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Determination of *In Vitro* Antibacterial Activity of Plant Oils Containing Medium-Chain Fatty Acids against Gram-Positive Pathogenic and Gut Commensal Bacteria

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ABSTRACT

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Increasing antibiotic resistance has led to a ban on antibiotic use in feed additives in the EU. Therefore, new non-antibiotic, pathogen-inhibiting agents are urgently needed. Inhibitory effects of eight plant oils containing medium-chain fatty acids (MCFAs) were evaluated against Gram-positive pathogenic and beneficial bacteria. The oils tested were palm, red palm, palm kernel (*Elaeis guineensis*), coconut (*Cocos nucifera*), babassu (*Attalea speciosa*), murumuru (*Astrocaryum murumuru*), tucuma (*Astrocaryum vulgare*), and *Cuphea* oil (*Cuphea ignea*); the method used was broth microdilution, and the findings were expressed as minimum inhibitory concentration (80%). Both hydrolyzed and unhydrolyzed forms of the oils were tested. MCFA hydrolysis was catalyzed by porcine pancreas lipase. The selective effect of the hydrolyzed forms of tested oils was highly evident. While the hydrolyzed oils were active against all tested bacteria (*Clostridium perfringens, Enterococcus cecorum, Listeria monocytogenes*, and *Staphylococcus aureus*), at 0.14–4.5 mg/ml, the same oils did not show any effect on commensal bacteria (*Bifidobacterium* spp. and *Lactobacillus* spp.). Tucuma and *Cuphea* seed oils showed the strongest antibacterial activity. Unhydrolyzed forms of all tested oils exerted no antibacterial effect against any test bacteria. This study, thus, forms a basis for the development of selective inhibitors in animal husbandry.

Keywords: antimicrobial effect; gastrointestinal tract; pathogens; palm oil; lauric acid; capric acid

Industrialized countries have been reporting an ever-increasing burden of zoonoses and foodborne diseases. Although fresh products have represented a very important source of foodborne infections in recent years, infections caused by consumption and mishandling of food of animal origin are still a major concern (Havelaar et al. 2010).

Gram-positive bacteria represent the causative agents of both animal intestinal diseases and potentially lethal foodborne diseases in humans. For instance, *Clostridium perfringens* can cause food poisoning and necrotic enteritis, sometimes with fatal outcomes (Songer 2010). *Enterococcus cecorum* has recently been identified as a significant problem

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in broilers, and is increasingly being reported as an important cause of arthritis and osteomyelitis in chickens (Boerlin et al. 2012). Similarly, *Listeria monocytogenes* and *Staphylococcus aureus* are considered to be the widespread pathogens causing serious illnesses and systematic disorder both in animals and humans (McLauchlin and Rees 2009).

Food-producing animals are an important reservoir of some of these serious pathogens. Therefore, there is increasing concern about controlling the spread of bacterial pathogens in animal husbandry. "Pathogen reduction" strategies in the food chain can also result in significant economic gain. The incidence of foodborne pathogens is usually controlled with antibiotics. However, because of the public health concern, the use of antibiotics in both human and veterinary medicine has been viewed with caution. Consequently, there is widespread interest in the research and development of alternative antibacterial compounds. A valuable property of these antibacterial compounds is selective activity without causing harm to the commensal microbiota (Huyghebaert et al. 2011).

Phytogenic additives, including organic acid, present a plausible alternative as they enhance a number of important processes in the animal body as well as they can be used also in the food industry because of their antibacterial properties (Karaskova et al. 2015). Organic acids can also be used as feed or drinking water additives (Windisch et al. 2008). Medium-chain fatty acids (MCFAs) form a promising group of antibacterial agents. These saturated and unbranched monocarboxylic acids are present in various feed materials, especially coconut, palm, and *Cuphea* seed oils (Dierick et al. 2003). The MCFA group consists of caproic acid (C6:0, hexanoic acid), caprylic acid (C8:0, octanoic acid), capric acid (C10:0, decanoic acid), and lauric acid (C12:0, dodecanoic acid) (Bach and Babayan 1982). MCFAs have long been used in feed preservation, especially in silage and foods, owing to their antibacterial effects, especially against Gram-positive bacteria (Kabara 1983).

Although the effects of plant oils in animal nutrition have been already observed, for example on ruminal fermentation and protozoal populations (Majewska et al. 2017) or milk performance in dairy cows (Dai et al. 2011), only few studies have discussed the antibacterial activity of plant oils against pathogenic and commensal bacteria present in gastrointestinal tract (Lee et al. 2015).

The aim of this study was to evaluate the *in vitro* antibacterial activity of plant oils containing MCFAs against pathogenic and gut commensal Gram-positive bacteria, including five foodborne and six beneficial intestinal strains.

MATERIAL AND METHODS

Bacterial strains and culture conditions. Thirteen bacterial strains were used to assess the antibacterial properties of plant oils, including seven pathogenic and six beneficial intestinal strains. All strains were grown and maintained in appropriate broth (Oxoid, UK). These strains were incubated at 37°C for 24/48 h under aerobic or anaerobic conditions, as appropriate (Table 1).

The sources of bacterial strains (listed in Table 2) were as follows: CCM, Czech Collection of Microorganisms (Brno, Czech Republic); ATCC, American Type Culture Collection (Manassas, USA); CNCTC, Czech National Collection of Type Cultures (National Institute of Public Health, Prague, Czech Republic); and CIP, Collections of Pasteur Institute (Paris, France). *C. perfringens* No. 56 was kindly provided by prof. F. Van Immerseel from the Ghent University (Belgium). *Bifidobacterium animalis* MA5 is an isolate from the culture collection of the Czech University of Life Sciences Prague.

Plant oils. Plant oils known to contain high percentage of MCFAs (Bach and Babayan 1982),

Table 1. Growth media and conditions

Bacteria	Medium	Conditions	Incubation
Enterococcus cecorum	Wilkins-Chalgren	aerobic	24 h
Clostridium perfringens	Wilkins-Chalgren	anaerobic	48 h
Listeria monocytogenes	Brain-Heart Infusion	aerobic	24 h
Staphylococcus aureus	Wilkins-Chalgren	aerobic	24 h
Bifidobacterium spp.	Wilkins-Chalgren + BifiBuffer	anaerobic	48 h
Lactobacillus spp.	de Man, Rogosa and Sharpe (MRS)	microaerophilic	48 h

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namely coconut (*Cocos nucifera*) and palm kernel (*Elaeis guineensis*) oils, were purchased from Sigma-Aldrich (USA). Palm, red palm (*Elaeis guineensis*), babassu (*Attale aspeciosa),* tucuma (*Astrocaryum vulgare*) oils, and murumuru butter (*Astrocarum murumuru*) were purchased from Sweet Natural Botanicals (USA). *Cuphea* seeds were purchased from the US Department of Agriculture – Agricultural Research Service (ARS; Plant Germplasm Inspection Station, USA), and dried and extracted using 80 g/kg methanol for 24 h. This extract was filtered and dried at 40°C by using a vacuum dryer Rotavapor R-200 (Buchi, Switzerland).

Determination of fatty acid composition of oils. The fatty acid (FA) composition of the oils was determined by gas chromatography/flame ionization detection (GC-FID) at the Institute of Animal Science Prague-Uhříněves, Czech Republic. Alkaline trans-methylation of the extracted FAs was performed according to the standard ISO 5509 (1994) procedure. For GC analysis of methyl esters, a HP 6890 gas chromatograph (Agilent Technologies, Inc., USA) with a programmed 60-m DB-23 capillary column (J&W Scientific, USA) was used. FAs were identified based on retention times by comparing with the retention times of FAME Mix 37 standards (Sigma-Aldrich).

Preparation of plant oils for microdilution tests. The oils were weighed and diluted in the same amount of dimethylsulfoxide (DMSO) (Lach-Ner, Czech Republic), followed by the addition of a detergent Tween 80 (Sigma-Aldrich) to form an emulsion.

After preliminary antibacterial tests with unhydrolyzed oils (data not shown), we concluded that in order to examine the antimicrobial properties of the oils, FA moieties esterified to the glycerol backbone of triacylglycerol must first be hydrolyzed into their free forms (free FAs). This hydrolysis was catalyzed by lipase from porcine pancreas (Sigma-Aldrich). The minimum amount of lipase required was calculated based on the molecular weight of the predominant FA (i.e. lauric or capric acid), as well as the enzyme activity specified by the manufacturer.

The emulsion obtained as previously described was diluted in appropriate medium (depending on microorganism) containing lipase, resulting in a final oil concentration of 4.5 mg/ml. The final concentrations of DMSO and Tween 80 did not exceed 1% and 0.1%, respectively.

The resulting emulsion of the medium, lipase, and oil was warmed to 37°C and shaken for 1 h.

Determination of in vitro antibacterial activity of plant oils. The antibacterial activity of the tested oils was evaluated *in vitro* by the broth microdilution method using 96-well microtiter plates, modified according to the proposed recommendations for effective assessment of the anti-infective potential of natural products (Cos et al. 2006). Samples were two-fold diluted in broth with porcine lipase, as stated in previous section, starting with an initial concentration of 4.5 mg/ml. The dilutions of each compound were prepared in appropriate medium (Table 1) recommended for the cultivation of certain bacteria (Oxoid). The bacterial inoculum was standardized to a density of 1×10^5 CFU/ml by using the McFarland scale, and inoculated into the wells (10 µl/well). The plates containing anaerobic bacteria were prepared in an anaerobic chamber (Bugbox; BioTrace, UK). The plates were incubated at 37°C for 24/48 h under aerobic, anaerobic, or microaerophilic conditions (Table 1). Microbial growth was assessed by culture turbidity, determined by the Infinite® 200 PRO Microplate Reader (Tecan, Switzerland) at 405 nm. The minimum inhibitory concentration for 80% (MIC_{80}) was expressed as the lowest concentration of the compound that resulted in 80% reduction in growth compared to that in the extract-free, blank medium, without microorganisms. The susceptibility of all microorganisms to penicillin G was evaluated as a control. The positive control tubes contained 10 µl of the bacterial suspension and 90 µl of broth, while the negative control tubes contained 100 µl of broth. Both controls contained 1% DMSO.

All samples were tested in three independent experiments, each carried out in triplicate.

Table 2. Bacterial strains used in this study

Species	Strain
Enterococcus cecorum	CCM 3659, CCM 4285
Clostridium perfringens	CIP 105178, CNCTC 5454, UGent 56
Listeria monocytogenes	ATCC 7644
Staphylococcus aureus	ATCC 25923
Bifidobacterium animalis	CCM 4988, MA5
Bifidobacterium longum	TP 1, CCM 4990
Lactobacillus fermentum	CCM 91
Lactobacillus acidophilus	CCM 4833

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RESULTS

Fatty acid composition of plant oils. Results of FA profile analysis are shown in Table 3. The composition of both palm oil and red palm oil was very similar, with the predominance of palmitic (C16:0; 41%) and oleic (C18:1; 40%) acids, while only trace amounts of MCFAs were noted. Lauric acid was detected as the dominant FA in tucuma oil (53%), murumuru butter (46%), palm kernel (45%), babassu (44%), and coconut oil (42%). In *Cuphea* oil, capric acid (C10:0) was found dominant (54%).

In vitro antibacterial activity of plant oils. Minimum inhibitory concentrations (MIC_{80}; 80% reduction of bacterial growth) of selected plant oils are shown in Table 4. As it is evident from our results, plant oils were ineffective toward the Gram-positive commensals (*Bifidobacterium* and *Lactobacillus* spp.). Also some of oil samples with high lauric acid content displayed antibacterial activity in our study. However, low or no antibacterial activity was observed in the case of coconut, red palm, and palm oils. Tucuma oil was effective against both strains of *E. cecorum* (2.25 mg/ml), two strains of *C. perfringens* (0.14–2.5 mg/ml), and *S. aureus* (0.56 mg/ml). Murumuru butter inhibited both all strains of *E. cecorum* (1.13–2.25 mg/ml) and *C. perfringens* (0.28–1.13 mg/ml), as well as *S. aureus* (1.13 mg/ml). Palm kernel oil had the inhibitory effect against *E. cecorum* (2.25 mg/ml), two strains of *C. perfringens* (1.13–2.25 mg/ml), and *S. aureus* (1.13 mg/ml). Babassu oil inhibited *E. cecorum* (2.25–4.5 mg/ml), *C. perfringens* (0.56 mg/ml), and *S. aureus* (1.13 mg/ml). *L. monocytogenes* was inhibited only by *Cuphea* oil (13 mg/ml). No oil samples exhibited considerable effect against pathogenic bacteria before hydrolysis (> 4.5 mg/ml; data not shown).

DISCUSSION

The plant oils are a heterogeneous mixture of FAs and it is necessary to know their prevalences in observed samples. Therefore, the analysis of the fatty acids composition in samples was performed prior to the antibacterial testing of plant oils. Contrary to other oil samples, in *Cuphea* seed oil (*Cuphea ignea*), capric acid was determined as a dominant fatty acid (Table 3). This percentage is lower compared to results of Zentek et al. (2011) (i.e. ± 85%). The fatty acid composition depends on the stability of the patterns under varying geographical and ecological conditions, as well as on the selected method and conditions of extraction.

Lauric acid was detected as the dominant FA in samples of tucuma, murumuru, palm kernel, babassu, and coconut oil (Table 3), which is generally in agreement with previous findings (Dubois et al. 2007). This FA is considered the most effective

Table 3. Fatty acid profile of selected oils (%)[1]

Fatty acid	Oil							
	palm	red palm	palm kernel	coconut	babassu	murumuru	tucuma	*Cuphea*
Caproic	nd	nd	0.25	0.55	0.43	0.10	0.21	0.01
Caprylic	nd	nd	3.48	6.73	6.05	1.29	2.47	0.82
Capric	nd	nd	3.27	5.29	5.52	1.30	2.15	54.04
Lauric	0.20	0.23	45.24	41.31	43.98	46.34	53.37	3.63
Myristic	1.10	1.05	15.85	16.5	15.56	28.72	24.82	11.31
Palmitic	42.93	41.96	9.46	9.05	8.72	7.30	5.41	8.74
Palmitoleic	0.20	0.18	0.03	nd	nd	0.04	0.02	0.06
Stearic	4.43	4.84	2.66	2.92	3.73	2.93	1.89	1.40
Oleic	40.28	40.6	16.54	11.72	13.62	7.98	6.47	12.47
Linoleic	10.29	10.43	2.68	4.79	2.39	3.55	2.77	5.98
α-Linolenic	0.19	0.31	0.03	0.88	nd	0.05	0.06	0.53
Arachidonic	0.40	0.41	0.13	0.13	nd	0.12	0.07	0.12
Eicosenoic	nd	nd	0.10	0.15	nd	0.05	0.05	0.25

nd = not detected

[1]average of two analyses, each performed in triplicate

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Table 4. Minimum inhibitory concentrations of selected plant oils (mg/ml)[1]

Species	Strain	Oil								
		palm	coconut	babassu	red palm	*Cuphea* seed	palm kernel	murumuru	tucuma	penicillin G (mg/ml)
E. cecorum	CCM 3659	> 4.5	2.25	4.5	> 4.5	2.25	2.25	2.25	2.25	0.001
	CCM 4285	> 4.5	1.13	2.25	> 4.5	1.13	2.25	1.13	2.25	0.001
	CNCTC 5454	> 4.5	> 4.5	0.56	> 4.5	4.5	1.13	1.13	2.50	0.00003
C. perfringens	UGent 56	> 4.5	> 4.5	0.56	> 4.5	2.25	> 4.5	0.56	1.13	0.00003
	CIP 105178	> 4.5	> 4.5	0.56	> 4.5	0.56	2.25	0.28	0.14	0.00003
L. monocytogenes	ATCC 7644	> 4.5	> 4.5	> 4.5	> 4.5	1.13	> 4.5	> 4.5	> 4.5	0.001
S. aureus	ATCC 25923	> 4.5	0.56	1.13	> 4.5	2.25	1.13	1.13	0.56	0.00003
B. animalis	CCM 4988	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	0.00025
	MA5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	0.00025
B. longum	CCM 4990	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	0.0005
	TP1	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	0.001
L. fermentum	CCM 91	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	0.000125
L. acidophilus	CCM 4833	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5	0.000125

[1]modus of three analyses, each performed in triplicate

MCFA against pathogenic bacteria (Galbraith et al. 1971).

Reduction of foodborne pathogens can be both costly and challenging, particularly in the light of the current microbial resistance status in both animal and human medicine. Antibiotics also frequently affect not only a certain pathogenic agent, but also the beneficial microbiota of the host (WHO 2017). In our study, none of the oils at tested concentrations showed any inhibition towards commensal bacteria (*Bifidobacterium* and *Lactobacillus* spp.), while all tested oils, excluding palm and red palm oil, possessed some degree of inhibitory activity against pathogens (Table 4). The resistance of *Bifidobacterium* to MCFAs can be caused by the lack of ferredoxin system responsible for the reduction of the nitro group in metronidazole, resulting in higher resistance to metronidazole, an antibiotic effective against most obligatory anaerobes (Pelissier et al. 2010). The negative antibacterial effect against *Lactobacillus acidophilus* and *L. fermentum* observed in our study corresponds to the findings of a previous study by Kodicek and Worden (1945), wherein they observed only low inhibition of *L. helveticus* inoculated in the medium containing riboflavin and salt. This finding represents the beneficial effects of MCFAs. In general, the antimicrobial susceptibility testing of anaerobic and slowly growing bacteria is complicated, and warrants further studies.

All of the effective oils were active only after hydrolysis. These findings are in agreement with the generally accepted mechanism of their antibacterial action, since the free FAs are believed to be the effective compounds (Lee et al. 2015). Fatty acids can pass across the bacterial cell membrane only in free form and subsequently, they can cause the intracellular acidification and bacterial growth inhibition (Sun et al. 1998). FAs can also cause the conformational changes in the plasma membrane structure, thus affect the membrane permeability and disrupt the electron transport chain. The balancing of the membrane potential using ion pumps is energy-exhausting for the cell. Other processes contributing to bacterial growth inhibition may be the influence of enzyme activity, impairment of nutrient uptake, and generation of toxic peroxidation and autooxidation products (Desbois and Smith 2010). Based on the above mentioned facts it is evident that the hydrolysis (the releasing of bounds on glycerol, respectively)

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represents the key process of antibacterial properties of the oils.

In the current trial, the broadest range of antibacterial activity was observed in hydrolyzed *Cuphea* oil, where all tested bacteria, excluding beneficial microbiota, were inhibited (0.56–4.5 mg/ml) (Table 4). *Cuphea* oil, containing high amounts of capric acid (Table 3), was effective against all Gram-positive pathogenic strains examined (*E. cecorum*, *C. perfringens*, *L. monocytogenes*, and *S. aureus*). The beneficial effect of whole *Cuphea* seeds, combined with exogenic lipase, was already observed by Dierick et al. (2003) in the experiments with piglets, where not only an antibacterial activity was observed, but also the enlargement of the villi in the small intestine was documented. This positive attribute was assigned to the high capric acid content of *Cuphea* seed oil.

In addtition, *Cuphea* seed oil was the only one which inhibited *L. monocytogenes* in this trial. In some previous studies, *L. monocytogenes* was inhibited by capric acid at 0.15 mg/ml (Mbandi et al. 2004). Lauric acid also possessed anti-listerial activity in these studies. In Parfene et al. (2013), coconut oil exerted an inhibitory effect on *L. monocytogenes* at 0.312 mg/ml. However, we observed no inhibition of this pathogen by coconut oil. This difference could be due to different exposure times or higher percentage of lauric acid in the coconut oil used in the previous study (70% vs 41% in the current study).

Several previous studies were focused on the inhibition of *C. perfringens* using MCFAs. Skrivanova et al. (2005) estimated the effective concentration of lauric acid for the inhibition of this pathogen at 0.04 mg/ml, in another study (Skrivanova et al. 2014) a 30-minute incubation with lauric acid (2 mg/ml) revealed disintegration of the cell wall in some cells of *C. perfringens*. However, cytoplasmic membrane appeared to remain intact. Galbraith et al. (1971) also reported that the most effective MCFA inhibiting *C. perfringens* was lauric acid (0.05 mg/ml).

S. aureus, a pathogen listed in Global priority list of antibiotic-resistant bacteria (WHO 2017), was susceptible to all oils with the predominance of lauric acid (Table 4). Coconut and tucuma oil were the most effective (0.56 mg/ml). Ruzin and Novick (2000) also reported that lauric acid might be, at least, partially responsible for the inhibitory effect of glycerol monolaurate against *S. aureus*.

Very little is known about the antibacterial effect of FAs on *E. cecorum*. However, Orhan et al. (2011) observed the significant inhibitory effect of edible oils rich in long-chain fatty acids against *E. faecalis*. A lag period in the growth of *E. faecalis* in the presence of monolaurin, a monoacylglycerol of lauric acid, was also observed (Bunkova et al. 2011). Based on these facts and our results, it can be assumed that lauric acid and other MCFAs represent the significant inhibitors of *E. cecorum*.

In conclusion, murumuru butter, tucuma, palm kernel, and babassu oils seem to be the compounds with most promising antibacterial effect (after hydrolysis), probably because they represent the compounds with lauric acid being the predominant FA. *Cuphea* seed oil with high capric acid content, on the other hand, displayed the broadest spectrum of antibacterial activity in our study. Low or no antibacterial activity was observed in the case of coconut, red palm, and palm oil. As mentioned above, no oil sample inhibited the commensal bacteria (*Bifidobacterium* spp. and *Lactobacillus* spp.). Since the effect would be highly undesirable, the inactivity against these bacterial strains poses the considerable benefit.

CONCLUSION

In this study, a pronounced antibacterial effect of MCFA-containing plant oils was observed in the case of Gram-positive bacteria, without any inhibitory effect on the beneficial commensal bacteria. Since only a limited number of strains were used in our study, the antibacterial effect of these oils should be verified using additional bacterial strains in subsequent experiments. MCFAs are commonly available and can be developed as promising suitable alternatives to the banned growth-promoting antibiotics for use in animal husbandry; however, *in vivo* experiments are necessary prior to their practical implementation.

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