ANTIBACTERIAL EFFECT OF CARVACROL AND COCONUT OIL ON SELECTED PATHOGENIC BACTERIA*

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Essential oils play a prominent role as flavouring agents and fragrances in the food and perfume industries. Carvacrol is a major component of various essential oils, such as oregano and thyme oils, and is responsible for their antimicrobial activity. Lauric acid is a medium-chain fatty acid (MCFA) with a high antibacterial potential. Both carvacrol and MCFAs have been used empirically as antimicrobial agents. Here, we tested the inhibitory properties of carvacrol and coconut (*Cocos nucifera* L.) oil containing a high percentage of MCFAs against 5 harmful bacterial pathogens: *Escherichia coli, Salmonella* Enteritidis, *Staphylococcus aureus, Listeria monocytogenes,* and *Enterococcus cecorum*. Gas chromatography (GC-FID) analysis of coconut oil showed a high concentration of lauric acid (41%). Microdilution antimicrobial assays showed that the combination of carvacrol and coconut oil had a stronger antibacterial effect against all tested bacteria than both agents separately. We conclude that carvacrol could significantly improve the antibacterial effect of coconut oil.

terpene, coconut, foodborne pathogens

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INTRODUCTION

Plant volatiles, or essential oils, are plant secondary metabolites, which are biosynthesized by specialized secretory cells. These aromatic oily liquids represent a complex mixture of natural substances obtained from various parts of the plant. They are often responsible for a plant's distinctive scent or taste and are traditionally used to enhance the taste or aroma of food. Nowadays, essential oils play a prominent role as flavouring agents and fragrances in the food and perfume industries (Adlard, 2010). Essential oils are known to possess antibacterial and antifungal properties and are used as antimicrobial agents (Burt, 2004; Bakkali et al., 2008; Kloucek et al., 2012; Seow et al., 2014). Carvacrol (PubChem CID:10364) is the major component of various essential oils, such as oregano and thyme oils, and its inhibitory effect on the growth of various microorganisms is well documented (Lambert et al., 2001; Si et al., 2006; Michiels et al., 2010; Guarda et al., 2011; Castillo et al., 2014). It is also known to have significant antioxidant activity (Hazzit et al., 2009; Shaaban et al., 2012; Kourimska et al., 2014).

The antimicrobial properties of many essential oils are well established (Nielsen, Rios, 2000; Fisher, Phillips, 2008; Nedorostova et al., 2009; Lv et al., 2011; Hyldgaard et al., 2012; Seow et al., 2014) and an area of particular interest is their potential to inhibit the growth of some of the most serious foodborne pathogens, such as *Salmonella* spp., *Escherichia coli* O157:H7, *Listeria*

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monocytogenes, and *Staphylococcus aureus* (Kim et al., 1995; Friedman et al., 2002; Burt, 2004). These bacterial pathogens are well-established causative agents of zoonoses and foodborne outbreaks, and although most of them are highly monitored, a transmission through food is still commonly observed. Illnesses caused by the consumption of contaminated foods have a significant economic and public health impact worldwide.

Another class of natural compounds with antibacterial properties are the medium-chain fatty acids (MCFAs), saturated unbranched monocarboxylic acids with 6–12 carbons (Marten et al., 2006) like coconut oil and dairy fat. Compared with long-chain fatty acids (LCFAs). They are natural components of milk and various feed materials, especially coconut and palm oils, and the seed oil of some species of *Cuphea* (Marten et al., 2006). Because of its high content of saturated fatty acids, coconut oil is very stable. Moreover, it is rich in MCFAs, as well as easily digestible (Marina et al., 2009). The three most valuable MCFAs in coconut fat are lauric acid ($C_{12:0}$), capric acid ($C_{10:0}$), and caprylic acid ($C_{8:0}$) (Oyi et al., 2010). Lauric acid may reach a proportion of up to 50% in coconut oil (Marten et al., 2006; Arlee et al., 2013) and is the MCFA with the highest potential effect on harmful microorganisms: it inhibits the growth of algae (McGrattan et al., 1976), Gram-negative (Bergsson et al., 1998, 1999) as well as Gram-positive bacteria (Galbraith et al., 1971; Kabara et al., 1972; Feldlaufer et al., 1993), fungi (Kabara et al., 1972; Bergsson et al., 2001) protozoa (Dohme et al., 2001; Thormar et al., 2006).

To date, the effect of a combination of essential oils with fatty acids as an antibacterial agent has still been poorly studied. The goal of this study was therefore to investigate the synergetic antibacterial effect of carvacrol and coconut oil against selected pathogenic bacteria.

MATERIAL AND METHODS

Bacterial strains

In this study, we used five potentially pathogenic bacterial strains: *Escherichia coli* (ATCC 29522), *Salmonella* Enteritidis (ATCC 13076), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 7644), and *Enterococcus cecorum* (CCM 3659). Bacterial strains were obtained from the American Type Culture Collection (ATCC) (Manassas, USA) and The Czech Collection of Microorganisms (CCM) (Masaryk University, Faculty of Science, Brno, Czech Republic) and kept in Wilkins-Chalgren broth (Oxoid, UK) with glycerine at –80°C. Before testing, a morphological identification was made.

Coconut oil and carvacrol

Carvacrol and coconut oil containing a high percentage of MCFAs were purchased from Sigma-Aldrich (USA) and stored at 4°C. Carvacrol was stored in air-tight sealed glass bottles.

Gas chromatography analysis of coconut oil

The fatty acids composition of coconut oil was determined by gas chromatography (GC-FID). Alkaline trans-methylation of extracted fatty acids was carried out according to standard ISO 5509 (1994). First 100 mg of coconut oil were weighed to a clean 15 ml glass tube. Then 5 ml of isooctane (Sigma-Aldrich) were added and sample was mixed well. Alkaline trans-methylation was performed by adding 0.5 ml of 2M methanolic KOH solution (Sigma-Aldrich) and sample was left at room temperature for 20 min. Using a Pasteur pipette, 1 ml of the upper (isooctane) layer was transferred into a GC vial for analysis. Gas chromatography analysis of methyl esters was carried out on a HP 6890 gas chromatograph (Agilent Technologies, Inc., USA) with a 60 m DB-23 capillary column (J&W Scientific, USA). One µl of the sample was injected in the split mode 25 : 1, the injector temperature was 250°C, and detector temperature 300°C. The oven temperature started at 150°C and was programmed to 230°C at a rate of 5°C per min, and second ramp to 245°C at a rate 15°C per min, then kept constant for 8 min. Fatty acids were identified based on their retention times by comparison to FAME Mix 37 standards (Sigma-Aldrich).

Preparation of coconut oil for microdilution tests

The coconut oil was weighed and diluted in dimethylsulfoxide (DMSO), (Lach-Ner, Czech Republic) followed by a detergent (Tween 80, Sigma-Aldrich) in order to form an emulsion. This emulsion was diluted in Wilkins-Chalgren medium with lipase, resulting in a final concentration of 1 mg per ml of coconut oil. The final concentration of DMSO and Tween 80 did not exceed 1%.

In order to examine whether an oil possesses antimicrobial properties, the fatty acid moieties that are esterified to the glycerol backbone of triacylglycerol must first be hydrolyzed into their free forms (free fatty acids). This hydrolysis was catalyzed by lipase from porcine pancreas (Sigma-Aldrich). The minimum necessary amount of lipase was calculated based on the molecular weight of the predominant fatty acid (i.e. lauric acid), as well as on the declared activity of this type of lipase. The enzyme was then added in surplus. The resulting emulsion of medium, lipase, and oil was warmed to 37°C and shaken for 1 h.

Table 1: Minimum inhibitory concentrations (g/L)

	carvacrol	coconut oil	carvacrol + coconut oil			ATB (mg/L)
			1:1	1:2	2:1	
Escherichia coli	0.50	> 1.00	0.13	0.50	0.25	1.5
Salmonella Enteritidis	0.25	> 1.00	0.03	1.00	0.13	3
Staphylococcus aureus	0.25	0.50	0.03	1	0.25	0.375
Listeria monocytogenes	0.50	1.00	0.25	0.25	0.25	1.5
Enterococcus cecorum	0.50	> 1.00	0.50	> 1.00	0.50	> 6

Antimicrobial assays

Stock cultures of bacterial strains had been grown in Wilkins-Chalgren broth at 37°C for 24 h before the tests. An inoculum was then created by dilution in the same medium to a final cell concentration of 10^6 colony forming units (CFU) ml^{-1}, which was confirmed by density measurement in McFarland units (densitometer McFarland type DEN-1B; Biosan, Latvia). A modification of the EUCAST microdilution method (E U C A S T, 2003) was used for antimicrobial testing. A two-fold serial dilution ranging from 1.00 to 0.03125 mg ml^{-1} was prepared from a stock solution in Wilkins-Chalgren broth with 1% of Tween 80 in 96-well microtitration plates. Carvacrol and coconut oil were used separately, as well as a combination in the following ratios: 1 : 1, 2 : 1, and 1 : 2. As a control, the antibiotic tetracycline was used in concentrations ranging from 6 to 0.1875 mg l^{-1}. Then 10 µl of the inoculum were added to 190 µl Wilkins-Chalgren broth containing 1% of Tween 80. The microtitration plates were incubated at 37°C for 24 h. After incubation, the minimum inhibitory concentrations (MICs) were recorded. MICs were expressed as the lowest concentration at which a compound inhibited bacterial growth by ≥ 80% compared to an agent-free control. Bacterial growth was checked visually before carrying out a turbidity measurement by absorbance at 405 nm on a microplate reader Infinite M200 (Tecan Group Ltd., Switzerland). Each plate contained two negative and two positive controls. For each compound or combination of compounds, the microdilution assay was carried out in triplicate, and the resulting median MICs were recorded.

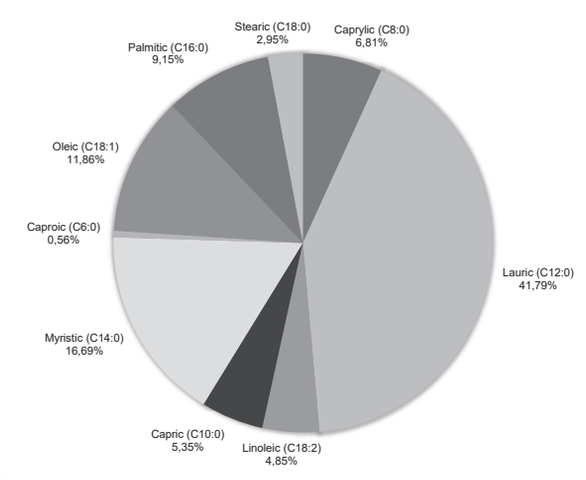

Fig. 1. The fatty acid profile of coconut oil

RESULTS

Coconut oil composition

The results of the gas chromatography analysis are shown in Fig. 1. The coconut oil contained a mixture of saturated MCFAs (C_6–C_{14}), with a predominant presence of lauric acid (41%), followed by myristic (16.5%), caprylic (6.7%), and capric acid (5.3%). It also contained long-chain fatty acid, in particular oleic (11.7%) and palmitic acid (9.1%).

Determination of minimum inhibitory concentrations

We performed a microdilution assay to determine the MICs of carvacrol, coconut oil, and their combinations against *Escherichia coli, Salmonella* Enteritidis, *Staphylococcus aureus, Listeria monocytogenes,* and *Enterococcus cecorum.* Our results are shown in Table 1.

DISCUSSION

Compared to previous studies (K o s m a t k a, 2003; D u b o i s et al., 2007; M a r i n a et al., 2009), our analysis showed a slightly lower percentage of MCFAs in coconut oil. This could be due to variations in geographical and ecological conditions (G r a h a m et al., 1981). The antimicrobial activity of essential oils, as well as the effectiveness of their active compounds, has been extensively investigated (B u r t, 2004; S i et al., 2006; T r i p a t h i et al., 2011). Nowadays, their bioactivity is generally attributed to phenolic compounds, which are soluble in the lipid bilayer of the membrane

and alter membrane fluidity (Ultee et al., 2002). An antibacterial effect of carvacrol against *Salmonella* Typhimurium, *E. coli* O157:H7, and *L. monocytogenes* was observed by Kim et al. (1995) and Cosentino et al. (1999). For instance, Cosentino et al. (1999) estimated the MIC of carvacrol against *Salmonella* Typhimurium, *E. coli,* and *Enterococcus faecalis* at 0.23 mg ml^{-1}, and that against *L. monocytogenes* and *S. aureus* at 0.45 mg ml^{-1}. MIC values for carvacrol obtained in this study were in agreement with these and other previous studies (Si et al., 2006; Bajpai et al., 2012; Hyldgaard et al., 2012; Seow et al., 2014) The antibacterial activity of free MCFAs is also well-established. The MIC values obtained for coconut oil in this trial were in agreement with previous studies (Graham et al., 1981; Bergsson et al., 2001; Oyi et al., 2010).

In general, the antibacterial activity of a fatty acid depends on its chain length, and its effectiveness varies depending on the bacterial strain it is used against (Kabara et al., 1972; Sprong et al., 2001). Nieman (1954) concluded that (a) fatty acids with a chain length of around C_{12} are the most active, (b) inhibitory properties of unsaturated fatty acids are more pronounced than those of saturated fatty acids, and (c) Gram-positive bacteria are more susceptible to the action of fatty acids than Gram-negative bacteria.

Mbandi et al. (2004) performed a screening of antilisterial effects of fatty acids *in vitro*. Lauric acid (0.04 mg ml^{-1}) exhibited the highest activity against *L. monocytogenes* (at an inoculum concentration of 10^3 CFU ml^{-1}), followed by monolaurin (0.08 mg ml^{-1}), and capric acid (0.15 mg ml^{-1}). Ruzin, Novick (2000) found that lauric acid might be at least partially responsible for the inhibitory effect of glycerol monolaurate against *Staphylococcus aureus.*

In Gram-positive bacteria, Skrivanova et al. (2004) found caprylic acid to be the only acid inhibiting glucose utilization by *Salmonella* Enteritidis, Infantis, and Typhimurium. The concentration of caprylic acid, at which 50% of the initial glucose in cultures was utilized (IC_{50}), ranged from 0.75 to 1.17 mg ml^{-1}. Similarly, the MIC of caprylic acid against three strains of *Salmonella* sp. in Skrivanova et al. (2004) was 3 mg ml^{-1}. *Salmonella* strains were resistant (MIC > 5 mg ml^{-1}) to other MCFAs. In another work, the count of *Salmonella* Enteritidis was decreased from 7.30 $\log_{10}$ to 4.68 ± 0.05 $\log_{10}$ CFU ml^{-1} by using capric acid and to 6.41 ± 0.03 $\log_{10}$ CFU ml^{-1} by using 0.5 mM (0.1 mg ml^{-1}) lauric acid *in vitro*. Sprong et al. (2001) considered the bactericidal activity of lipids as biologically significant when a reduction to > 0.5 $\log_{10}$ CFU ml^{-1} was observed.

Marounek et al. (2003) determined the antimicrobial activity of C_2–C_{18} fatty acids *in vitro* in cultures of two strains of *E. coli* grown on glucose. Glucose utilization was inhibited by caprylic acid (IC_{50} 0.30–0.45 g l^{-1}) and capric acid (IC_{50} 1.25–2.03 mg ml^{-1}). No other fatty acids were found to have an antimicrobial effect. In another study, Skrivanova et al. (2006) determined the effective concentration of caprylic acid at 2 mg ml^{-1}. These two strains of *E. coli* were also susceptible to capric acid at 5 mg ml^{-1}.

Many studies have reported the effects of essential oils and/or their components combined with chemical or physical preservation methods (Valero, Frances, 2006) such as nisine (Periago, Moezelaar, 2001; Rajkovic et al., 2005), organic acids (Chai et al., 2016), low pressure (Frankova et al., 2014), conventional antibiotics (Langeveld et al., 2014; Magi et al., 2015), or thermal treatment (Knight, McKellar, 2007). The treatment with the combination of 0.5% w/v lactic acid and 0.02% w/v carvacrol completely inactivated *Shigella sonnei* at 6.52 log CFU ml^{-1} *in vitro* within 10 min (Chai et al., 2016). Kim, Rhee (2013) demonstrated synergistic effects of three MCFAs combined with four weak organic acids (acetic, lactic, malic, and citric acid). In some recent studies, the combined effects of MCFAs and essential oils have not been studied in great detail. Hulankova, Borilova (2011) reported inhibitory effects of caprilic acid combined with oregano oil obtained from *Origanum vulgare* L. using the determination of minimum inhibitory concentration. Kim, Rhee (2016) tested antibacterial effects of medium chain fatty acids (caprylic, capric, and lauric acid) combined with essential oil components (carvacrol, eugenol, β-resorcylic acid, *trans*-cinnamaldehyde, thymol, and vanillin) against *Escherichia coli* O157:H7. The improved synergistic bactericidal effect was achieved by the combination of MCFAs and essential oils, where bacterial cells were completely inactivated when treated with certain combinations. Combined treatment achieved an additional > 7.05 log reduction CFU ml^{-1} compared with the sum of the individual effects.

The use of essential oils in real foods is often limited, since the concentrations required for effective antimicrobial capacity may exceed organoleptically acceptable levels and negatively affect the properties of the foods (Burt, 2004; Bakkali et al., 2008). This problem may be overcome by combined treatments showing synergistic bactericidal effects. The effective concentration of antimicrobials can be reduced in combined treatment compared to the concentrations that would be required for individual treatment.

CONCLUSION

In the present study, we evaluated and compared the antimicrobial activities of carvacrol, coconut oil, and various combinations of the two against a range of pathogenic bacteria. A 1 : 1 combination of the two compounds was more effective than each compound

alone, thereby showing a significant synergistic effect in their antibacterial activity. Further studies are required to better understand the mechanism of the synergistic combined effects of MCFAs and essential oils. In conclusion, carvacrol in combination with coconut oil can act effectively against pathogens at low concentrations. The combined treatment can overcome the disadvantages of MCFAs and essential oils such as unpleasant odour and high cost because the required concentrations can be reduced. Our results indicate that the combined treatments applied here could be successfully used to eliminate foodborne pathogens, significantly improving the microbiological safety of foods.

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