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Antimicrobial activity of essential oils and five terpenoid compounds against *Campylobacter jejuni* in pure and mixed culture experiments

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ABSTRACT

The aim of this study was to examine the antimicrobial potential of three essential oils (EOs: tea tree oil, lemon myrtle oil and *Leptospermum* oil), five terpenoid compounds (α-bisabolol, α-terpinene, cineole, nerolidol and terpinen-4-ol) and polyphenol against two strains of *Campylobacter jejuni* (ACM 3393 and the poultry isolate C338), *Campylobacter coli* and other Gram negative and Gram positive bacteria. Different formulations of neem oil (*Azadirachta indica*) with these compounds were also tested for synergistic interaction against all organisms. Antimicrobial activity was determined by the use of disc diffusion and broth dilution assays. All EOs tested were found to have strong antimicrobial activity against *Campylobacter* spp. with inhibitory concentrations in the range 0.001–1% (v/v). Among the single compounds, terpinen-4-ol showed the highest activity against *Campylobacter* spp. and other reference strains. Based on the antimicrobial activity and potential commerciality of these agents, lemon myrtle oil, α-tops (α-terpineol + cineole + terpinen-4-ol) and terpinen-4-ol were also evaluated using an *in vitro* fermentation technique to test antimicrobial activity towards *C. jejuni* in the microbiota from the chicken-caecum. EO compounds (terpinen-4-ol and α-tops) were antimicrobial towards *C. jejuni* at high doses (0.05%) without altering the fermentation profile. EOs and terpenoid compounds can have strong anti-*Campylobacter* activity without adversely affecting the fermentation potential of the chicken-caeca microbiota. EOs and their active compounds may have the potential to control *C. jejuni* colonisation and abundance in poultry.

1. Introduction

Campylobacter jejuni is a Gram negative bacterium considered to be a common cause of human gastrointestinal illness (Zilbauer et al., 2008), resulting in bloody diarrhoea, abdominal cramps, fever and vomiting (Black et al., 1988; Gray and Pedler, 1991). Although in most subjects, the mortality rate is low and the illness is simply treated with antibiotics, infection in the elderly and in young children may be severe (Butzler, 2004). Moreover, Guillain-Barré syndrome and reactive arthritis are rare post-infection complications of this illness (Nachamkin et al., 1998). Chickens are healthy carriers of *C. jejuni* and have been reported to be the main vehicle of human infection based on epidemiological evidence (Mullner et al., 2010; Sheppard et al., 2009). Once a chicken is infected in a commercial poultry flock, *C. jejuni* spreads rapidly to all birds in the flock (Sahin et al., 2002). Prevalence of *C. jejuni* in the chicken carcass can be high due to contamination from intestinal contents during slaughter (Gregory et al., 1997). Pointon et al. (2008) reported that up to 90% of retail chicken meat in Australia was surface contaminated with *Campylobacter* spp. Several strategies have been employed to control or reduce the spread of *Campylobacter* spp. in poultry including biosecurity measures, competitive exclusion in the gut, vaccination and bacteriophage therapy (Gibbens et al., 2001; Stern et al., 2006; Wagenaar et al., 2005), but none of these attempts proved to be effective in commercial flocks. Furthermore, there is a rising public concern regarding the use of antibiotics as a feed additive in animal production systems. In response to such concerns, the European Union banned the use of antibiotics as a growth promoter in animal nutrition as a part of their regulation (EC) 1831/2003 in 2006 (Castanon, 2007). In Australia, although there has been a big concern to use antimicrobials in food-producing animals to improve growth, there are still some products including antimicrobial for this purposes authorised by Australian Pesticide and Veterinary Medicines Authority (available at http://www.apvma.gov.au/products/review/completed/virginiamycin.php). However, it has been reported that the withdrawal of antibiotics from animal feedstuffs can lead to reduction of animal productivity and an increase in the incidence of some bacterial diseases (Andreasen et al., 2005). In

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this regard, replacement compounds for antibiotics are urgently needed and the use of plant extracts in feedstuffs could be used as a strategy to reduce food-borne pathogens including *C. jejuni*.

Plant essential oils (EOs) are volatile materials that protect plants from diseases and insect attack (Valero, 2008). Most are highly aromatic due to the volatile nature of their component parts (Valero, 2008) and have been used since the start of human civilization for therapeutic and medicinal purposes (Oyen and Dung, 1999). Antimicrobial properties of certain plant EOs and/or their constituents towards human and animal pathogens have been well documented and reviewed by several authors (Burt, 2004; Carson et al., 2006; González-Lamothe et al., 2009). Plants are one of the more popular traditional antimicrobial sources in many societies because they are marketed and perceived as a healthy alternative to antibiotics due to favourable natural properties and perceived lack of side effects. Therefore, in recent years, the use of EOs as antimicrobial agents has become increasingly popular.

The *Myrtaceae* is one of the most important plant families of the Australian flora (Stanley and Ross, 2002). Various *Myrtaceae* spp., namely, *Melaleuca alternifolia*, *Backhousia citriodora* and *Leptospermum petersonii*, are considered as medicinal plants and mostly found in Australia (Weiss, 1996). The antibacterial properties of EOs derived from these plants, particularly *M. alternifolia* (tea tree oil, TTO) and *B. citriodora* (lemon myrtle oil, LMO) have now been documented, and there are susceptibility data on a wide range of bacteria (Carson et al., 2006; Wilkinson et al., 2003). With some exceptions the emphasis in most studies has focused on pathogenic bacteria like *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Candida albicans*. However, there are no studies available for antimicrobial activities of these oils against *Campylobacter* spp. In addition, there are few studies about the antibacterial activity of *L. petersonii* oil (Demuner et al., 2011; Hutton et al., 2012), though anti-fungal activity has been well investigated (Hood et al., 2010a; Hood et al., 2010b; Lee et al., 2008). Thus, the objective of the current study was to evaluate the antimicrobial activity of some EOs (TTO, LMO and *Leptospermum* oil), major compounds of TTO and olive polyphenols against *C. jejuni*, *Campylobacter coli* and some other Gram positive and Gram negative bacteria. Two different methods, broth microdilution and disc diffusion for pure cultures and an *in vitro* fermentation system using a mixed culture of the chicken caecal microflora were used to evaluate the compounds.

2. Materials and methods

2.1. Test compounds and microorganisms

All oils, compounds and blend of compounds used in this study were provided by BioAust, Australia. The oils were extracted from the biomass using steam distillation and then partitioned using super critical carbon dioxide to obtain the bioactives (BioAust, Australia) and the components of these oils are listed in Table 1. Compounds and formulations are listed in Table 2. α-Bisabolol obtained from traditional medical plant chamomile (*Matricaria chamomilla*) was supplied by BASF Aktiengesellschaft, Germany. α-Terpinene and terpinen-4-ol obtained from tee tree oil were supplied by Nanning Venusson Bio-Technology Co. LTD, China. Cineole and nerolidol also supplied by the latter company were obtained from eucalyptus oil and *Melaleuca quinquenervia* oil, respectively. For dilution, ethanol/polyoxyethylene fatty glyceride (EtOH/PFG) (1/1 v/v) (Huntsman, Brooklyn, Victoria, Australia) was used as it increased the solubility of test agents in liquid medium without resulting in foam. *C. jejuni* poultry strain C338 was obtained from Dr Pat Blackall (DEEDI, Australia; Merchant-Patel et al., 2008). *C. jejuni* ACM 3393 (equivalent to NCTC 11351) was purchased from the Australian Collection of Microorganisms (QLD, Australia) and *C. coli* (ATCC 43484) was obtained from Leonie Hodgers (CSIRO-Land & Water). *Enterococcus faecalis* (ATCC 29212), *Salmonella typhimurium* (ATCC 14028), *Bacillus cereus* (ATCC 11778) were purchased from Oxoid, Australia. *E. coli* 026 was obtained as a human clinical isolate (Al-Ajmi et al., 2006). All cultures were grown as previously described (Kurekci et al., 2012). *Campylobacter* isolates were cultured on blood agar plates [40 g/L blood agar base no. 2, containing 5% (v/v) defibrinated horse blood and *Campylobacter* growth supplement; Oxoid, Australia] and incubated under microaerobic atmosphere, created by self-contained gas generating

Table 1
Chemical composition of essential oils tested.

L. petersonii oil	Lemon myrtle oil	Tea tree oil
α-Thujene (trace)	6-Methyl-5-hepten-2-one (0.2%)	α-Pinene (2.6%)
α-Pinene (0.8%)	2,3-Dehydro-1.8-cineole (0.2%)	Sabinene (0.1%)
Sabinene (trace)	Myrcene (trace)	α-Terpinene (9.0%)
β-Pinene (0.3%)	Linalool (0.3%)	*para*-Cymene (2.9%)
Myrcene (2.7%)	*exo*-Isocitral (trace)	Limonene (1.1%)
Limonene (0.2%)	Citronellal (0.1%)	1,8-Cineole (2.9%)
Methyl heptanone (1.6%)	*cis*-Isocitral (0.5%)	γ-Terpinene (20.8%)
trans-β-Ocimene (0.1%)	*trans*-Isocitral (0.9%)	Terpinolene (3.4%)
Terpinolene (0.2%)	Neral (42.6%)	Terpinen-4-ol (39.9%)
Linalool (3.7%)	Geranial (54.6%)	α-Terpineol (3.1%)
iso-Pulegol (5.5%)	$Citral_{total}$ (98.7%)[a]	Aromadendrene (1.4%)
Citronellal (18.1%)		Delta-cadinene (1.0%)
Terpinen-4-ol (0.3%)		Globulol (0.1%)
Citral (55.1%)		Viridiflorol (trace)
Citronellol (3.5%)		

The composition of *L. petersonii* was analysed by New Directions Laboratory Pty. Lmt. Australia while others were analysed by Australian Essential Oil Company Pty. Ltd. Australia.

[a] $Citral_{total}$ is sum of components *exo*-isocitral, *cis*-isocitral, *trans*-isocitral, neral and geranial.

Table 2
Compounds and formulations used in this study.[a]

Compounds			
α-Bisabolol		Sesquiterpene alcohol	Alves et al. (2010)
α-Terpinene		Monocyclic terpene	Carson et al. (2006)
Cineole		Monoterpenoid ether	Lassak and McCarthy (2001)
Nerolidol		Sesquiterpene alcohol	Madyastha and Gururaja (1993)
Olive polyphenol			
Terpinen-4-ol		Monoterpenoid alcohol	Lassak and McCarthy (2001)
Formulations			
α-Tops	α-Terpineol	Monoterpenoid alcohol	Lassak and McCarthy (2001)
	Cineole		
	Terpinen-4-ol		
γ-Tops	γ-Terpinene	Monocyclic terpene	Carson et al. (2006)
	α-Terpinene		
	Terpinolene	Monocyclic terpene	Carson et al. (2006)
Formula 1	Sunflower oil 55%		
	γ-Tops 12.5%		
	Neem oil 12.5%		
	Surfactant[b] 20%		
Formula 2	Soy oil 55%		
	γ-Tops 12.5%		
	Neem oil 12.5%		
	Surfactant 20%		
Fungatol + neem oil	Neem oil 50%		
	α-Tops 50%		
Gammatol + neem oil	Neem oil 50%		
	γ-Tops 50%		

[a] Obtained from BioAust, Australia.

[b] Surfactant is a hydrogenated castor oil ethoxylated in 40 mol l^{-1} ethylene oxide (BioAust, Australia).