presence of chemical preservatives and naturally occuring antimicrobial compounds. *International Journal of Food Microbiology,* **99,** 119-128.

Lucas, R., Grande, M. J., Abriouel, H., Maqueda, M., Ben Omar, N., Valdivia, E., Martinez-Canamero, M. & Galvez, A. (2006) Application of the broad-spectrum bacteriocin enterocin AS-48 to inhibit *Bacillus coagulans* in canned fruit and vegetable foods. *Food and Chemical Toxicology,* **44,** 1774-1781.

Lucet, J. C., Armand-Lefevre, L., Laurichesse, J. J., Macrez, A., Papy, E., Ruimy, R., Deblangy, C., Lozach, A., Lolom, I., Jarlier, V., Andremont, A. & Leport, C. (2007) Rapid control of an outbreak of vancomycin-resistant enterococci in a French university hospital. *Journal of Hospital Infection,* **67,** 42-48.

Mahmoud, B. S. M., Yamazaki, K., Miyashita, K., Il-Shik, S., Dong-Suk, C. & Suzuki, T. (2004) Bacterial microflora of carp (*Cyprinus carpio*) and its shelf-life extension by essential oil compounds. *Food Microbiology,* **21,** 657-666.

Maki-Arvela, P., Kumar, N., Eranen, K., Salmi, T. & Murzin, D. Y. (2006) Inverse temperature dependence due to catalyst deactivation in liquid phase citral hydrogenation over Pt/Al2O3. *Chemical Engineering Journal,* **122,** 127-134.

Mandalari, G., Bennett, R. N., Bisignano, G., Trombetta, D., Saija, A., Faulds, C. B., Gasson, M. J. & Narbad, A. (2007) Antimicrobial activity of flavonoids extracted from bergamot (*Citrus bergamia* Risso) peel, a byproduct of the essential oil industry. *Journal of Applied Microbiology,* **103,** 2056-2064.

Mannu, L., Paba, A., Daga, E., Comunian, R., Zanetti, S., Dupre, I. & Sechi, L. A. (2003) Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. *International Journal of Food Microbiology,* **88,** 291-304.

Martinez, S., Lopez, M. & Bernardo, A. (2003) Thermal inactivation of *Enteroccocus faecium:* effect of growth temperature and physiologicalm state of microbial cells. *Letters in Applied Microbiology,* **37,** 475-481.

Maruzzella, J. C. & Sicurella, N. A. (1960) Antibacterial activity of essential oil vapours. *Journal of American Pharmaceutical Association.,* **49,** 692-4.

Metan, G., Zarakolu, P. & Unal, S. (2005) Rapid detection of antibacterial resistance in emerging Gram-positive cocci. *Journal of Hospital Infection,* **61,** 93-99.

Montecalvo, M. A., Jarvis, W. R., Uman, J., Shay, D. K., Petrullo, C., Rodeney, K., Gedris, C., Horowitz, H. W. & Wormser, P. (1999) Infection-control measures reduce transmission of vancomycin-resistant enterococci in an endemic setting. *Annals of Internal Medicine,* **131,** 269-272.

Moreira, M. R., Ponce, A. G., del Valle, C. E. & Roura, S. I. (2005) Inhibitory parameters of essential oils to reduce a foodborne pathogen. *LWT - Food Science and Technology,* **38,** 565-570.

Motohashi, K., Yohda, M., Odaka, M. & Yoshida, M. (1997) K+ is an indispensable cofactor for GrpE stimulation of ATPase activity of DnaK·DnaJ complex from *Thermus thermophilus. FEBS Letters,* **412,** 633-636.

Moufida, S. & Marzouk, B. (2003) Biochemical characterization of blood orange, sweet orange, lemon, bergamot and bitter orange. *Phytochemistry,* **62,** 1283-1289.

Mouly, P., Gaydou, E. M. & Auffray, A. (1998) Simultaneous separation of flavanone glycosides and polymethoxylated flavones in citrus juices using liquid chromatography. *Journal of Chromatography A,* **800,** 171- 179.

Murdoch, H., Taylor, D., Dickinson, J., Walker, J. T., Perrett, D., Raven, N. D. H. & Sutton, J. M. (2006) Surface decontamination of surgical instruments: an ongoing dilemma. *Journal of Hospital Infection,* **63,** 432-438.

Mutnick, A. H., Biedenbach, D. J. & Jones, R. N. (2003) Geographic variations and trends in antimicrobial resistance among *Enterococcus faecalis* and *Enterococcus faecium* in the SENTRY Antimicrobial Surveillance Program (1997-2000). *Diagnostic Microbiology and Infectious Disease,* **46,** 63-68.

Nakajo, K., Iwami, Y., Komori, R., Ishikawa, S., Ueno, T., Suzuki, Y. & Takahashi, N. (2005) The resistance to acidic and alkaline environments of endodontic pathogen *Enterococcus faecalis. International Congress Series,* **1284,** 191-192.

National Nosocomial Infection Surveillance (2004) System report, data summary from January 1992 through June 2004, issued October 2004. *A report from the NNIS System. American Journal of Infection Control,* 470-485.

Nazer, A. I., Kobilinsky, A., Tholozan, J. L. & Dubois-Brissonnet, F. (2005) Combinations of food antimicrobials at low levels to inhibit the growth of *Salmonella sv.* Typhimurium: a synergistic effect? *Food Microbiology,* **22,** 391-398.

Nedorostova, L., Kloucek, P., Kokoska, L., Stolcova, M. & Pulkrabek, J. (2008) Antimicrobial properties of selected essential oils in vapour phase against foodborne bacteria. *Food Control,* **In Press.**

Nguefack, J., Budde, B. B. & Jakobsen, M. (2004) Five essential oils from aromatic plants of Cameroon: their antibacterial activity and ability to permeabilize the cytoplasmic membrane of *Listeria innocua* examined by flow cytometry. *Letters in Applied Microbiology,* **39,** 395-400.

Nimura. K, Yoshikawa, H. & Takahashi, N. (1996) DnaK3, one of the three DnaK proteins of Cyanobacterium *Synechococcus* sp. PCC7942, is quantitatively detected in the thylakoid membrane. *Biochemical and Biophysical Research Communications,* **229,** 334-340.

Nogata, Y., Sakamoto, K., Shiratsuchi, H., Ishii, T., Yano, M. & Ohta, H. (2006) Flavonoid compostion of fruit tissue of citrus species. *Bioscience Biotechnology and Biochemistry,* **70,** 178-192.

O'Sullivan, L., Ross, R. P. & Hill, C. (2002) Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie,* **84,** 593-604.

Omar, N. B., Castro, A., Lucas, R., Abriouel, H., Yousif, N. M. K., Franz, C. M. A. P., Holzapfel, W. H., Ruben, P.-P., Martinez-Canamero, M. & Galvez, A. (2004) Functional and safety aspects of *Enterococci* isolated from different spanish foods. *Systematic and Applied Microbiology,* **27,** 118-130.

Opalchenova, G. & Obreshkova, D. (2003) Comparative studies on the activity of basil--an essential oil from *Ocimum basilicum L.* against multidrug resistant clinical isolates of the genera *Staphylococcus, Enterococcus* and *Pseudomonas* by using different test methods. *Journal of Microbiological Methods,* **54,** 105-110.

Ostrowsky, B. E., Trick, W. E., Sohn, A. H., Quirk, S. B., Holt, S., Carson, L. A., Hill, B. C., Arduino, M. J., Kuehnert, M. J. & Jarvis, W. R. (2001) Control of Vancomycin-Resistant Enterococcus in Health Care Facilities in a Region. *N Engl J Med,* **344,** 1427-1433.

Oussalah, M., Caillet, S. & Lacroix, M. (2006) Mechanism of action of spanish oregano, chinese cinnamon, and savory oils against cell membrane and walls of *Escherichia coli* O157:H7 and *Listeria monocytogenes. Journal of Food Protection,* **69,** 1046-1055.

Oussalah, M., Caillet, S., Saucier, L. & Lacroix, M. (2007) Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella Typhimurium, Staphylococcus aureus* and *Listeria monocytogenes*. *Food Control*, **18**, 414-420.

Ozturk, S. & Ercisli, S. (2006) The chemical composition of essential oil and in vitro antibacterial activities of essential oil and methanol extract of *Ziziphora persica* Bunge. *Journal of Ethnopharmacology*, **106**, 372-376.

Palhano, L. P., Vilches, T., Santos, R. B., Orlando, M., Ventura, J. & Fernandes, P. (2004) Inactivation of *Colletotrichum gloeosporioides* spores by high hydrostatic pressure combined with citral and lemongrass essential oil. *International Journal of Food Microbiology*, **95**, 61-66.

Papagianni, M. (2003) Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. *Biotechnology Advances*, **21**, 465-499.

Parish, E. P., Baum, D., Kryger, R., Goodrich, R. & Baum, R. (2003) Fate of *Salmonellae* in citrus oils and aqueous aroma. *Journal of Food Protection*, **66**, 1704-1707.

Pasquarella, C., Sansebastiano, G. E., Ferretti, S., Saccani, E., Fanti, M., Moscato, U., Giannetti, G., Fornia, S., Cortellini, P., Vitali, P. & Signorelli, C. (2007) A mobile laminar airflow unit to reduce air bacterial contamination at surgical area in a conventionally ventilated operating theatre. *Journal of Hospital Infection*, **66**, 313-319.

Peana, A. T., Marzocco, S., Popolo, A. & Pinto, A. (2006) (-)-Linalool inhibits in vitro NO formation: Probable involvement in the antinociceptive activity of this monoterpene compound. *Life Sciences*, **78**, 719-723.

Periago, P. M., Abee, T. & Wouters, J. A. (2002) Analysis of the heat-adaptive response of psychrotrophic *Bacillus weihenstephanensis*. *International Journal of Food Microbiology*, **79**, 17.

Periago, P. M. & Moezelaar, R. (2001) Combined effect of nisin and carvacrol at different pH and temperature levels on the viability of different strains of *Bacillus cereus*. *International Journal of Food Microbiology*, **68**, 141-148.

Peters, J., Mac, K., Wichmann-Schauer, H., Klein, G. & Ellerbroek, L. (2003) Species distribution and antibiotic resistance patterns of *Enterococci* isolated from food of animal origin in Germany. *International Journal of Food Microbiology*, **88**, 311-314.

Peterson, J. & Dwyer, J. (1998) Flavonoids: Dietary occurrence and biochemical activity. *Nutrition Research*, **18**, 1995-2018.

Peterson, J. J., Beecher, G. R., Bhagwat, S. A., Dwyer, J. T., Gebhardt, S. E., Haytowitz, D. B. & Holden, J. M. (2005) Flavanones in grapefruit, lemons, and limes: A compilation and review of the data from the analytical literature. *Journal of Food Composition and Analysis*, **19**, S74-S80.

Poeta, P., Costa, D., Rodrigues, J. & Torres, C. (2006) Antimicrobial resistance and the mechanisms implicated in faecal *Enterococci* from healthy humans, poultry and pets in Portugal. *International Journal of Antimicrobial Agents*, **27**, 131-137.

Poh, C. H., Oh, H. M. L. & Tan, A. L. (2006) Epidemiology and clinical outcome of Enterococcal bacteraemia in an acute care hospital. *Journal of Infection*, **52**, 383-386.

Prabuseenivasan, S., Jayakumar, M. & Ignaciumuth, S. (2006) *In vitro* antibacterial activity of some plant essential oils. *BMC Complementary and Alternative Medicine*, **6**.

Puupponen-Pimia, R., Nohynek, L., Meier, C., Kahkonen, M., Heinonen, M., Hopia, A. & Oksman-Caldentey, K. M. (2001) Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology,* **90,** 494-507.

Rackabrand, D., Arthur. T, G., K., Livers, K. & Blum, P. (1995) An essential role of *Escherichia coli* DnaK protein in starvation-induced thermotolerance, H_2O_2, and reductive division. *Journal of Bacteriology,* **177,** 3695-3703.

Radulovic, N., Misic, M., Aleksic, J., Dokovic, D., Palic, R. & Stojanovic, G. (2007) Antimicrobial synergism and antagonism of salicylaldehyde in *Filipendula vulgaris* essential oil. *Fitoterapia,* **78,** 565-570.

Rasooli, I., Rezaei, M. B. & Allameh, A. (2006a) Growth inhibition and morphological alterations of *Aspergillus niger* by essential oils from *Thymus eriocalyx* and *Thymus x-porlock. Food Control,* **17,** 359-364.

Rasooli, I., Rezaei, M. B. & Allameh, A. (2006b) Ultrastructural studies on antimicrobial efficacy of thyme essential oils on *Listeria monocytogenes. International Journal of Infectious Diseases,* **10,** 236-241.

Raybaudi-Massilis, R.M., Rojas-Grau, M., Mosqueda-Melgar, J. & Martin-Belloso, O. (2008) Comparative study on essential oils incorporated into an alginate-based edible coating to assure the saftey and quality of fresh-cut fuji apples. *Journal of Food Protection,* **71,** 1150-1161.

Raybaudi-Massilis, R. M., Mosqueda-Melgar, J. & Martin-Belloso, O. (2006) Antimicrobial activity of essential oils on *Salmonella enteritidis, Escherichia coli,* and *Listeria innocua* in fruit juices. *Journal of Food Protection,* **69,** 1579-1586.

Reuter, G. (1992) Culture media for *Enterococci* and group *D-streptococci. International Journal of Food Microbiology,* **17,** 103-114.

Rince, A., Flahaut, S. & Auffray, Y. (2000) Identification of general stress genes in *Enterococcus faecalis. International Journal of Food Microbiology,* **55,** 87-91.

Rios, J. L. & Recio, M. C. (2005) Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology,* **100,** 80-84.

Rodriguez-Bano, J., Ramirez, E., Muniain, M. A., Santos, J., Joyanes, P., Gonzalez, F., Garcia-Sanchez, M. & Martinez-Martinez, L. (2005) Colonization by high-level aminoglycoside-resistant enterococci in intensive care unit patients: epidemiology and clinical relevance. *Journal of Hospital Infection,* **60,** 353-359.

Rojas-Grau, M. A., Avena-Bustillos, R. J., Olsen, C., Friedman, M., Henika, P. R., Martin-Belloso, O., Pan, Z. & McHugh, T. H. (2007) Effects of plant essential oils and oil compounds on mechanical, barrier and antimicrobial properties of alginate-apple puree edible films. *Journal of Food Engineering,* **81,** 634-641.

Ross, A. I. V., Griffiths, M. W., Mittal, G. S. & Deeth, H. C. (2003) Combining nonthermal technologies to control foodborne microorganisms. *International Journal of Food Microbiology,* **89,** 125-138.

Russell, A. D. (1999) Bacterial resistance to disinfectants: present knowledge and future problems. *Journal of Hospital Infection,* **43,** S57.

Saiyasombati, P. & Kasting, G. B. (2003) Two-stage kinetic analysis of fragrance evaporation and absorption from the skin. *International Journal of Cosmetic Science,* **25,** 235-243.

Sakagami, Y. & Kajimura, K. (2002) Bactericidal activities of disinfectants against vancomycin-resistant enterococci. *Journal of Hospital Infection,* **50,** 140.

Salvat, A., Antonacci, L., Fortunato, R. H., Suarez, E. Y. & Godoy, H. M. (2004) Antimicrobial activity in methanolic extracts of several plant species from northern Argentina. *Phytomedicine,* **11,** 230-234.

Sandri, I. G., Zacaria, J., Fracaro, F., Delamare, A. P. L. & Echeverrigaray, S. (2007) Antimicrobial activity of the essential oils of Brazilian species of the genus *Cunila* against foodborne pathogens and spoiling bacteria. *Food Chemistry,* **103,** 823-828.

Satake, A., Furukawa, K., Ueno, T., Ukeda, H. & Sawamura, M. (2004) MS fragment isotope ratio analysis for the evaluation of citrus essential oils by HRGC-MS. *Bioscience, Biotechnology and Biochemistry,* **68,** 312-316.

Sato, K., Krist, S. & Buchbauer, G. (2006) Antimicrobial effect of trans-cinnamaldehyde, (-)-perillaldehyde (-)-citronellal, citral, eugenol and carvacrol on airborne microbes using an airwasher. *Biological Pharmaceutical Bulletin,* **29,** 2292-2294.

Schelz, Z., Molnar, J. & Hohmann, J. (2006) Antimicrobial and antiplasmid activities of essential oils. *Fitoterapia,* **77,** 279-285.

Scott, E. (1999) Hygiene issues in the home. *American Journal of Infection Control,* **27,** S22.

Shanks, O. C., Santo Domingo, J. W. & Graham, J. E. (2006) Use of competitive DNA hybridization to identify differences in the genomes of bacteria. *Journal of Microbiological Methods,* **66,** 321-330.

Shaw, P. E. (1979) Review of quantitative analyses of citrus essential oils. *American Chemical Society.* Florida.

Shekarforoush, S. S., Nazer, A. H. K., Firouzi, R. & Rostami, M. (2007) Effects of storage temperatures and essential oils of oregano and nutmeg on the growth and survival of *Escherichia coli* O157:H7 in barbecued chicken used in Iran. *Food Control,* **18,** 1428-1433.

Shelef, L. A., Jyothi, E. K. & Bulgarelli, M. A. (1984) Growth of enteropathogenic and spoilage bacteria in sage-containing broth and foods. *Journal of Food Science,* **49,** 737-740.

Si, W., Gong, J., Tsao, R., Zhou, T., Yu, H., Poppe, C., Johnson, R. & Du, Z. (2006) Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria. *Journal of Applied Microbiology,* **100,** 296-305.

Skandamis, P.N., Tsigarida, E. & Nychas, G.-J. E. (2002) The effect of oregano essential oil on survival/death of *Salmonella typhimurium* in meat stored at 5 °C under aerobic, VP/MAP conditions. *Food Microbiology,* **19,** 97-103.

Skandamis, P. N. & Nychas, G. J. E. (2001) Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres. *Journal of Applied Microbiology,* **91,** 1011-1022.

Skarstad, K., Boye, E. & Steen, H. B. (1986) Timing of initiation of chromosome replication in individual *Escherichia coli* cells. *EMBO Journal,* **5,** 1711-7.

Smith-Palmer, Stewart & Fyfe (1998) Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Letters in Applied Microbiology,* **26,** 118-122.

Smith-Palmer, A., Stewart, J. & Fyfe, L. (2001) The potential application of plant essential oils as natural food preservatives in soft cheese. *Food Microbiology,* **18,** 463-470.

Smith, D., Forland, S., Bachanos, E., Matejka, M. & Barrett, V. (2001) Qualitive anaylysis of citrus fruits extracts by GC/MS: An undergraduate experiment. *Chemical Educator,* **6,** 28-31.

Stashenko, E. E., Martinez, R., Pinzon, M. H. & Ramirez, J. (1996) Changes in chemical composition of catalytically hydrogenated orange oil (*Citrus sinensis*). *Journal of Chromatography A,* **752,** 217-222.

Stiles, M. E. & Holzapfel, W. H. (1997) Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology,* **36,** 1-29.

Strompfova, V., Marcinakova, M., Simonova, M., Gancarcikova, S., Jonecova, Z., Scirankova, L., Koscova, J., Buleca, V., Cobanova, K. & Laukova, A. (2006) *Enterococcus faecium* EK13--an enterocin A-producing strain with probiotic character and its effect in piglets. *Anaerobe,* **12,** 242-248.

Su, H.-J., Chao, C.-J., Chang, H.-Y. & Wu, P.-C. (2007) The effects of evaporating essential oils on indoor air quality. *Atmospheric Environment,* **41,** 1230.

Svoboda, K. & Greenaway, R. I. (2003) Lemon scented plants. *International Journal of Aromatherapy,* **13,** 23-32.

Tacconelli, E. & Cataldo, M. A. (2008) Vancomycin-resistant enterococci (VRE): transmission and control. *International Journal of Antimicrobial Agents,* **31,** 99-106.

Tassou, C.C., Koutsoumanis, K. & Nychas, G. J. E. (2000) Inhibition of *Salmonella enteritidis* and *Staphylococcus aureus* in nutrient broth by mint essential oil. *Food Research International,* **33,** 273-280.

Tassou, C. C. & Nychas, G. J. E. (1995) Antimicrobial activity of the essential oil of mastic gum (*Pistacia lentiscus var. chia*) on Gram positive and Gram negative bacteria in broth and in Model Food System. *International Biodeterioration & Biodegradation,* **36,** 411-420.

Tejedor-Junco, M. T., Afonso-Rodriguez, O., Martin-Barrasa, J. L. & Gonzalez-Martin, M. (2005) Antimicrobial susceptibility of *Enterococcus* strains isolated from poultry faeces. *Research in Veterinary Science,* **78,** 33-38.

Temmerman, R., Pot, B., Huys, G. & Swings, J. (2003) Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *International Journal of Food Microbiology,* **81,** 1-10.

The Hospital Infection Control Practices Advisory Committe (1995) Recommendations for preventing the spread of vancomycin resistance: Recommendations of the Hospital Infection Control Practices Advisory Committee (HICPAC). *American Journal of Infection Control,* **23,** 87-94.

Thomas, R., Anjaneyulu, A. S. R. & Kondaiah, N. (2008a) Development of shelf stable pork sausages using hurdle technology and their quality at ambient temperature (37 ± 1 °C) storage. *Meat Science,* **79,** 1-12.

Thomas, R., Anjaneyulu, A. S. R. & Kondaiah, N. (2008b) Effect of hot-boned pork on the quality of hurdle treated pork sausages during ambient temperature (37 ± 1 °C) storage. *Food Chemistry,* **107,** 804-812.

Timmerman, H. M., Koning, C. J. M., Mulder, L., Rombouts, F. M. & Beynen, A. C. (2004) Monostrain, multistrain and multispecies probiotics--A comparison of functionality and efficacy. *International Journal of Food Microbiology,* **96,** 219-233.

Tirado, C. B., Stashenko, E. E., Combariza, M. Y. & Martinez, J. R. (1995) Comparative study of Colombian citrus oils by high-resolution gas chromatography and gas chromatography-mass spectrometry. *Journal of Chromatography A,* **697,** 501-513.

Todorov, S. D., Meincken, M. & Dicks, L. M. (2006) Factors affecting the adsorption of bacteriocins ST194BZ and ST23LD to *Lactobacillus sakei* and *Enterococcus* spp. *Journal of General Applied Microbiology,* **52,** 159-67.

Townsend, J., Nielsen, K., Fisher, D. & Hart, D. (2003) Horizontal Acquisition of Divergent Chromosomal DNA in Bacteria: Effects of Mutator Phenotypes *Genetics,* **164,** 13-21.

Tsai, C.-C., Liu, T.-H., Chen, M.-H., Tsai, C.-C. & Tsen, H.-Y. (2004) Toxicity evaluation for an *Enterococcus faecium* strain TM39 in vitro and in vivo. *Food and Chemical Toxicology,* **42,** 1601-1609.

Tzortzakis, N. G. (2007) Maintaining postharvest quality of fresh produce with volatile compounds. *Innovative Food Science & Emerging Technologies,* **8,** 111-116.

Ultee, A., Bennik, M. H. J. & Moezelaar, R. (2002) The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus. Applied and Environmental Microbiology,* **68,** 1561-1568.

Ultee, A., Kets, E. P. & Smid, E. J. (1999) Mechanisms of action of carvacrol on food-borne pathogen *Bacillus cereus. Applied and Environmental Microbiology,* **65,** 4606-4610.

Valero, M., Fernández, P. S. & Salmerón, M. C. (2003) Influence of pH and temperature on growth of Bacillus cereus in vegetable substrates. *International Journal of Food Microbiology,* **82,** 71.

Valero, M. & Frances, E. (2006) Synergistic bactericidal effect of carvacrol, cinnamaldehyde or thymol and refrigeration to inhibit *Bacillus cereus* in carrot broth. *Food Microbiology,* **23,** 68-73.

Van den Berghe, E., De Winter, T. & De Vuyst, L. (2006) Enterocin A production by *Enterococcus faecium* FAIR-E 406 is characterised by a temperature- and pH-dependent switch-off mechanism when growth is limited due to nutrient depletion. *International Journal of Food Microbiology,* **107,** 159-170.

Van Griethuysen, A. J. A., Rooijen, N. H. S.-v. & Hoogenboom-Verdegaal, A. M. M. (1996) Surveillance of wound infections and a new theatre: unexpected lack of improvement. *Journal of Hospital Infection,* **34,** 99-106.

Van Merode, A. E. J., Duval, J. F. L., van der Mei, H. C., Busscher, H. J. & Krom, B. P. (2008) Increased adhesion of *Enterococcus faecalis* strains with bimodal electrophoretic mobility distributions. *Colloids and Surfaces B: Biointerfaces,* **64,** 302-306.

Vattem, D. A., Lin, Y. T., Ghaedian, R. & Shetty, K. (2005) Cranberry synergies for dietary management of *Helicobacter pylori* infections. *Process Biochemistry,* **40,** 1583-1592.

Verkkala, K., Eklund, A., Ojajärvi, J., Tiittanen, L., Hoborn, J. & Mäkelä, P. (1998) The conventionally ventilated operating theatre and air contamination control during cardiac surgery - bacteriological and particulate matter control garment options for low level contamination. *European Journal of Cardio-Thoracic Surgery,* **14,** 206-210.

Wannissorn, B., Jarikasem, S., Siriwangchai, T. & Thubthimthed, S. (2005) Antibacterial properties of essential oils from Thai medicinal plants. *Fitoterapia,* **76,** 233-236.

Wilcox, M. H. & Hodgson, G. (2005) Gram-positive hospital acquired infection in the ITU setting. *Current Anaesthesia & Critical Care,* **16,** 201-207.

Williams, G. J., Denyer, S. P., Hosein, I. K., Hill, D. W. & Maillard, J. Y. (2007) The development of a new three-step protocol to determine the efficacy of disinfectant wipes on surfaces contaminated with *Staphylococcus aureus. Journal of Hospital Infection,* **67,** 329-335.

Wolford, R. W., Kesterson, J. W. & Attaway, J. A. (1971) Physicochemical properties of citrus essential oils from Florida. *Journal of Agricultural and Food Chemistry,* **19,** 1097-1102.

Wu, C. C., Chung, J. G., Tsai, S. J., Yang, J. H. & Sheen, L. Y. (2004) Differential effects of allyl sulfides from garlic essential oil on cell cycle regulation in human liver tumor cells. *Food and Chemical Toxicology,* **42,** 1937-1947

Yea, S. S., Jeong, H.-S., Choi, C. Y., Park, K.-R., Oh, S., Shin, J.-G. & Yun, C.-H. (2006) Inhibitory effect of anethole on T-lymphocyte proliferation and interleukin-2 production through down-regulation of the NF-AT and AP-1. *Toxicology in Vitro,* **20,** 1098-1105.

Zotta, T., Ricciardi, A., Ciocia, F., Rossano, R. & Parente, E. (2008) Diversity of stress responses in dairy thermophilic streptococci. *International Journal of Food Microbiology,* **124** (1), 34-42.

Appendices

TEXT BOUND INTO

THE SPINE

NPar Tests

One-Sample Kolmogorov-Smirnov Test

		L15	L30	L45	CCON	C15	C30	C45
		6	6	6	6	6	6	6
Parameters(a,b)	Mean	4.4667	5.1350	4.5950	4.9717	4.6600	4.8000	4.4533
	Std. Deviation	.12565	.24970	.14460	.10381	.03521	.10973	.05645
xtreme Differences	Absolute	.140	.315	.208	.226	.278	.167	.348
	Positive	.140	.315	.208	.226	.222	.167	.158
	Negative	-.135	-.281	-.206	-.124	-.278	-.118	-.348
orov-Smirnov Z		.344	.771	.509	.553	.682	.408	.853
. Sig. (2-tailed)		1.000	.592	.958	.920	.741	.996	.460

a Test distribution is Normal.
b Calculated from data.

NPar Tests

One-Sample Kolmogorov-Smirnov Test

		DATA
N		48
Normal Parameters[a,b]	Mean	4.7567
	Std. Deviation	.26816
Most Extreme Differences	Absolute	.093
	Positive	.093
	Negative	-.065
Kolmogorov-Smirnov Z		.642
Asymp. Sig. (2-tailed)		.804

a. Test distribution is Normal.

b. Calculated from data.

Oneway

Test of Homogeneity of Variances

DATA

Levene Statistic	df1	df2	Sig.
1.806	7	40	.113

ANOVA

DATA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.694	7	.385	22.465	.000
Within Groups	.685	40	.017		
Total	3.380	47			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: DATA
Tukey HSD

(I) CODE	(J) CODE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.5050(*)	.07557	.000	.2634	.7466
	3.00	-.1633	.07557	.396	-.4049	.0782
	4.00	.3767(*)	.07557	.000	.1351	.6182
	5.00	.0000	.07557	1.000	-.2416	.2416
	6.00	.3117(*)	.07557	.004	.0701	.5532
	7.00	.1717	.07557	.334	-.0699	.4132
	8.00	.5183(*)	.07557	.000	.2768	.7599
2.00	1.00	-.5050(*)	.07557	.000	-.7466	-.2634
	3.00	-.6683(*)	.07557	.000	-.9099	-.4268
	4.00	-.1283	.07557	.688	-.3699	.1132
	5.00	-.5050(*)	.07557	.000	-.7466	-.2634
	6.00	-.1933	.07557	.202	-.4349	.0482
	7.00	-.3333(*)	.07557	.002	-.5749	-.0918
	8.00	.0133	.07557	1.000	-.2282	.2549
3.00	1.00	.1633	.07557	.396	-.0782	.4049
	2.00	.6683(*)	.07557	.000	.4268	.9099
	4.00	.5400(*)	.07557	.000	.2984	.7816
	5.00	.1633	.07557	.396	-.0782	.4049
	6.00	.4750(*)	.07557	.000	.2334	.7166
	7.00	.3350(*)	.07557	.002	.0934	.5766
	8.00	.6817(*)	.07557	.000	.4401	.9232
4.00	1.00	-.3767(*)	.07557	.000	-.6182	-.1351
	2.00	.1283	.07557	.688	-.1132	.3699
	3.00	-.5400(*)	.07557	.000	-.7816	-.2984
	5.00	-.3767(*)	.07557	.000	-.6182	-.1351
	6.00	-.0650	.07557	.988	-.3066	.1766
	7.00	-.2050	.07557	.148	-.4466	.0366
	8.00	.1417	.07557	.575	-.0999	.3832
5.00	1.00	.0000	.07557	1.000	-.2416	.2416
	2.00	.5050(*)	.07557	.000	.2634	.7466
	3.00	-.1633	.07557	.396	-.4049	.0782

	4.00	.3767(*)	.07557	.000	.1351	.6182
	6.00	.3117(*)	.07557	.004	.0701	.5532
	7.00	.1717	.07557	.334	-.0699	.4132
	8.00	.5183(*)	.07557	.000	.2768	.7599
6.00	1.00	-.3117(*)	.07557	.004	-.5532	-.0701
	2.00	.1933	.07557	.202	-.0482	.4349
	3.00	-.4750(*)	.07557	.000	-.7166	-.2334
	4.00	.0650	.07557	.988	-.1766	.3066
	5.00	-.3117(*)	.07557	.004	-.5532	-.0701
	7.00	-.1400	.07557	.590	-.3816	.1016
	8.00	.2067	.07557	.142	-.0349	.4482
7.00	1.00	-.1717	.07557	.334	-.4132	.0699
	2.00	.3333(*)	.07557	.002	.0918	.5749
	3.00	-.3350(*)	.07557	.002	-.5766	-.0934
	4.00	.2050	.07557	.148	-.0366	.4466
	5.00	-.1717	.07557	.334	-.4132	.0699
	6.00	.1400	.07557	.590	-.1016	.3816
	8.00	.3467(*)	.07557	.001	.1051	.5882
8.00	1.00	-.5183(*)	.07557	.000	-.7599	-.2768
	2.00	-.0133	.07557	1.000	-.2549	.2282
	3.00	-.6817(*)	.07557	.000	-.9232	-.4401
	4.00	-.1417	.07557	.575	-.3832	.0999
	5.00	-.5183(*)	.07557	.000	-.7599	-.2768
	6.00	-.2067	.07557	.142	-.4482	.0349
	7.00	-.3467(*)	.07557	.001	-.5882	-.1051

* The mean difference is significant at the .05 level.

Homogeneous Subsets

DATA

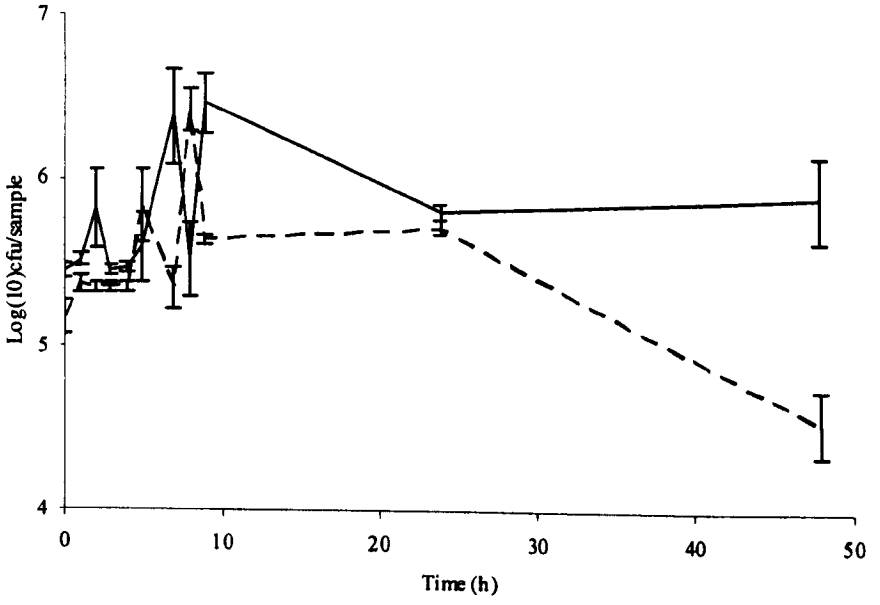
Tukey HSD[a]

CODE	N	Subset for alpha = .05			
		1	2	3	4
8.00	6	4.4533			
2.00	6	4.4667			
4.00	6	4.5950	4.5950		
6.00	6	4.6600	4.6600		
7.00	6		4.8000	4.8000	
1.00	6			4.9717	4.9717
5.00	6			4.9717	4.9717
3.00	6				5.1350
Sig.		.142	.148	.334	.396

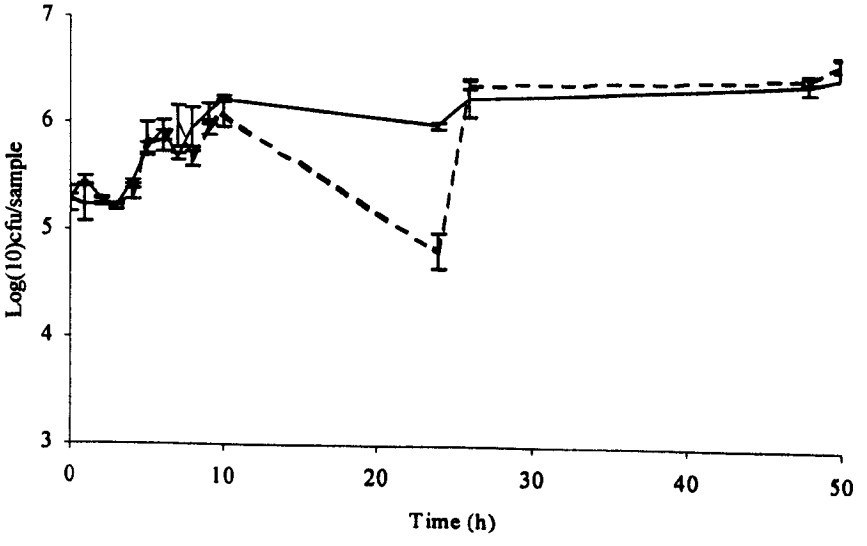
Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

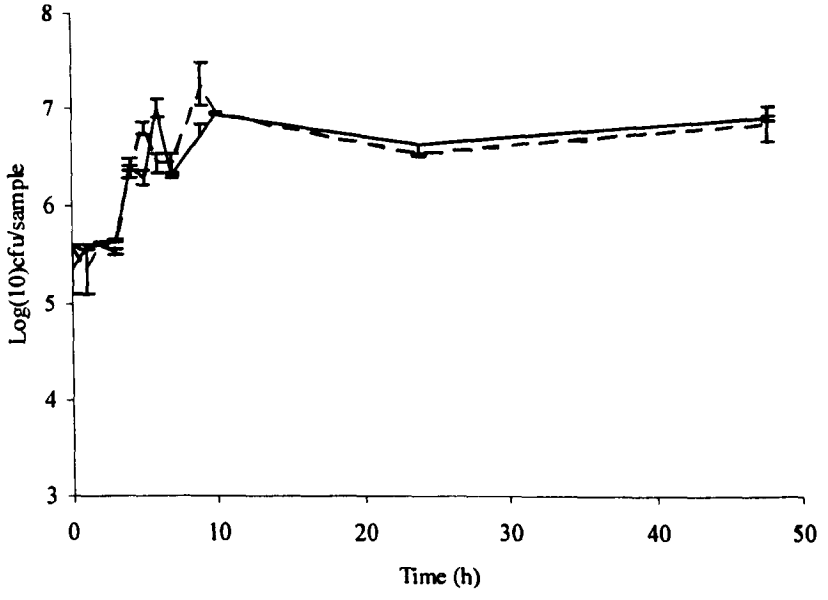
a)


Log(10)cfu/sample
7
6
5
4
0
10
20
30
40
50
Time (h)

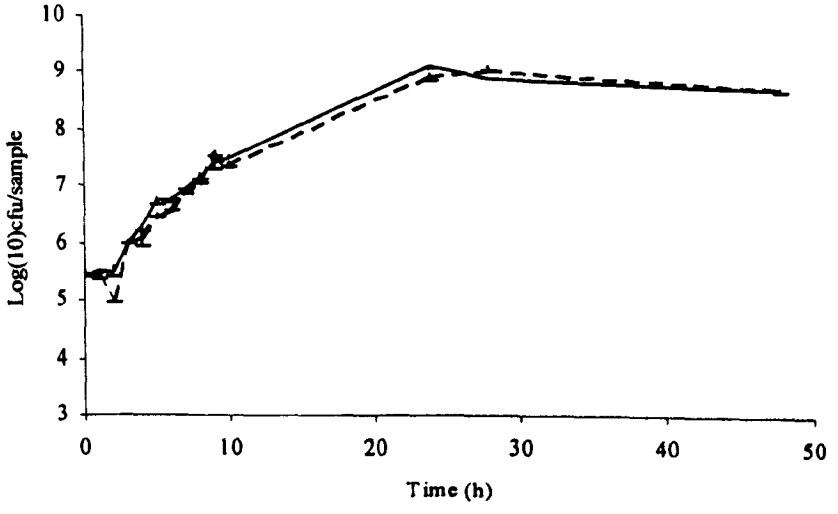
b)


Log(10)cfu/sample
7
6
5
4
3
0
10
20
30
40
50
Time (h)

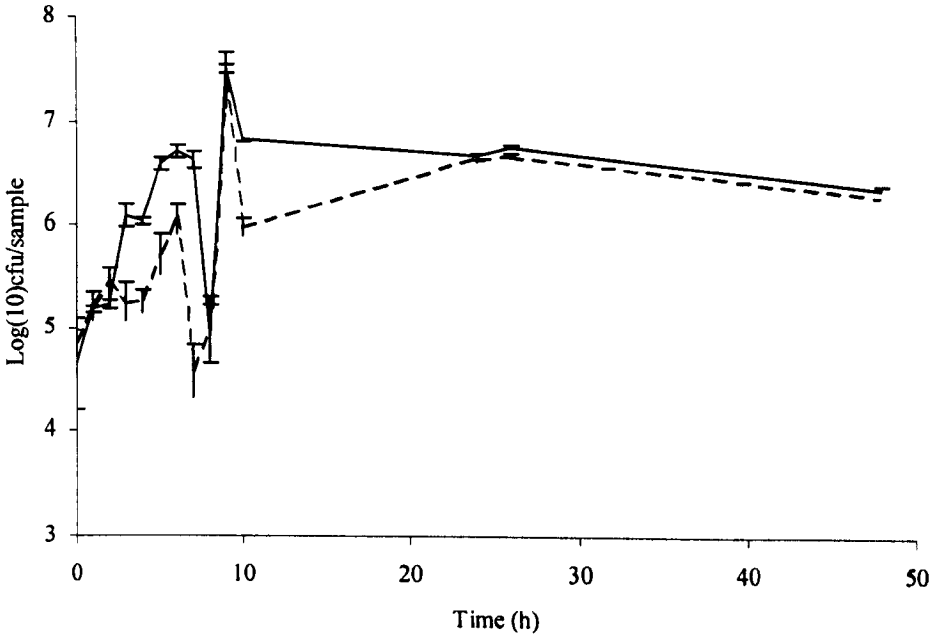
c)


8
7
6
5
4
3
Log(10)cfu/sample
0
10
20
30
40
50
Time (h)

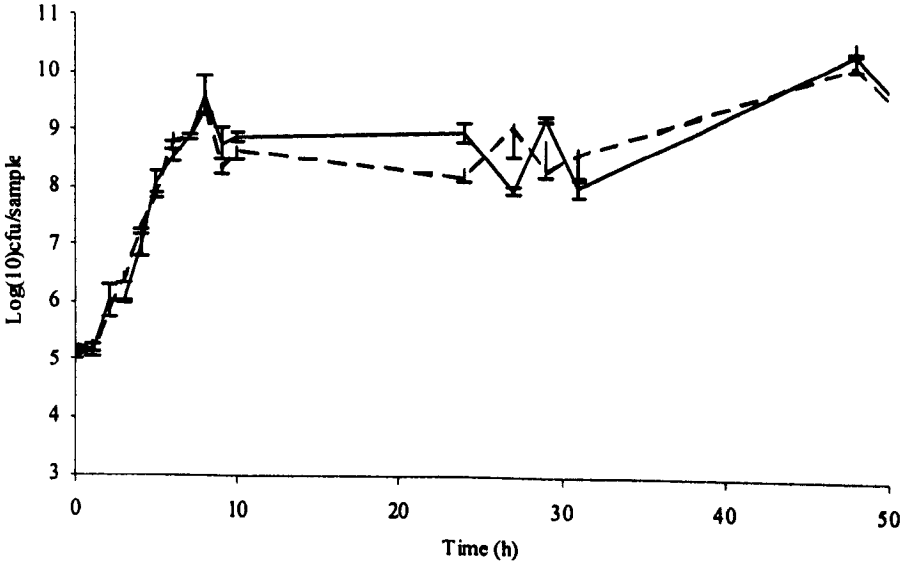
d)


10
9
8
7
6
5
4
3
Log(10)cfu/sample
0
10
20
30
40
50
Time (h)

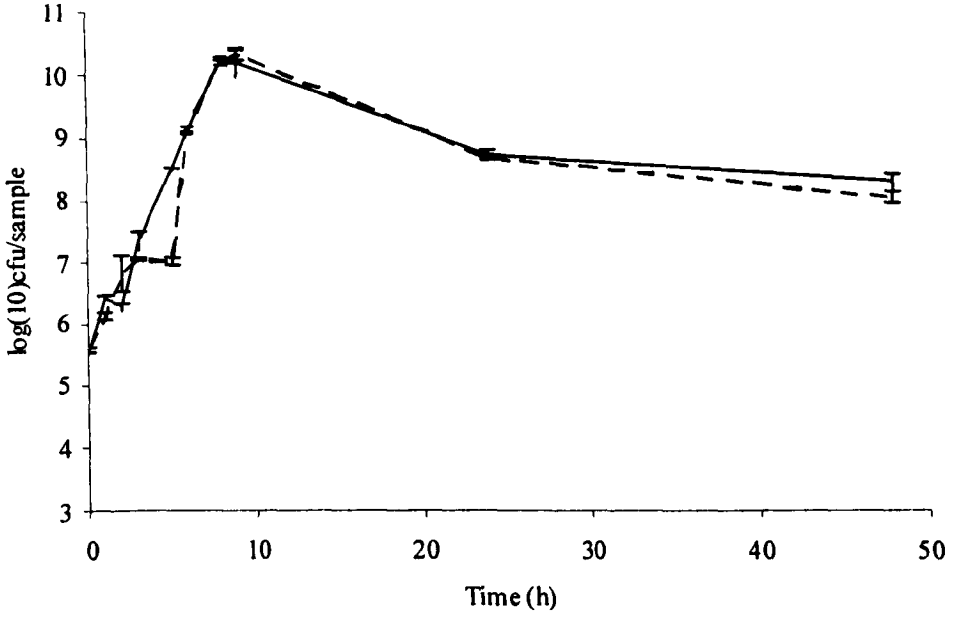
e)


Log(10)cfu/sample
8
7
6
5
4
3
0
10
20
30
40
50
Time (h)

f)


Log(10)cfu/sample
11
10
9
8
7
6
5
4
3
0
10
20
30
40
50
Time (h)

g)



h)

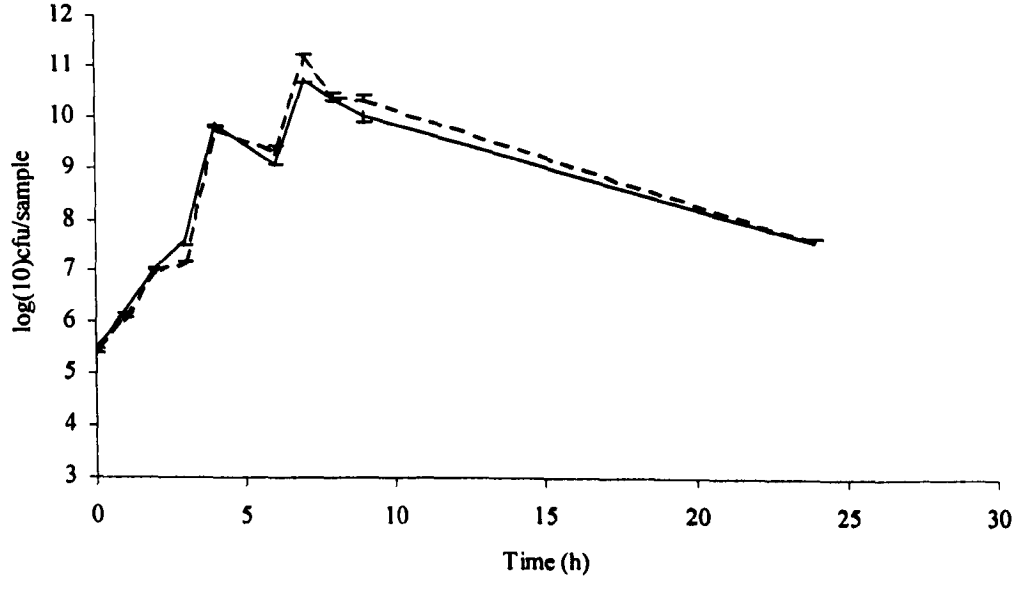


Figure appendix II: Growth curves of vancomycin susceptible *E. faecium* (- - -) and *E. faecalis* (—) at a) 5°C, b) 10°C, c) 15°C, d) 20°C, e) 25°C, f) 37°C, g) 45°C and h) 50°C.

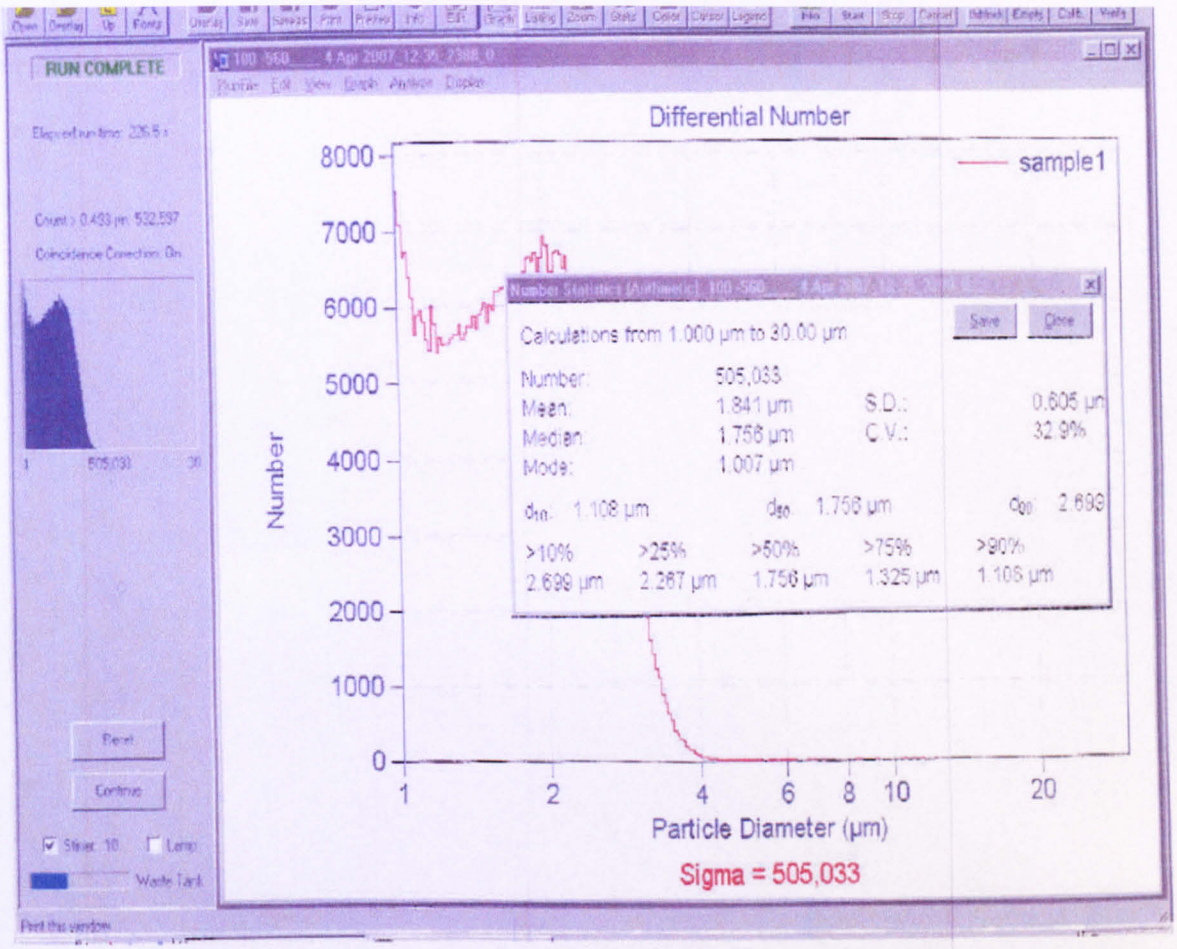
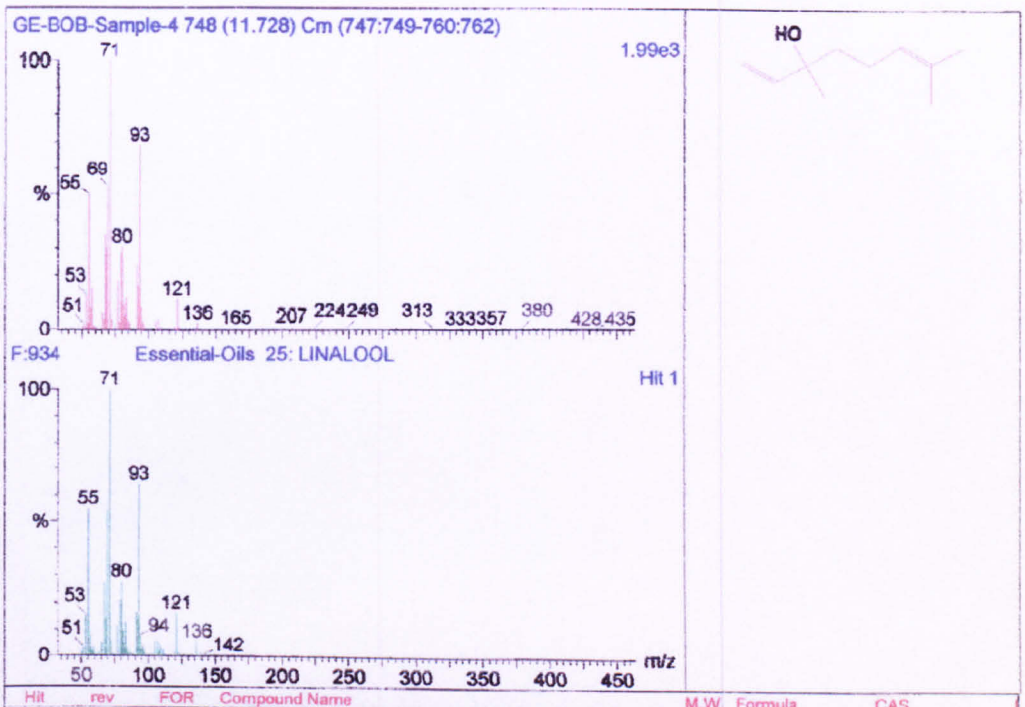
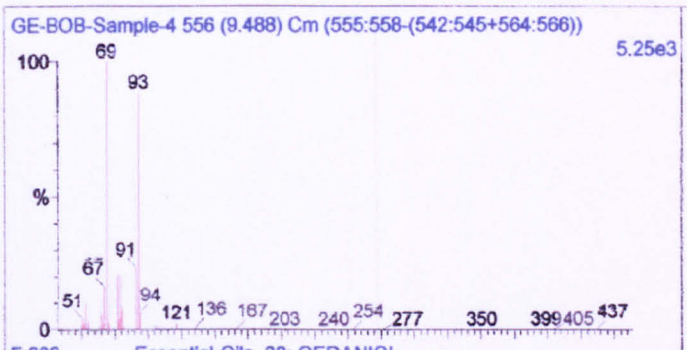
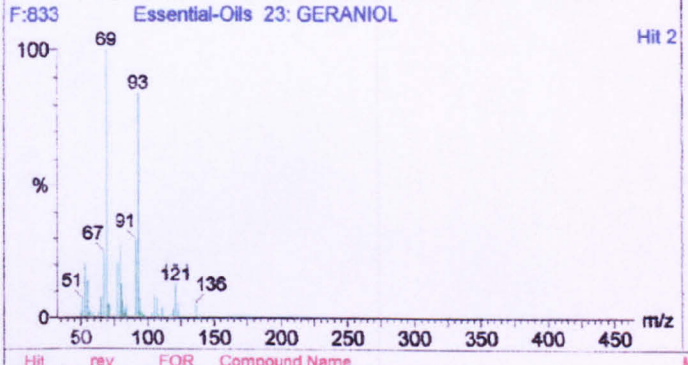


Figure appendix III: Output of data from coulter counter of *E. faecalis* control at 6 hours growth.

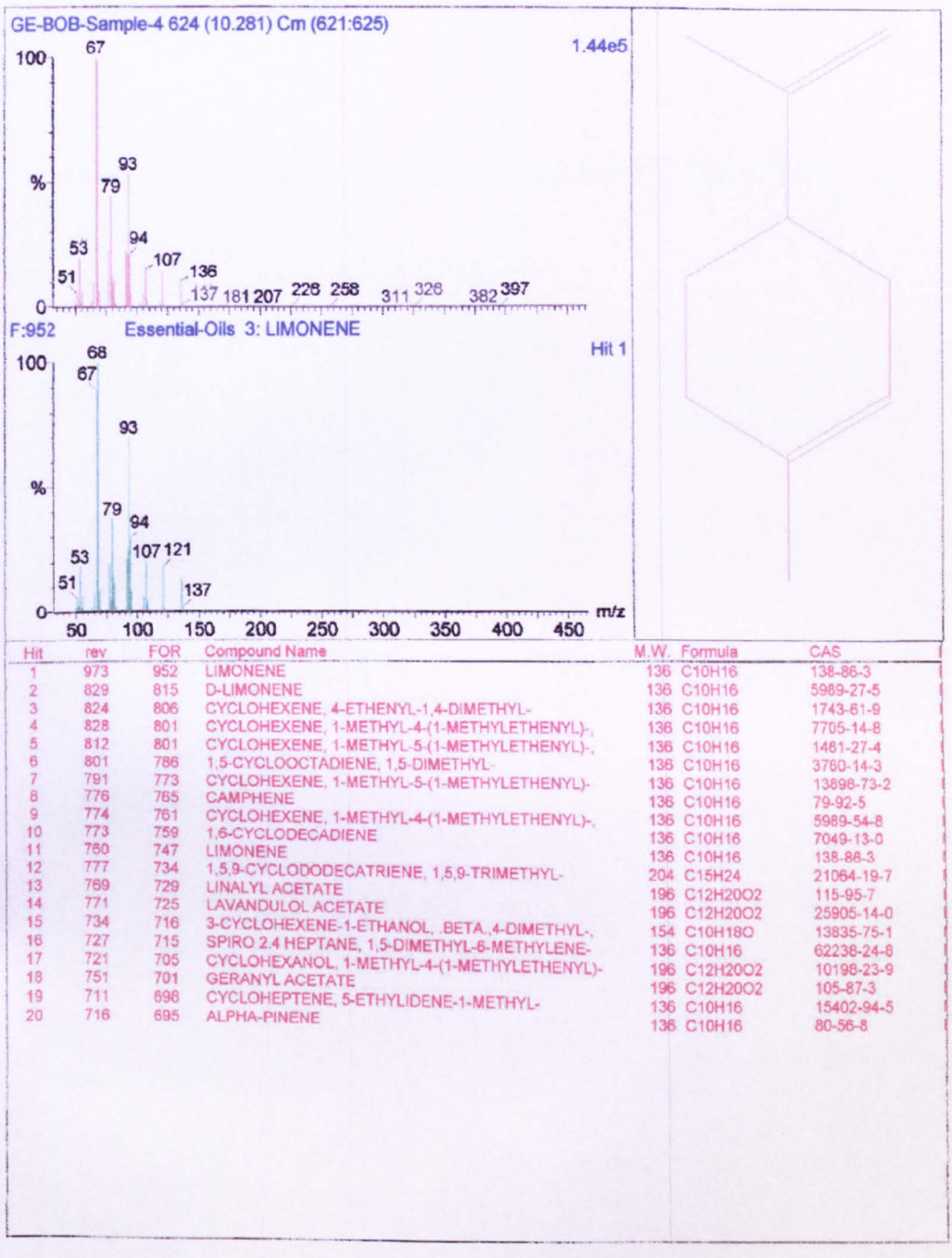


Hit	rev	FOR	Compound Name	M.W.	Formula	CAS
1	953	934	LINALOOL	154	C10H18O	78-70-6
2	831	744	LINALYL ACETATE	196	C12H20O2	115-95-7
3	741	706	1,6-OCTADIEN-3-OL, 3,7-DIMETHYL-	154	C10H18O	78-70-6
4	692	667	1-CYCLOHEXYL-2-BUTEN-1-OL (C,T)	154	C10H18O	79605-62-2
5	703	659	1,6-OCTADIEN-3-OL, 3,7-DIMETHYL-, FORMATE	182	C11H18O2	115-99-1
6	751	658	GERANIOL	154	C10H18O	106-24-1
7	688	631	TERPINEN-4-OL	154	C10H18O	20126-76-5
8	852	624	LAVANDULOL ACETATE	196	C12H20O2	25905-14-0
9	854	617	GERANYL ACETATE	196	C12H20O2	105-87-3
10	835	611	BETA-PINENE	136	C10H16	127-91-3
11	682	598	FARNESOL, CIS	222	C15H26O	3790-71-4
12	634	590	1,6-OCTADIEN-3-OL, 3,7-DIMETHYL-, 2-AMINOBENZO	273	C17H23O2N	7149-26-0
13	670	587	FARNESOL, TRANS	222	C15H26O	106-28-5
14	793	582	LIMONENE	136	C10H16	138-86-3
15	607	573	1,6-OCTADIEN-3-OL, 3,7-DIMETHYL-, (.+/-.)-	154	C10H18O	22564-99-4
16	600	562	8,11,14-EICOSATRIENOIC ACID, (Z,Z,Z)-	306	C20H34O2	1783-84-2
17	591	544	4-CYCLOOCTENE-1-METHANOL	140	C9H16O	13366-81-9
18	596	543	ALPHA-TERPINEOL	154	C10H18O	10482-56-1
19	624	542	9,12-OCTADECADIENAL, DIMETHYL ACETAL	310	C20H38O2	1599-51-5
20	727	541	CAMPHENE	136	C10H16	79-92-5





Hit	rev	FOR	Compound Name	M.W.	Formula	CAS
2	869	833	GERANIOL	154	C10H18O	106-24-1
3	758	725	LINALYL ACETATE	196	C12H20O2	115-95-7
4	755	720	LAVANDULOL ACETATE	196	C12H20O2	25905-14-0
5	755	719	GERANYL ACETATE	196	C12H20O2	105-87-3
6	706	697	BICYCLO 3.1.1 HEPTANE, 6,6-DIMETHYL-2-METHYLE	136	C10H16	18172-67-3
7	715	674	FARNESYL ACETATE (TRANS, TRANS)	264	C17H28O2	4128-17-0
8	674	669	.BETA.-PINENE	136	C10H16	127-91-3
9	695	655	FARNESOL, TRANS	222	C15H26O	106-28-5
10	681	651	BICYCLO 3.1.0 HEX-2-ENE, 4-METHYL-1-(1-METHYLE	136	C10H16	28634-89-1
11	681	642	FARNESOL, CIS	222	C15H26O	3790-71-4
12	656	638	TRANS-OCIMENE	136	C10H16	13877-91-3
13	644	637	.BETA.-MYRCENE	136	C10H16	123-35-3
14	648	637	.BETA.-PHELLANDRENE	136	C10H16	555-10-2
15	650	629	1,6-OCTADIEN-3-OL, 3,7-DIMETHYL-, PROPANOATE	210	C13H22O2	144-39-8
16	634	624	CIS-OCIMENE	136	C10H16	13877-91-3
17	624	615	CYCLOHEXANE, 1-METHYLENE-4-(1-METHYLETHENY	136	C10H16	499-97-8
18	631	606	BICYCLO 3.1.0 HEXANE, 4-METHYLENE-1-(1-METHYL	136	C10H16	3387-41-5
19	591	584	CAMPHENE	136	C10H16	79-92-5
20	593	581	BICYCLO 2.2.1 HEPTANE, 2,2-DIMETHYL-3-METHYLE	136	C10H16	5794-03-6



Hit	rev	FOR	Compound Name	M.W.	Formula	CAS
1	973	952	LIMONENE	136	C10H16	138-86-3
2	829	815	D-LIMONENE	136	C10H16	5989-27-5
3	824	806	CYCLOHEXENE, 4-ETHENYL-1,4-DIMETHYL-	136	C10H16	1743-61-9
4	828	801	CYCLOHEXENE, 1-METHYL-4-(1-METHYLETHENYL)-,	136	C10H16	7705-14-8
5	812	801	CYCLOHEXENE, 1-METHYL-5-(1-METHYLETHENYL)-,	136	C10H16	1461-27-4
6	801	786	1,5-CYCLOOCTADIENE, 1,5-DIMETHYL-	136	C10H16	3760-14-3
7	791	773	CYCLOHEXENE, 1-METHYL-5-(1-METHYLETHENYL)-	136	C10H16	13898-73-2
8	776	765	CAMPHENE	136	C10H16	79-92-5
9	774	761	CYCLOHEXENE, 1-METHYL-4-(1-METHYLETHENYL)-,	136	C10H16	5989-54-8
10	773	759	1,6-CYCLODECADIENE	136	C10H16	7049-13-0
11	760	747	LIMONENE	136	C10H16	138-86-3
12	777	734	1,5,9-CYCLODODECATRIENE, 1,5,9-TRIMETHYL-	204	C15H24	21064-19-7
13	769	729	LINALYL ACETATE	196	C12H20O2	115-95-7
14	771	725	LAVANDULOL ACETATE	196	C12H20O2	25905-14-0
15	734	716	3-CYCLOHEXENE-1-ETHANOL, .BETA.,4-DIMETHYL-,	154	C10H18O	13835-75-1
16	727	715	SPIRO 2.4 HEPTANE, 1,5-DIMETHYL-6-METHYLENE-	136	C10H16	62238-24-8
17	721	705	CYCLOHEXANOL, 1-METHYL-4-(1-METHYLETHENYL)-	196	C12H20O2	10198-23-9
18	751	701	GERANYL ACETATE	196	C12H20O2	105-87-3
19	711	698	CYCLOHEPTENE, 5-ETHYLIDENE-1-METHYL-	136	C10H16	15402-94-5
20	716	695	ALPHA-PINENE	136	C10H16	80-56-8

Figure Appendix IV: Data output from GC-MS for linalool, citral and limonene.

Publications Associated with Thesis

Patent

United Kingdom Patent Application No. 0809935.0: Antimicrobial citrus essential oil blend vapour.

Publications

Fisher, K. and Phillips, C. (2006). The effect of lemon, orange and bergamot essential oils and their components on the survival of *Campylobacter jeuni, Escherichia coli* O157, *Listeria monocytogenes, Bacillus cereus* and *Staphylococcus aureus* in vitro and in food systems. Journal of Applied Microbiology. 101. (6) 1232-1240.

Fisher, K., Rowe C. and Phillips C. (2007) The survival of three strains of *Arcobacter butzleri* in the presence of lemon, orange and bergamot essential oils and their components *in vitro* and on food. Letters in Applied Microbiology. 44, 495-499

Fisher, K. & Phillips, C. (2008). Potential antimicrobial uses of essential oils in food: is citrus the answer? Trends in Food Science & Technology, 19, 156-164.

Fisher, K. & Phillips C. (2008). The mechanism of action of a citrus oil blend against *Enterococcus faecium* and *Enterococcus faecalis*. Journal of Applied Microbiology. In Press.

Food micro Conference – Italy September 2006: The effect of citrus essential oils and vapours and their components on the survival of foodborne pathogenic bacteria *in vitro* and in food systems.

House of Commons, Set for Britain – March 2007: Citrus essential oils: a potential bactericide in both the clinical and food arena.

ASM Conference – Toronto, May 2007: The use of citrus essential oils, temperature and pH (hurdle technology) against *Enterococcus* sp. & Boston, USA, June 2008: The mechanism of action of a citrus oil blend against *Enterococcus* sp.

SfAM Conference – Cardiff, June 2007: The use of citrus essential oils against *Enterococcus faecium* and *E. faecalis*