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Sensitivity of pathogenic and commensal bacteria from the human colon to essential oils

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The microbiota of the intestinal tract plays an important role in colonic health, mediating many effects of dietary components on colonic health and during enteric infections. In the context of the increasing incidence of antibiotic resistance in gut bacteria, complementary therapies are required for the prevention and treatment of enteric infections. Here we report the potential application of essential oils (EO) and pure EO compounds to improve human gut health. Nerolidol, thymol, eugenol and geraniol inhibited growth of the pathogens *Escherichia coli* O157 : H7(VT$^-$), *Clostridium difficile* DSM1296, *Clostridium perfringens* DSM11780, *Salmonella typhimurium* 3530 and *Salmonella enteritidis* S1400 at a half-maximal inhibitory concentration (IC_{50}) varying from 50 to 500 p.p.m. Most EO showed greater toxicity to pathogens than to commensals. However, the beneficial commensal *Faecalibacterium prausnitzii* was sensitive to EO at similar or even lower concentrations than the pathogens. The EO showed dose-dependent effects on cell integrity, as measured using propidium iodide, of Gram-positive bacteria. These effects were not strongly correlated with growth inhibition, however, suggesting that cell membrane damage occurred but was not the primary cause of growth inhibition. Growth inhibition of Gram-negative bacteria, in contrast, occurred mostly without cell integrity loss. Principal component analysis showed clustering of responses according to bacterial species rather than to the identity of the EO, with the exception that responses to thymol and nerolidol clustered away from the other EO. In conclusion, the selective effects of some EO might have beneficial effects on gut health if chosen carefully for effectiveness against different species.

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INTRODUCTION

Essential oils (EO) are steam-volatile or organic solvent extracts of plants, comprising mainly terpene, terpenoid and other aromatic and aliphatic constituents of fairly low molecular mass (Bakkali *et al.*, 2008). Typically, EO contain a mixture of about 20–60 different compounds with two or three at high concentration (20–70 %). Throughout history, herbs and spices and their constituent EO have been used as antiseptics, to preserve food and to enhance flavour (Shelef, 1984). More recently, EO have been used in animal feeds to combat infection and improve productivity (Wallace *et al.*, 2002, 2010; Newbold *et al.*, 2004; Wallace, 2004; Franz *et al.*, 2010). For example, EO and EO compounds, including thymol, carvacrol and eugenol, reduced the bacterial load in different parts of the gut and lowered the shedding of *Clostridium perfringens* in broiler chickens (Mitsch *et al.*, 2004). EO also improved productivity in ruminants by altering the community of commensal bacteria in the rumen, altering protein degradation and reducing ammonia losses (Wallace *et al.*, 2002; McIntosh *et al.*, 2003; Wallace, 2004). In man, however, the roles of EO in microbial manipulation and gut fermentation have not been evaluated.

The main targets relating to gut health in man are enteric infection, inflammation and carcinogenesis. Enteric infections by gut pathogens, such as *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp. and *Clostridium* spp., occur when host defences fail to prevent colonization and proliferation (Sekirov & Finlay, 2009). The complex community of commensal bacteria has a role to play in protecting against this type of infection. They also have a role in gastrointestinal homeostasis. Dysbiosis of intestinal microbiota occurs in ulcerative colitis, irritable bowel syndrome and colon cancer (Moore & Moore, 1995; Flint *et al.*, 2007; Flint & Wallace, 2010; Noor *et al.*, 2010; Sekirov *et al.*, 2010). In a recent cohort study, the microbial community of patients with Crohn's disease was found to be markedly different from healthy controls and their unaffected relatives (Joossens *et al.*, 2011). Density gradient gel electrophoresis (DGGE) of ribosomal PCR amplicons indicated that numbers of *Faecalibacterium prausnitzii* and *Bifidobacterium*

Abbreviations: EO, essential oils; PCA, principal component analysis; PI, propidium iodide; RFU, relative fluorescence units.

(*Bif.*) *adolescentis* were lower and *Ruminococcus gnavus* higher than in healthy relatives. Thus, if EO are to be useful in promoting gut health in man, their effects on commensal as well as pathogenic bacteria must be determined.

The antimicrobial properties of EO have been demonstrated against a wide range of food micro-organisms, including bacteria, protozoa and fungi (Burt, 2004). Several studies also found that the most pathogenic gut bacteria, *E. coli* O157 : H7 (Burt & Reinders, 2003; Delaquis *et al.*, 2002), *Salmonella typhimurium* (Si *et al.*, 2006), *Clostridium perfringens* (Ouwehand *et al.*, 2010), *Campylobacter jejuni* (Anderson *et al.*, 2009) and *Helicobacter pylori* (Bergonzelli *et al.*, 2003) are inhibited by EO *in vitro*. The aim of the present study was to compare the effects of a range of EO and EO compounds on human pathogenic and commensal intestinal bacteria. Further studies were undertaken to explain the nature of the selectivity of EO against different bacterial species.

METHODS

Chemicals and reagents. The pure oils of clove, coriander and curcuma, a commercial blend of EO ('Agolin'), analytical grade eugenol, geraniol, geranylacetate, linalool, methylisoeugenol, nerolidol and thymol, and chestnut extract were provided by Agolin SA, Bière, Switzerland. The test materials were selected on the basis of traditional and potential commercial usefulness, on their published effects on pathogens and their safety. The EO and EO compounds were >98 % pure, while the chestnut extract contained >75 % tannins. Stock solutions (100 mg ml^{-1} in methanol) were stored in air-tight capped bottles at 4 °C in the dark. Propidium iodide (PI) was purchased from Sigma-Aldrich, UK. All other reagents were of analytical grade.

Bacteria. Five species of recognized pathogens were investigated, along with 11 species of commensal bacteria. *Clostridium difficile* DSM 1296, *C. perfringens* DSM 11780, *Propionibacterium shermanii* DSM 4902, *Propionibacterium freudenreichii* DSM 20271 and *Bacteroides* (*Bac.*) *thetaiotaomicron* 5482 (DSM 2079) were obtained from Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). *Bifidobacterium breve* NCFB 2258 and *Bif. adolescentis* NCFB 2204 were from the National Collection of Food Bacteria (Reading, UK). *Lactobacillus plantarum* NCIMB 7220 was from the National Collection of Industrial, Food and Marine Bacteria (Aberdeen, UK). *Salmonella typhimurium* 3530 and *Salmonella enteritidis* S1400 were kindly provided by George Grant (University of Aberdeen). *E. coli* O157 : H7 NCTC 12900, a verotoxin-deleted strain, is a human isolate. *Anaerostipes caccae* L1-92 (DSM 14662^T), *Eubacterium* (*Eu.*) *hallii* L2-7 (DSM 17630), *Roseburia inulinivorans* A2-194 (DSM 16841^T), *Roseburia hominis* A2-181 and *F. prausnitzii* L2-6 were isolated from human faeces (Barcenilla *et al.*, 2000) and are maintained at the Rowett Institute of Nutrition and Health. *Salmonella* spp. and *E. coli* were grown in LB medium aerobically, and all others were grown in the liquid form of medium 2 (Hobson, 1969) under CO_2.

Influence of EO on bacterial growth. The influence of EO on the growth of *E. coli*, *S. typhimurium* and *S. enteritidis* was tested by a broth dilution method on 96-well plates. A range of concentrations of EO in methanol was prepared on a dilution plate from the stock solution (100 mg l^{-1}) and 10 µl was transferred to a culture plate to give final concentrations of 50, 100, 200, 300, 500, 750 and 1000 p.p.m. A 200 µl volume of LB medium, containing 5 % overnight culture as inoculum, was transferred to corresponding wells in triplicate. Positive control wells contained only 10 µl methanol. Plate control and negative control wells were set up using sterile water and uninoculated medium, respectively. Plates were sealed with plastic adhesive tape (Fasson S695, catalogue no. SH 236269, Nunc) and growth at 37 °C was measured spectrophotometrically (SpectraMax spectrophotometer, Molecular Devices Corporation) as OD_{650} for 24 h at 30 min intervals. All other bacteria were inoculated as a 5 % inoculum in the liquid form of medium 2 (Hobson, 1969) in Hungate-type tubes containing different concentrations of EO in methanol. Incubation was carried out at 37 °C and growth was measured spectrophotometrically (Novaspec II spectrophotometer, Amersham Biosciences) as OD_{650} at different incubation times. The methanol concentration was 5 %, which had no effect on the growth of the tested bacteria. The calculation of percentage growth and percentage inhibition by EO treatment was based on the growth of positive controls (Sultanbawa *et al.*, 2009):

$$\text{Percentage growth} = (OD_{tt} - OD_{t0})/(OD_{ct} - OD_{c0}) \times 100$$

$$\text{Percentage inhibition} = 100 - \text{percentage growth}$$

where OD_{tt} is the OD_{650} of test samples at incubation time t, OD_{t0} for test samples at time zero (0), OD_{ct} is the positive control at incubation time t and OD_{c0} is the positive control at time zero (0).

The half-maximum inhibitory concentration (IC_{50}) of EO and EO compounds was calculated by linear interpolation of triplicate observations:

$$d = d_1 + (p - p_1) \times (d_2 - d_1)/(p_2 - p_1)$$

where d is IC_{50}, d_1 is a first dose lower than 50 % inhibition, d_2 is a first dose higher than 50 % inhibition, p is equal to 50 (for 50 % inhibition), p_1 is percentage inhibition at dose d_1, and p_2 is percentage inhibition at dose d_2.

Measurement of cell integrity. The influence of EO on the cell integrity of bacteria was determined by a PI uptake method based on Amor *et al.* (2002) and later modified by Maia *et al.* (2007). Briefly, 1 ml of overnight culture was inoculated into 9 ml M2 medium and incubated at 37 °C until it reached mid-exponential phase (OD_{650} approx. 0.6). The bacterial culture was centrifuged at 3000 ***g*** for 10 min at 4 °C. The pellet was washed twice with anaerobic potassium phosphate buffer (100 mM, pH 7.0) containing 1 mM DTT. Cells were resuspended to OD_{650} 0.4 in the same buffer for assay. Dilution series of EO were prepared in methanol in separate 96-well plates and 10 µl was added to 200 µl of cell suspension. Control cultures contained the same volume of methanol, which did not affect measurements. The suspension was incubated at 37 °C for 30 min. Cell suspensions without EO and sonicated cells (10 µm amplitude, 3 min; MSE Soniprep 150) served as controls. A working solution of PI (1.5 mM) was prepared in distilled water and stored at 4 °C in the dark. Fifty microlitres of each sample was added to 149 µl anaerobic potassium phosphate buffer (100 mM, pH 7.0, containing 1 mM DTT) in the presence of 1 µl PI solution. The mixtures were incubated for 5 min at 37 °C in the dark. Fluorimetry measurements were done using a Gemini XPS Microplate Reader (Molecular Devices Corporation) at λ_{EX}=530 nm and λ_{EM}=620 nm. Calculation of percentage cell integrity loss was based on the relative fluorescence units (RFU) of the positive control (sonicated damaged cells):

$$\text{Percentage cell integrity loss} = (RFU_{\text{treatment}}/RFU_{\text{positive control}}) \times 100$$

Principal component analysis (PCA). PCA (Jolliffe, 2002) of the data was carried out in order to look for patterns of similarity and clustering in the organisms and compounds. For the PCA, each of the

99 combinations of organisms and compounds was considered as an observation. The growth inhibition and cell membrane damage at each of the seven doses were considered as variables, leading to 14 variables in total, and so a 99 by 14 data matrix. Scores plots of the first two components then show a map of the similarities and differences among the organism and compound combinations.

Statistical analysis. Growth inhibition data are presented as mean ± SD of triplicate observations. Data on membrane integrity loss were transformed to log values and analysed using one-way ANOVA, and means for the treatments were separated by Bonferroni post-hoc multiple comparisons in SPSS 19 software with significance set at $P<0.05$. The PCA was performed with R (R Foundation for Statistical Computing, ISBN 3-900051-07-0; http://www.R-project.org).

RESULTS

Differential effects of EO on bacterial growth

The growth of human gut bacteria in the presence of a range of EO or EO compounds at concentrations ranging from 50 to 1000 p.p.m. was investigated. Most pathogenic strains of *E. coli* O157 : H7, *Salmonella* spp., *Clostridia* spp. and the abundant commensal Firmicutes and Bacteroidetes were affected to different extents by different EO in a dose-dependent manner. Fig. 1 illustrates the type of growth data that were obtained. The measurement of percentage growth compares the optical density of bacteria in EO-containing medium with that of a parallel non-amended culture at the entry into stationary phase. Thymol showed the strongest effect on the growth of all bacteria, with most of the bacteria unable to grow at 300 p.p.m. (Fig. 1a). In contrast, clove oil was less toxic to all the bacteria at similar concentrations (Fig. 1b). Eugenol, the major EO compound present in clove oil, had similar effects on the growth of *E. coli*, *S. typhimurium* and *C. difficile*, while the commensal species *L. plantarum*, *Eu. hallii* and *Bif. adolescentis* were less affected (Fig. 1c).

In order to condense large quantities of growth data to values that enable comparison between species and their response to different EO, IC_{50} values of EO were calculated by linear interpolation. Most of the EO showed an IC_{50} below 500 p.p.m. for pathogenic bacteria, while there was a wide range of effectiveness with different EO. Nerolidol and thymol were the most active EO, with IC_{50} values of 41 and 108 p.p.m. for *C. difficile* and 156 and 111 p.p.m. for *E. coli* O157 : H7, respectively (Table 1). Clove oil, coriander oil, curcuma oil and Agolin blend were effective at higher concentration ranges of 234 and up to >1000 p.p.m. Similar effects were observed with pure compounds of EO such as eugenol, geraniol, linalool and methylisoeugenol, which are major constituents of a number of EO, particularly clove oil (eugenol) and geranium oil (geraniol). Nerolidol also had strong growth-inhibitory effects against most of the commensals, with IC_{50} values of 33–244 p.p.m. depending upon the bacterial species. Thymol had similar effects against pathogens, and most commensals were affected by low concentrations. *F. prausnitzii* L2-6, in particular, was highly sensitive to all EO relative to all other commensals and the pathogens. However, most other commensals were insensitive to many EO at the concentrations that were effective against pathogens.

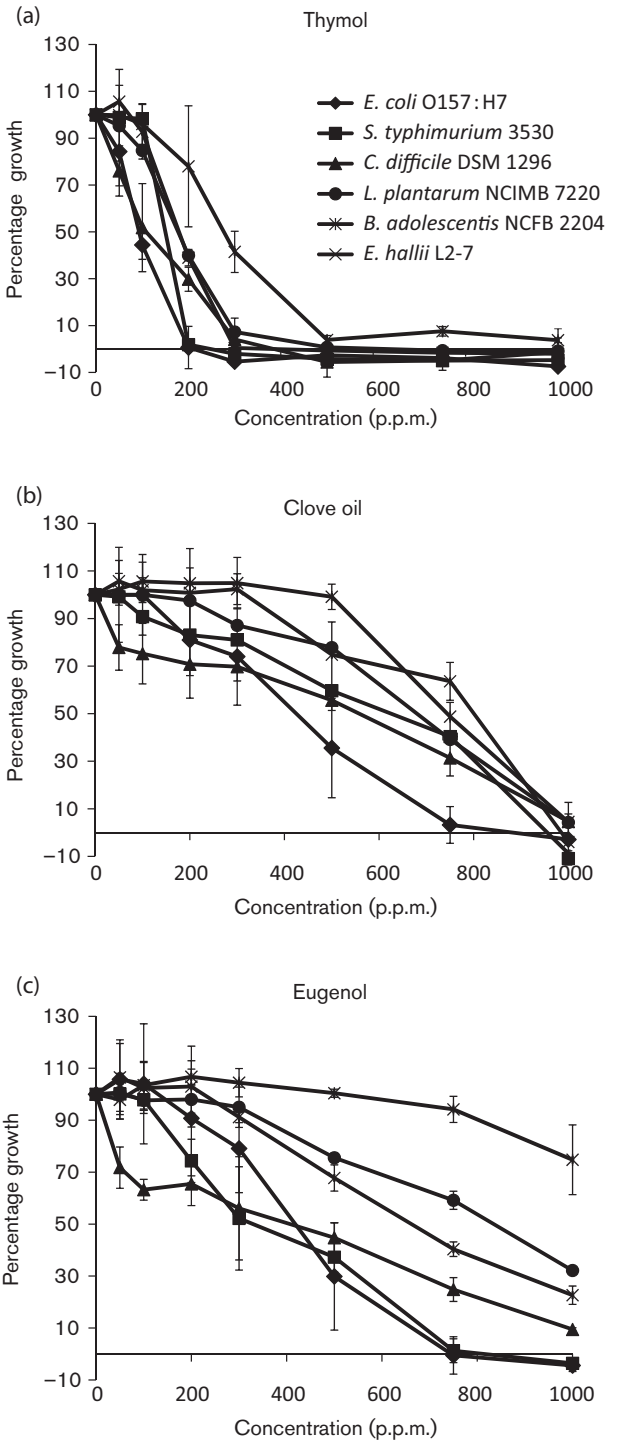

Fig. 1. Differential inhibitory effect of EO on human intestinal pathogenic and commensal bacteria; (a) thymol, (b) clove oil and (c) eugenol. Percentage growth due to treatment was determined by taking growth in medium containing no EO as 100 % at stationary phase. Results are mean ± SD of triplicate observations.

Table 1. IC_{50} values of EO on pure cultures of human gut bacteria

ND, Not determined.

Micro-organism	Eugenol	Geraniol	Geranylacetate	Linalool	Methylisoeugenol	Nerolidol	Thymol	Clove oil	Coriander oil	Curcuma oil	Agolin blend	Chestnut extract
Pathogens												
C. difficile DSM 1296	464	182	365	464	251	41	108	562	323	305	563	149
C. perfringens DSM 11780	>1000	632	>1000	>1000	608	78	388	878	>1000	614	890	600
E. coli O157 : H7 (VT$^-$) NCTC 12900	426	238	>1000	521	339	156	111	466	234	829	340	784
S. typhimurium 3530	297	239	>1000	871	823	590	147	568	824	ND	971	960
S. enteritidis S1400	477	422	>1000	913	>1000	ND	233	833	950	ND	>1000	ND
Commensals												
A. caccae L1-92	814	854	>1000	>1000	686	80	202	552	>1000	620	>1000	689
Bac. thetaiotaomicron 5482	488	214	35	342	233	39	172	456	773	527	400	44
Bif. breve NCFB 2258	779	860	>1000	>1000	>1000	199	246	790	>1000	706	>1000	551
Bif. adolescentis NCFB 2204	>1000	863	902	>1000	>1000	162	299	750	>1000	653	>1000	615
Eu. hallii L2-7	661	747	>1000	>1000	770	65	179	799	>1000	610	>1000	739
F. prausnitzii L2-6	71	121	>1000	111	108	42	128	100	109	496	180	735
L. plantarum NCIMB 7220	833	823	>1000	>1000	852	188	178	680	>1000	722	>1000	856
P. freudenreichii DSM 20271	221	156	ND	356	217	ND	68	215	301	ND	298	ND
P. freudenreichii subsp. *shermanii* DSM 4902	486	759	151	>1000	522	33	142	392	825	255	695	105
R. hominis A2-181	>1000	763	>1000	>1000	608	244	248	848	>1000	860	955	703
R. inulinivorans A2-194	934	516	>1000	>1000	>1000	151	259	886	>1000	740	>1000	589
Variability (SEM as percentage of mean)*	5.9	6.9	5.7	15.0	6.8	5.2	5.8	8.2	9.8	12.2	7.9	7.3

*Variability of means was not considered for doses >1000 p.p.m.

Influence of EO on cell integrity

The influence of EO on the cell integrity of different species of pathogenic and commensal bacteria was investigated using PI, which fluoresces when it reacts with DNA. The relative fluorescence was compared between undamaged cells, sonicated cells and cells exposed to EO. Typical results are illustrated in Fig. 2, where thymol or nerolidol were added to suspensions of *E. coli* O157 : H7 at 50, 100, 200 and 300 p.p.m. for 30 min. Thymol caused a dose-dependent loss of cell integrity in *E. coli* (Fig. 2a). In contrast, nerolidol had no effect at any concentration (Fig. 2b), despite having an IC_{50} of 156 p.p.m. for growth (Table 1).

When the full range of bacteria, including both pathogens and commensals, was compared for cell integrity loss and growth inhibition by EO, a clear differential effect was observed (Fig. 3). In general, Gram-negative bacteria clustered along the *y* axis (Fig. 3a), indicating that cell integrity loss was not high and that growth inhibition occurred without loss of cell integrity. Gram-positive bacteria, on the other hand, tended to cluster along the *x*-axis, with only a few clusters at the top right of the graph (Fig. 3b). Thus, Gram-positive bacteria were much more susceptible than Gram-negative species to cell envelope disruption by EO, although this in itself was insufficient to cause growth inhibition.

When the results were analysed by PCA (Fig. 4), cell integrity loss explained 76 % of the variability in growth inhibition. Patterns linking organisms and EO were identified by ellipses drawn on the PCA, based on our biological understanding of the species and compounds, rather than a statistically based cluster analysis, which would lack the biological interpretation. The observed effects could be explained with five clusters: cluster 1 revealed high growth inhibition of *Bac. thetaiotaomicron* relative to cell integrity loss; clusters 2 and 3 were mainly due to *C. perfringens* and *R. inulinivorans*, respectively, showing growth inhibition mainly due to high cell integrity. Cluster 4 was mainly due to *F. prausnitzii*, and cluster 5 revealed a subset of most other clusters and mainly contained the effects of thymol, nerolidol and geraniol on most of the bacteria. This plot also visualized the distinction between Gram-positive and Gram-negative species, as they clustered far from each other.

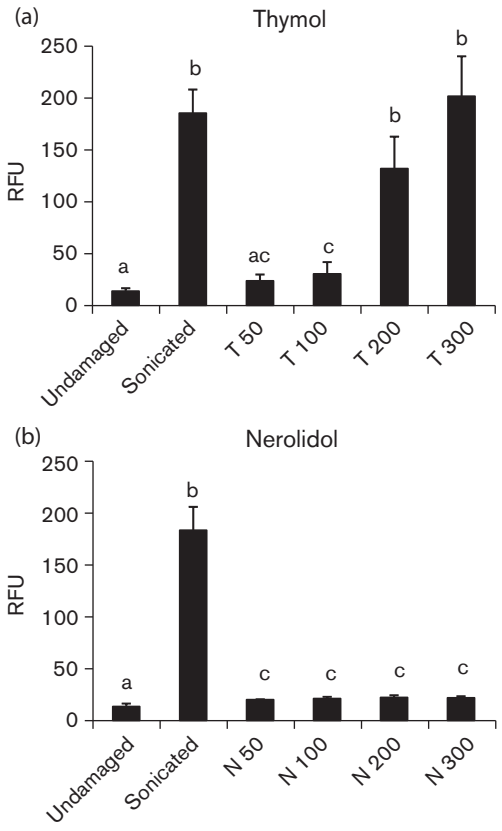

Fig. 2. Relative fluorescence of PI as a measure of cell integrity loss of *E. coli* O157 : H7 treated with (a) thymol (T 50, T 100, T 200 and T 300: 50, 100, 200 and 300 p.p.m.) and (b) nerolidol (N 50, N 100, N 200 and N 300: 50, 100, 200 and 300 p.p.m.), at 30 min exposure (n=4). Different letters differ significantly (P<0.05).

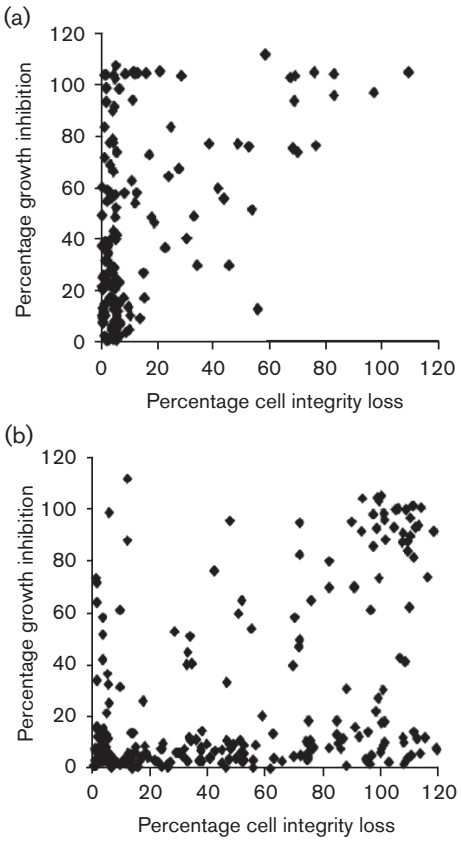

Fig. 3. Correlation plots of the effect of EO on growth inhibition and on cell integrity loss. Plots were obtained from mean observations of 11 EO at seven doses in duplicate. (a) Gram-negative bacteria (*E. coli*, *S. typhimurium*, *S. enteritidis*, *Bac. thetaiotaomicron*), (b) Gram-positive bacteria (*C. perfringens*, *Bif. adolescentis*, *Bif. breve*, *R. inulinivorans* and *F. prausnitzii*).

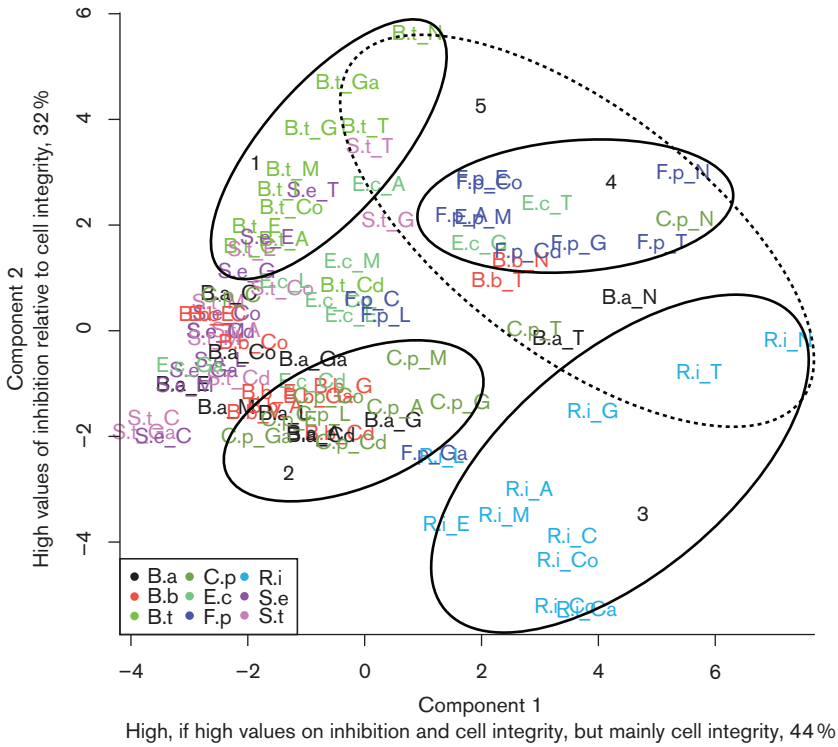

Fig. 4. PCA scores plot to show the patterns of similarity and clustering in organisms and compounds based on growth inhibition and cell integrity loss by 11 EO at seven doses in duplicate. Each value represented by the first two letters is specific for the genus and species of bacterium, and the last letter(s) for the EO. Gram-positive species: B.a, *Bif. adolescentis*; C.p, *C. perfringens*; R.i, *R. inulinivorans*; B.b, *Bif. breve*; F.p, *F. prausnitzii*. Gram-negative species: E.c, *E. coli*; S.e, *S. enteritidis*; S.t, *S. typhimurium*; B.t, *Bac. thetaiotaomicron*. A, Agolin blend; E, eugenol; M, methylisoeugenol; C, curcuma oil; G, geraniol; N, nerolidol; Cd, coriander oil; Ga, geranylacetate; T, thymol; Co, clove oil; L, linalool.

DISCUSSION

EO and their component compounds have been used by mankind for many centuries, and their antibacterial effects have been determined on various microbial species during recent years. The present work reports the results of an investigation of an unusually wide range of EO and bacterial species not investigated previously, in a depth that enabled PCA analysis to reveal correlations between different EO and different types of bacteria. This is also the first report of the influence of EO on commensal gut bacteria, which, if EO are to be used therapeutically or for prophylaxis, is an essential element of background information. The results form the first phase of possible development of EO as health-enhancing food additives/supplements in man.

The potency of the different EO depended on their chemical structure. The categories of EO compounds included (A) phenol derivatives (e.g. thymol), (B) alcohol derivatives [e.g. geraniol (Bg), linalool (B_l)] and (C) ester derivatives (e.g. geranylacetate) of monoterpenes; (D) alcohol derivatives (e.g. nerolidol) of sesquiterpenes; (E) phenol derivatives (e.g. eugenol) and (F) methoxy derivatives (e.g. methylisoeugenol) of aromatic compounds. The IC_{50} values and thus the overall antibacterial effects of EO compounds against *C. difficile* were in the order D>A>B_g>F>C>E>B_l, for *C. perfringens* D>A>F>B_g>C=E=B_l, for *E. coli* A>D>B_g>F>E>B_l>C, for *S. typhimurium* A>B_g>E>D>G>B_l>C and for *S. enteritidis* A>B_g>E>B_l>F>C. These relative potencies were quite similar when tested against most of the commensals (Table 1). Thus, sesquiterpenes and phenolic monoterpenes were the most active class of antibacterial agents followed by other phenolic aromatic compounds. A twofold lower activity of geranylacetate, compared with geraniol, which is an ester derivative of geraniol, indicates the importance of the free -OH group for this activity. Similar observations were made before, whereby the inhibitory effects of carvacrol against *Bacillus cereus* (Ultee *et al.*, 2002), *E. coli* (Veldhuizen *et al.*, 2006) and *Staphylococcus aureus* (Veldhuizen *et al.*, 2006) were completely abolished in its methyl ester derivative. In those studies, the substituted groups and delocalized electrons in the benzene ring were postulated to be responsible for this difference in activity. It is important to note, however, that EO mixtures and natural EO may have activities different from those that might be expected based on the potency of pure EO compounds. Here we have established that such interactions depend on the bacterial species. For *E. coli*, *C. difficile* and *L. plantarum*, the results for eugenol and clove oil were very similar, while for *S. typhimurium* eugenol alone was clearly more effective. On the other hand, the sensitivity of *Bif. adolescentis* and *Eu. hallii* to clove oil was greater than that to eugenol, suggesting that a minor component of clove oil may act synergistically with eugenol. These observations have implications for the use of natural EO, in which changes in the chemical composition can occur by seasonal variation, growth period and geographical region (Mabrouk *et al.*, 2011). Thus, careful quality control would be necessary when applying natural EO therapeutically or prophylactically.

The possible application of EO in manipulating the human gut microbiota to treat or prevent bowel diseases and enteric infections will depend on understanding the inhibitory

profile of both pathogenic micro-organisms and the more abundant normal flora of the gut. Here, different EO showed different inhibitory effects on different species of bacteria. However, in general, EO were more inhibitory towards pathogens than commensals, a finding that confirms a similar conclusion made for porcine intestinal bacteria (Si *et al.*, 2006). A very significant exception was, however, the greater sensitivity of *F. prausnitzii* to virtually all EO and EO compounds than the pathogens. *F. prausnitzii* plays an important anti-inflammatory role in the gut (Sokol *et al.*, 2008), and lower numbers of *F. prausnitzii* are associated with Crohn's disease (Marteau *et al.*, 2001). Thus, the use of EO in man would have to guard against the suppression of *F. prausnitzii* as well as proving efficacy against pathogens.

A study of the relationship between effects of EO on growth and their effects on cell integrity was undertaken in order to understand better the mechanisms by which different EO interact with and inhibit the growth of different bacterial species. While some of the data were difficult to explain, such as the difference between thymol and nerolidol in their effects on *E. coli* (Fig. 2), a general pattern emerged that the growth of Gram-negative bacteria was inhibited generally without a loss of cell integrity, while the opposite was true for Gram-positive bacteria. PI, which fluoresces when it interacts with DNA, was the indicator of cell integrity damage, as it has been in many other studies (Gill & Holley, 2006a). These results appear to contrast with observations reported in *E. coli* (Gill & Holley, 2006a), in which *E. coli* treated with 10 mM eugenol results in 100 % staining of the cell and 100 % cell death; however, this concentration corresponds to 1640 p.p.m., much higher than the concentrations used here. The same authors concluded that the primary action of EO in *E. coli* was against membrane-bound ATPases (Gill & Holley, 2006b). Our observations would also support the hypothesis that growth inhibition of gut bacteria by EO is not solely the result of membrane damage. The cellular membrane has a selective and low permeability for polar and charged particles. Lipophilic compounds such as cyclic hydrocarbons, including EO, can easily penetrate the membrane and increase the loss of ATP and intracellular metabolites, but more specific metabolic inhibition may occur with specific EO compounds (Di Pasqua *et al.*, 2007).

PCA was used to analyse in greater depth the relationship between growth inhibition and cell integrity loss by EO in different species. The intention of PCA is to take a high-dimensional set of observations (in this case the 14 values that specify the dose–responses in growth inhibition and membrane damage) and reduce the dimensionality by calculating summaries of them which aim to capture as much as possible of the total variability. By plotting the scores of each observation (organism/compound combination) from the first few components, a map was produced of the similarities and differences among the organism/compound combinations (Fig. 4). Thus, observations close in the score plot share similar dose–response characteristics. The orientation of the axes is arbitrary: only proximity or distance is relevant. The PCA scores plot confirmed that the antimicrobial effects due to loss in cell integrity were specific to bacterial species, as shown by clusters 1, 2, 3 and 4 (Fig. 4), while Gram-negative bacteria (clusters 1 and 4) and Gram-positive bacteria (clusters 2 and 3) clustered more tightly. Some EO, such as thymol, nerolidol and geraniol, showed generalized effects and were more strongly associated with cell integrity loss and growth inhibition than other EO and EO compounds (cluster 5). These differences in clusters due to both micro-organisms and the EO illustrate the importance of the chemical nature of EO and the type of bacterium to an understanding of specific inhibitory effects.

In conclusion, although pathogenic species were generally more sensitive to EO than most commensal bacteria, EO may compromise *F. prausnitzii*, one of the most beneficial of the commensal microbiota. The possible usefulness of EO therefore depends on many factors. If the site of inhibition of pathogens precedes the large intestine, i.e. the stomach or small intestine, EO might be selected that are absorbed before reaching the terminal ileum or are metabolized by the intestinal microbiota to avoid toxicity to *F. prausnitzii*. Alternatively, combinations of EO compounds may be sought that increase the selectivity towards pathogens. Further information is thus required on differential effects of EO in mixed cultures of gut microbiota and *in vivo* to formulate different dietary regimes for specific inhibition of pathogens during enteric infections, and to treat or prevent colonic dysbiosis (Kaefer & Milner, 2008; Moore & Moore, 1995).

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