In vitro inhibition of vancomycin-susceptible and vancomycin-resistant *Enterococcus faecium* and *E. faecalis* in the presence of citrus essential oils

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Accepted: 18 September 2009

ABSTRACT

Glycopeptide-resistant *Enterococcus* (GRE) is an important healthcare-acquired infection (HCAI) which costs the healthcare service many millions of pounds worldwide. In this study, lemon (*Citrus limon*), sweet orange (*Citrus sinensis*) or bergamot (*Citrus bergamia*) essential oils (EO) and their vapours, alone and in combination, are tested for their antimicrobial activity against vancomycin-resistant and vancomycin-sensitive strains of *E. faecium* and *E. faecalis*. A blend of 1:1 (v/v) orange and bergamot EO was the most effective of the oils and/or blends tested with a minimum inhibitory concentration (MIC), at 25°C and pH 5.5, of 0.25–0.5% (v/v) and a minimum inhibitory dose (MID) of 50 mg/L, at 50°C at pH 7.5, when viable counts reduced by 5.5–10 $\log_{10}$ colony forming units (cfu)/mL, suggesting that this blend of citrus oils is effective under a range of conditions for inhibiting the growth and survival of *E. faecalis, E. faecium* and VRE.

KEY WORDS: Enterococcus.
Inhibition.
Oils, volatile.

Introduction

The genus *Enterococcus* consists of Gram-positive, catalase-negative, non-spore forming facultative anaerobic bacteria. Enterococci will grow at a range of temperatures and pH levels (5–50°C, pH 4.5–10.5).[1] *Enterococcus faecalis* is able to grow in 6.5% (w/v) NaCl and has a cation homeostasis that is considered to contribute to its resistance to pH, salt, metal ions and desiccation. The resilience of *E. faecalis* to varying pH is probably due to the durability and impermeability of its membrane.[2] Temperature resistance is also associated with membrane structure and has been related to lipid and fatty acid content.[3]

Healthcare-associated infections (HCAIs) cost the NHS in the UK an estimated £1 billion per annum. Of the HCAIs causing bacteraemia, methicillin-resistant *Staphylococcus aureus* (MRSA) and glycopeptide-resistant *Enterococcus* (GRE) are the most important in terms of numbers of cases and mortality and morbidity rates.[4] In 2005 in the UK there were 7066 reported cases of bacteraemia caused by *Enterococcus* spp., an 8% increase from 2004, with 28% of all cases being resistant to antibiotics.[5] The risk of death from vancomycin-resistant *Enterococcus* (VRE) is 75% compared with 45% for those infected with a susceptible strain.[6] This increase in numbers of cases of *Enterococcus* spp. bacteraemia is mirrored in the USA where, in a 15-year period, there has been a 20-fold increase in VRE associated with nosocomial infections reported to the Centers for Disease Control and Prevention (CDC) National Nosocomial Infections Surveillance (NNIS).[7]

Subjecting cells to a number of sublethal stresses may either result in these stresses acting on the same element of the cell, in which case an additive inhibitory effect may be observed, or, if they act to disturb several different functions of the cell, a synergistic effect may occur, either of which can lead to cell death.[8]

Many plant extracts have antibacterial properties. For example, out of 39 plants from northern Argentina tested against a range of pathogens, 25% of the extracts showed inhibitory activity, and all inhibited *S. aureus*.[9] The use of oregano, carvacrol and thymol showed no significant difference in inhibition between methicillin-susceptible *S. aureus* and MRSA, with minimum inhibitory concentrations (MICs) of 0.03–0.125%.[10] A limited number of studies have been reported in the literature concerning the effect of essential oils (EOs) against *E. faecalis*, although basil EO has been shown to have antimicrobial properties against this organism, with an MIC of 0.05% (v/v) against multidrug-resistant *E. faecalis*, although this is a bacteriostatic effect.[11]

When tea tree oil was tested against a range of bacteria, *E. faecium* was found to be the most resistant, requiring an MIC and MID of >8% (v/v).[12] In one study, using a diffusion method to screen citral and linalool, inhibition zones of 21.6±0.1 and 16.7±1.1 mm, respectively, were produced on *E. faecalis*, with carvacrol, eugenol, geraniol and α-pinene also exhibiting inhibition.[13] *Cinnamomum osmophleum* has been shown to have an MIC of 250 µg/mL against *E. faecalis*.[14] The inhibitory activity of *Carlina acanthifolia* root EO, at an MIC of 2%, against *Enterococcus* spp. has been found to be more than that of ampicillin, with the antimicrobial exhibiting 18 mm and 16 mm inhibition zones, respectively, in a disc-diffusion assay.[15]

Piacentini first noted the antimicrobial properties of citrus oils in 1949.[16] Orange oil has been observed to have the same

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effect as tylosin against a variety of bacteria and yeasts, including *Bacillus* spp. and *Streptococcus faecalis* (now reclassified as *E. faecalis*), and citrus oils have antimicrobial properties against yeast, moulds and spore-forming bacteria and also bacteria responsible for food-poisoning.[17] The potential uses of citrus EOs on food has been recognised due to their acceptability to humans in terms of their fragrance and flavour, and this is especially the case with regard to use of the citrus EO vapours, where no contact is required, thus limiting any organoleptic changes.[18]

This study aims to establish the effectiveness of citrus essential oils and blends at different temperatures and pH levels in oil and in vapour form, against vancomycin-susceptible and vancomycin resistant strains of *E. faecium* and *E. faecalis*.

Materials and methods

Microorganisms and culture methods

All media were obtained from Oxoid (Basingstoke, Hampshire, UK) unless otherwise stated. *E. faecalis* NCTC 12697, *E. faecium* NCTC 07171, vancomycin-resistant *E. faecalis* NCTC 12203 and *E. faecium* NCTC 12202 were obtained from the Health Protection Agency, London, UK. The strains were grown in brain-heart infusion (BHI) broth (CM225) aerobically at 37°C, and BHI agar (CM0375) was used to culture the bacteria on a solid medium.

Essential oils and oil components

Sweet orange (*Citrus sinensis*), lemon (*C. limon*) and bergamot (*C. bergamia*) were obtained from AMPHORA, (Bristol. UK). Limonene (97%), linalool (97%), citral (95%), hersipidin (90%) and neoericitrin (95%) – all known components of citrus EOs – were obtained from Sigma-Aldrich (Dorset, UK). The blends were produced by combining orange and lemon, lemon and bergamot, and orange and bergamot (1:1 v/v) or orange, lemon and bergamot (1:1:1 v/v) oils.

Growth at different temperatures or pH

A culture of each of the strains was incubated at 37°C and duplicate samples were taken at various time intervals and plated on BHI agar using a spiral plater (Don Whitley, West Yorkshire, UK). Plates were incubated for 24 h at 37°C before enumeration. This experiment was repeated at 5°C intervals between 5°C and 50°C.

To assess the effect of pH on growth, cultures of each of the strains with pH adjusted to 4.5, 5.5, 6.5, 8.5, 9.5 or 10.5 using HCl or KOH were incubated at 25°C, 37°C or 50°C. Duplicate samples were taken at various time intervals, spiral-plated on BHI agar and incubated for 24 h at the original incubation temperature.

Screening of essential oils and oil components

Aliquots (0.1 mL) of the EO, component or blend were spotted on 2-cm diameter filter paper discs. Three discs were placed on the surface of a BHI agar plate previously spread with the test bacterial culture. This was carried out in duplicate, with a control containing no antimicrobial on the disc.

The effect of the vapour was assessed by placing three impregnated discs on the lid of a Petri dish, approximately 8 mm from the bacteria.[19] All plates were incubated at 37°C for 24 h, and the zones of inhibition measured using Vernier calipers. This was repeated at 25°C and 50°C and at pH levels of 4.5, 7.5 or 9.5.

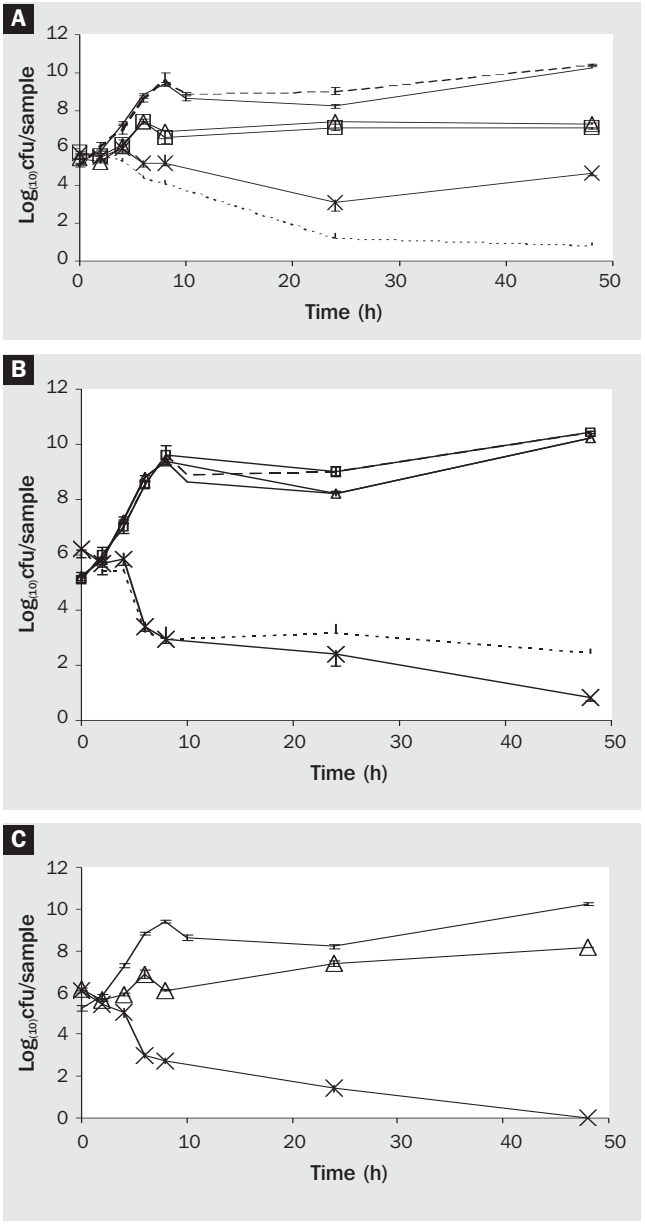

Fig. 1. Growth over 48 h in the presence of an orange/bergamot blend at 37°C: a) pH 5.5, MIC 0.5% *E. faecalis*, 1% *E. faecium*; b) pH 7.5, MIC 2% *E. faecalis* and *E. faecium*; and c) pH 9.5, MIC 2% *E. faecium* (mean±SE, $n=6$).
E. faecalis with oil/vapour (.....), *E. faecalis* with no oil/vapour (–□–), *E. faecalis* optimum conditions (-----), *E. faecium* with oil/vapour (–X–), *E. faecium* with no oil/vapour (–△–), *E. faecium* optimum conditions (——).

Determination of minimum inhibitory concentrations

Dilutions (0.03%, 0.06%, 0.12%, 0.5%, 1%, 2% and 4% [v/v]) of the oil/component or blend were added to BHI agar plates. Tween 20 (0.5%) was also added and the pH adjusted to 5.5, 7.5 or 9.5. Controls contained no oil in the agar. Plates were then spread with the organism and incubated at 25°C,

37˚C or 50˚C for 24 h. The MIC was defined as the lowest concentration of the oil inhibiting visible growth.

Determination of minimum inhibitory dose

Aliquots (0.1 mL) of oil/component or blend were spotted on 3-cm diameter filter paper discs in two-fold dilutions (1600, 800, 400, 200, 100 and 50 mg/L) placed on the lids of BHI agar plates inoculated with either *E. faecium* or *E. faecalis*. The pH of the agar was adjusted using either HCl or KOH to 4.5, 5.5, 6.5, 8.5, 9.5 or 10.5. The plates were placed in an airtight beaker (1.3 L) and incubated at 25˚C, 37˚C or 50˚C for 24 h. The MID was the lowest concentration that inhibited growth.

Assessment of effect on growth

Strains were grown in BHI broth with the most inhibitory combination of pH and MIC or MID of the orange and bergamot blend EO at 25˚C, 37˚C or 50˚C, as established from previous experiments. Samples were taken at various time intervals, plated on BHI agar using a spiral plater and incubated for 24 h at 37˚C.

To assess whether the inhibition of growth observed was due to a bacteriocidal or bacteriostatic effect, samples of growing cultures were taken after 24-h and 48-h exposure, washed (x2) in sterile deionised water and resuspended in BHI broth. After 24-h incubation at 37˚C, duplicate samples were spiral-plated on BHI agar and incubated for 24 h at 37˚C. All experiments were carried out in duplicate on at least two separate occasions.

Statistical analysis

Statistical analyses using independent *t*-tests for unpaired data were carried out using SPSS version 11.5, with significance set at $P=0.05$ to establish differences in growth at different temperatures or pH.

Results

From the disc-diffusion method, ranges were established for differences in inhibition or growth. These were temperatures ranges of 5–15˚C, 20–25˚C, 30–45˚C and 50˚C, and pH ranges of 4.5–6.5, 7.5 and 8.5–10.5 ($P<0.001$). Within each range there was no significant difference in inhibition produced by the EO or the components, but there was a significant difference in inhibition between the ranges. Mid-points of pH ranges at 25˚C and 37˚C were carried forward for testing the EOs, while 50˚C was used for vapour investigations, as this was the only temperature tested at which vapours were inhibitory (Table 1).

As demonstrated by the MICs, for the vancomycin-susceptible strains the lemon EO alone or blended and orange EO alone were not as effective as citral or linalool alone or bergamot blends (Table 1). Of the components tested, limonene, hersipidin and neoericitrin showed no inhibitory effect, with only citral and linalool in vapour form inhibiting growth (MID: 100 to >1600 mg/L at 25˚C and 37˚C). At 50˚C, at which temperature the oils vapourise, there was greater inhibition than at 25˚C or 37˚C, with the blends of orange and bergamot, and orange and lemon, being effective against *E. faecium* (MID: 50–400 mg/L). The orange and lemon blend was ineffective against *E. faecalis*, with all other EOs or blends having MIDs of 50–100 mg/L (Table 1).

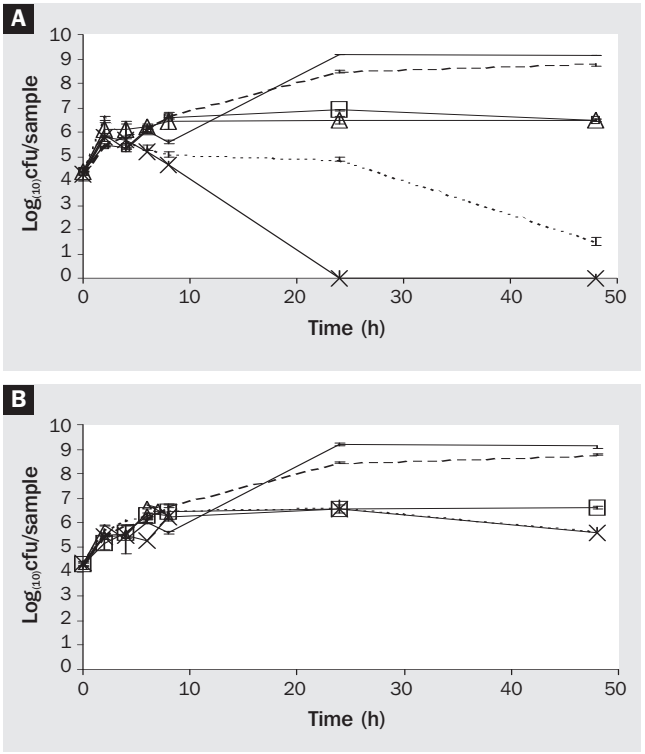

Fig. 2. Growth of vancomycin-resistant strains in the presence of an orange/bergamot blend over 48 h: a) 37˚C, pH 5.5 and MIC of 2%; and b) 50˚C, pH 7.5 and MID of 50 mg/mL (mean±SE, $n=6$). *E. faecalis* with oil/vapour (.....), *E. faecalis* with no oil/vapour (–□–), *E. faecalis* optimum conditions (-----), *E. faecium* with oil/vapour (–×–), *E. faecium* with no oil/vapour (–△–), *E. faecium* optimum conditions (——).

The MICs of the orange and bergamot blend against the vancomycin-resistant strains were higher than against the vancomycin-susceptible strains, with only those at pH 5.5 and 25˚C or 37˚C being below 4% (Table 2). The MIDs (50 mg/L) were the same for both resistant and susceptible strains, with the exception of *E. faecium* at pH 7.5 at 50˚C (MID: 200 mg/L) (Tables 1 and 2). Oils/blends/components with MICs of 4% (v/v) and above were not used in subsequent experiments because such levels would produce an intensity of odour unacceptable in a healthcare environment.

The reduction in numbers of antibiotic-susceptible strains in the presence of the orange and bergamot blend ranged from 5.5 to 10 $\log_{10}$ colony-forming units (cfu)/mL at 37˚C (Figs. 1a, b and c), compared with a reduction at 25˚C of approximately 5.5 $\log_{10}$ cfu/mL at pH 5.5, 7.5 and 9.5. No growth was detected after exposure to the vapour at 50˚C and pH 7.5 or pH 9.5 after 48 h (results not shown).

Log reductions in the presence of oils or vapours in optimum conditions (pH 7.5/37˚C) became significantly different between two and six hours under all conditions tested (Figs. 1 and 2). 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 numbers of viable cells, respectively. The vapours only reduced growth of the resistant strains by 4.5 $\log_{10}$ cfu/mL compared with 10 $\log_{10}$ cfu/mL for the susceptible strains (Fig. 2b).

The inhibition was a bacteriostatic effect. When removed

from the EO and grown under optimal conditions, cell numbers increased from 1–3 $\log_{10}$ cfu/mL in the presence of the orange and bergamot EO blend to log 6.5–7.5 cfu/mL under optimum conditions, and from undetectable levels in the presence of the vapours to a population of 4.5 $\log_{10}$ cfu/mL, although in the latter case recovery was lower.

Discussion

The ranges of temperature and pH for growth established in this study are similar to those used in previous studies that assessed the membrane permeability of *Enterococcus* in the presence of 3% NaCl (10–13˚C, 17–22˚C and 42–47˚C) or bacteriocins from *Lactobacillus plantarum* (4–25˚C, 30–37˚C and 45–60˚C, pH 2–4, 6–8 and 10).[3,20]

Although the strains of *Enterococcus* spp. tested were found to be less susceptible than other Gram-positive bacteria such as *Listeria monocytogenes* and *Bacillus cereus* to orange or bergamot EOs,[21] the results of this investigation demonstrate that the blend of orange and bergamot (1:1 v/v) EOs has antibacterial properties against vancomycin-resistant and vancomycin-susceptible *E. faecalis* and *E. faecium*, with citral, linalool and bergamot being the most inhibitory, and lemon the least inhibitory. Similar results have been demonstrated in previous studies.[17,21]

The concept of blending citrus EOs has yet to be explored, but the results from this study show that the blending of orange and bergamot oils results in a lower MIC than that achieved individually. It is essential to obtain the lowest MIC/MID possible because of potential changes in organoleptic properties of foodstuffs, if used as a preservative regime. For example, a tasting panel has found carvacrol, cinnamaldehyde and thymol to be unacceptable at any level when used in carrot juice.[22] In a healthcare environment, patient acceptability as well as antimicrobial activity is an important consideration, so a lower MIC/MID is key to any potential use in such situations.

The inhibition of growth over 48 h using oil for all strains at 25˚C was statistically lower than that at 37˚C. This may be considered unusual as, by subjecting bacteria to various sublethal stresses, additive inhibitory effects are generally observed, such as in *Salmonella typhimurium*, with combinations of low temperature, modified atmospheres and oregano EO.[23] One of the reasons why this was not observed may be that the enterococcal membrane is more stable near the minimal growth temperature, which is a specific mechanism associated with enterococci.[3] The ability of *Enterococcus* spp. to survive in such adverse conditions (i.e., large ranges of growth temperature [5–50˚C] and pH [4.5–10.5]) suggests that it contains proteins that are able to function at extremes of these parameters, and that it is also able to adapt. The proteins involved in temperature change response are DnaK and GroEL.[24]

The effect of pH on the inhibition of *Enterococcus* is dependent on the species, with *E. faecium* more resistant to pH 9.5 than *E. faecalis*, which at high temperatures does not grow. Unlike the cross-response of alkaline to acid stresses in *Escherichia coli*, it has been noted that *Enterococcus faecalis*

Table 1. Mean MIC (% v/v) and MID (mg/L) of citrus essential oils/components/blends against vancomycin-susceptible strains of *E. faecalis* and *E. faecium* at 25˚C and 37˚C using the agar dilution method (n = 4).

		E. faecalis						*E. faecium*					
		pH 5.5		pH 7.5		pH 9.5		pH 5.5		pH 7.5		pH 9.5	
		Oil	Vapour	Oil	Vapour	Oil	Vapour	Oil	Vapour	Oil	Vapour	Oil	Vapour
25˚C	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
	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
37˚C	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
	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

O: orange, L: lemon, B: bergamot.

NE: not effective.

does not have this ability; therefore, acid adaptation does not then result in alkaline tolerance as it involves different resistance pathways.[25]

Vapours are needed in lower concentrations than oils to produce the same inhibition and they produce a greater bacteriocidal effect. This suggests that there may be a potential for the use of the vapour in a clinical environment for reducing cross-contamination.

Vancomycin-resistant strains are not as susceptible to the blend of orange and bergamot EO vapours as are vancomycin-susceptible strains. However, the orange and bergamot EO has approximately the same inhibitory effect in both, 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 that alters the D-alanine in the pentapeptide of the murein precursor, resulting in the inability of vancomycin to bind to the cell.[26] This may suggest that vapours are using antibiotic docking sites to affect the cell membrane. Essential oils are believed to disrupt and penetrate the lipid structure of the cell wall of bacteria, resulting in the denaturing of proteins and destruction of the cell membrane, leading to cytoplasmic leakage and cell lysis.[27]

In conclusion, a blend of 1:1 (v/v) orange and bergamot EOs in both oil and vapour form has antimicrobial properties against vancomycin-resistant and vancomycin-susceptible strains of *E. faecium* and *E. faecalis* tested in this study, and this is amplified when used at the extremes of pH. However, temperature stress appears to make the cells more resistant to the citrus EOs tested. Patient acceptability tests will need to be carried out before the real potential of the blend can be ascertained. However, with antibiotic-resistant bacteria a real issue for the NHS, the low MIC/MID values demonstrated by the orange and bergamot blend in this study suggest that it may provide an alternative natural antimicrobial for use in the healthcare environment. □

Table 2. Mean MIC (% v/v) and MID (mg/L) of citrus essential oils/components/blends against vancomycin susceptible strains of *E. faecalis* and *E. faecium* using the agar dilution method at 50˚C (n=4).

	E. faecalis		*E. faecium*			
	pH 7.5		pH 7.5		pH 9.5	
	Oil	Vapour	Oil	Vapour	Oil	Vapour
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
Linalool	0.125	50	0.125	100	0.06	50
O/L	4	NE	>4	NE	>4	NE
O/B	1	50	2	200	1	50
B/L	4	100	4	400	4	100
O/L/B	2	50	4	NE	1	NE

O: orange, L: lemon, B: bergamot.

NE: not effective.

The authors would like to thank AMPHORA (Bristol, UK) for the oils and technical information.

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