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In vitro antimicrobial effect of palm oils rich in medium-chain fatty acids against mastitis-causing Gram-positive bacteria

*Klára Laloučková[1,2], Lucie Malá[1,2], Paula Slaničková[2], Eva Skřivanová[1,2]**

[1]Department of Nutritional Physiology and Animal Product Quality, Institute of Animal Science, Prague-Uhříněves, Czech Republic

[2]Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic

**Corresponding author: skrivanova.eva@vuzv.cz*

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Abstract: Various pathogens causing mastitis in dairy cattle are of serious concern due to their increasing antibacterial resistance and potential transmission to other cows, calves, and the environment, especially through the milking process. Therefore, alternative approaches to antimicrobial usage in the treatment or control of mastitis in dairy cattle are severely needed. The antibacterial effect of medium-chain fatty acids (MCFAs) is known to be significant for various pathogens, but there is only limited information about the activity of MCFAs on mastitis-causing pathogens. Moreover, no evidence about the antimicrobial effects of palm oils rich in MCFAs, such as coconut, palm kernel, and tucuma oil, can be found in the current literature. The aim of this study was to evaluate the *in vitro* antibacterial effect of palm oils rich in MCFAs, after cleavage by an exogenous lipase from *Mucor javanicus*, on bovine mastitis-causing strains (*Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*) by the broth microdilution method. All tested palm oils exerted antibacterial activity against eight tested bacterial strains in the range of 64–8192 µl/ml with *Str. agalactiae* being the most sensitive and *S. aureus* being the most resistant species. The results of the present study demonstrate that palm oils rich in MCFAs can serve as an alternative to the predominantly used predip and postdip procedures in bovine mastitis control, but further *in vivo* studies are needed to confirm the findings for their possible applications.

Keywords: antibacterial; bovine; *Staphylococcus; Streptococcus*; vegetable oil

Bovine mastitis affects the dairy industry worldwide. Defined as "inflammation of the mammary gland", mastitis is currently the most common reason for the use of antibacterials in lactating dairy cattle (Erskine et al. 2004). Van Boeckel et al. (2015) estimated that the global annual consumption of antimicrobials per kilogram of animal produced is 45 mg/kg in cattle. The economic impact of bovine mastitis is tremendous – the annual losses have been estimated to be approximately 2 billion dollars in the USA (Hossain et al. 2017). The loss comprises production losses connected with reduced milk production, treatment losses linked to necessary remedies, and loss of animal value due to the fibrotic changes in the udders (Jingar et al. 2017). The frequency of mastitis is usually higher in later lactations compared to that in the first lactation (Kasna et al. 2018).

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The classical approach to cure mastitis in cattle is the use of antibiotics, but this treatment is accompanied by various disadvantages, including a low cure rate, increasing occurrence of resistance, and the presence of antibiotic residues in milk (Gomes and Henriques 2016). Intramammary antibiotic therapy is a usual practice for mastitis treatment or control in dairy cattle herds. Commonly used drugs include betalactams, macrolides and lincosamides (Barkema et al. 2006). Cure rates scarcely reach 50% in mastitis-causing *S. aureus* strains with currently available therapies (Ster et al. 2013). The antibiotic therapy of bovine mastitis has been related to emerging antimicrobial resistance in various bacterial strains, including isolates from the infected cows, other animals from the same herd, and even from food products of infected/cured animal origin (Silva et al. 2018). The modern concepts of mastitis treatment and prevention include nonsteroidal anti-inflammatory drugs (Breen 2017) and intramammary teat seals (Kromker et al. 2014). Nevertheless, the occurrence of antibiotic resistance in dairy cattle mastitis isolates is still of serious concern and is a reason for seeking an alternative treatment in current research.

Organic acids are promising alternatives to antibiotics due to their antimicrobial activity in controlling bacterial contamination and their ability to boost animal production (Polycarpo et al. 2017). Unbranched saturated fatty acids with medium-chain lengths of carbons, namely caproic ($C_{6:0}$), caprylic ($C_{8:0}$), capric ($C_{10:0}$) and lauric ($C_{12:0}$) acids, are a group of antibacterials occurring naturally (e.g., in cow milk) (Legrand 2008). These fatty acids exhibit antimicrobial properties against a wide variety of pathogens, including Gram-positive bacteria (Hovorkova et al. 2018). Their bactericidal or bacteriostatic effect is believed to occur through their ability to incorporate into bacterial membranes, increasing the fluidity of the membrane and resulting in the leakage of internal content from the cells (Chamberlain et al. 1991). Medium-chain fatty acids (MCFAs) frequently occur in oils of tropical palms native to tropical and subtropical areas (Van der Vossen et al. 2001). It has been proven by previous research that the antibacterial effect of palm oils rich in MCFAs, such as babassu (*Attalea speciosa*), coconut (*Cocos nucifera*), murumuru (*Astrocaryum murumuru*), palm kernel (*Elaeis guineensis*), and tucuma (*Astrocaryum vulgare*) oils, is exerted only after their cleavage, meaning after their release from triglycerides; the most prevalent of the detected MCFAs is dodecanoic (lauric) acid followed by tetradecanoic (myristic) acid, with the total MCFA content higher than 50%, and saturated fatty acids content more than 80% in all the tested oils (Hovorkova et al. 2018). The antibacterial properties of free MCFAs and their monoesters against various pathogens (Bunkova et al. 2011), including staphylococci (Batovska et al. 2009) and streptococci (Schlievert and Peterson 2012), are well known. Nair et al. (2005) evaluated the *in vitro* antibacterial activity of capric acid and monocaprylin against major bacterial mastitis pathogens in milk and found it to be effective. Unlike free fatty acids, palm oils are complex substances with enhanced sensory properties and lower prices, but there are no studies about the inhibitory activity of palm oils rich in MCFAs against bovine mastitis-causing bacteria.

The present research was carried out to evaluate the possible application of palm oils rich in MCFAs with the aim of decreasing the undesirable bacterial colonization of udders of dairy cows, especially during the milking process.

MATERIAL AND METHODS

Chemicals. Coconut (*C. nucifera*) oil and palm kernel (*E. guineensis*) oil were purchased from Sigma-Aldrich (USA), while tucuma (*A. vulgare*) oil was obtained from Natural Sweet Botanicals (USA). The oils used in this experiment were prepared according to previous research (Hovorkova et al. 2018). Briefly, the oils were dissolved in dimethyl sulfoxide (DMSO) and emulsified with Tween 80 (both Sigma-Aldrich) to ensure sufficient dispersion into an emulsion with a final concentration of 819 200 μg/ml. The final concentration of solvents in the tested samples did not exceed 1%; thus, the bacterial viability could not be influenced (Wadhwani et al. 2009). Appropriate volumes of previously obtained emulsions with 100× higher concentrations were finally diluted in tryptic soy broth (TSB) (Oxoid, UK) or TSB enriched with yeast extract (Oxoid) to reach a final concentration of 8192 μg/ml. This oil-medium emulsion was further supplemented with a lipase from *Mucor javanicus* (Sigma-Aldrich), according to its lipolytic

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activity at 2.73 mg/ml in culture media. The solution consisting of tested oil, appropriate culture medium chosen according to the tested strain, and lipase from *M. javanicus* was then shaken in a water bath heated to 37°C for 1 h to release MCFAs from triglycerides and to facilitate their antibacterial action. Penicillin G (Sigma-Aldrich) was used as a growth control for bacterial cultures. A row of wells filled with medium only and a row with bacteria in medium without palm oil emulsion were included in every microtiter plate as a negative control and positive bacterial growth control, respectively.

Bacterial cultures and their maintenance. To evaluate the antibacterial potential of the above-mentioned palm oils rich in MCFAs against bovine mastitis pathogens, 8 strains of bacteria, that were proven mastitis-causing pathogens, were chosen. The strains listed in Table 1 were purchased from the Czech Collection of Microorganisms and from the German Collection of Microorganisms and Cell Cultures. Aliquots of bacterial cultures were stored at −80°C in 20% glycerol until use in TSB or TSB with yeast extract. Stock cultures of microorganisms were cultivated in medium at 37°C for 24 h prior to testing.

Determination of the minimum inhibitory concentrations. Minimum inhibitory concentrations (MICs) were determined using the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2013) to evaluate the antibacterial activity of coconut, palm kernel, and tucuma oil against chosen Gram-positive bovine mastitis-causing strains. The *in vitro* broth microdilution method performed in 96-well microtiter plates was used to test the antimicrobial susceptibility of palm oils rich in MCFAs. The initial concentration of palm oils for susceptibility testing was 8192 μg/ml. Plates were inoculated by bacterial suspension to a final density of 5×10^5 CFU/ml, which was con-

Table 1. Bacterial strains and their specification

Bacterium	Strain	Specification	Other designation
Staphylococcus aureus subsp. *aureus* Rosenbach 1884[AL]	CCM 4442	bovine mastitis isolate (CZ); production of β-haemolysin; atypical strain: phosphatase and clumping factor negative	P. Benda M 27/92
	CCM 6188	bovine mammary gland isolate; loss of haemolysins production	B. Skalka K 126
	DSM 6732	bovine udder isolate; murein: A11.3; no toxin genes present (PCR)	ATCC 25178
Streptococcus agalactiae Lehmann and Neumann 1896[AL]	CCM 6187	bovine mammary gland isolate (CZ); test organism for CAMP-test, production strain for CAMP-factor; control strain for PYR test	J. Smola 3767
	DSM 6784	bovine udder infection isolate; Lancefield group B; beta-haemolytic; recommended as reference strain for cAMPtest	ATCC 27956
Streptococcus dysgalactiae subsp. *dysgalactiae* (ex Diernhofer 1932) Garvie et al. 1983 emend. Vieira et al. 1998	DSM 20662	cow isolate	ATCC 43078, NCDO 2023
Streptococcus uberis Diernhofer 1932[AL]	CCM 4617	subclinical bovine mastitis isolate (CZ); control strain for STREPTOtest and HIPPURATEtest	P. Benda 1268
	DSM 20569	type strain	ATCC 19436 CCUG 17930 JCM 5709 NCDO 2038 NCTC 3858

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trolled using a McFarland Densitometer Biosan DEN 1 (BioTech, Czech Republic), and incubated at 37°C for 24 h. Bacterial growth before and after incubation was evaluated spectrophotometrically by a Tecan Infinite® 200 PRO microplate reader (Tecan Group Ltd., Switzerland) at a wavelength of 405 nm. MICs were expressed as the lowest palm oil concentration that resulted in 80% growth reduction compared to the oil-free growth control. In addition, a susceptibility test of all strains to penicillin G was also performed by MIC determination, like in the case of palm oils. All combinations of oil/penicillin G per strain were tested in three individual experiments, each of which was carried out in triplicate. The final MIC was determined as the mode of all values. In accordance with turbidity values measured in the wells of the microtiter plates before and after incubation of the stock solution of three tested palm oils and eight bacterial strains, MIC values for the selected oils and bacteria were calculated. No antimicrobial activity of palm oils was observed without prior lipase cleavage (Hovorkova et al. 2018); therefore, the results mentioned below apply only to oils after cleavage by a lipase from *M. javanicus*.

RESULTS

The minimum inhibitory concentrations (MICs) displayed in Table 2 show susceptibility of all tested Gram-positive bovine mastitis-causing pathogenic strains to the chosen palm oils in the concentration range of 64 (*S. aureus*)–8192 μg/ml (*Str. agalactiae*). The most sensitive of the tested bacterial strains according to the lowest measured modal MIC value was *Str. agalactiae* CCM 6178 (64 μg/ml in the case of palm kernel oil), followed by *Str. agalactiae* DSM 6784, and *Str. uberis* DSM 20569 (128 μg/ml for tucuma and palm kernel oil, respectively). In the inhibitory activity, viewed as the average of the MIC values for each oil against all strains, palm kernel oil showed to be the most effective (average MIC 1720 μg/ml), followed by tucuma oil (average MIC 1776 μg/ml), and coconut oil (average MIC 3040 μg/ml). The most sensitive species of the tested bacteria was *Str. agalactiae* with an average MIC value of 331 μg/ml for all tested oils, followed by *Str. uberis* (1045 μg/ml), *Str. dysgalactiae* (1707 μg/ml), and *S. aureus* (4323 μg/ml). *Str. agalactiae* was also the most sensitive species (average MIC 0.00037 μg/ml), and *S. aureus* was the most resistant species (average MIC 0.00138 μg/ml) to antibiotic control as demonstrated by penicillin G. The susceptibility pattern of the species to palm oils was similar to that of penicillin G, except for the switch between susceptibility of *Str. dysgalactiae* and *Str. uberis.*

DISCUSSION

The increasing resistance to drugs in many bacteria has resulted in a ban on the use of antibiotic growth promoters in livestock farming among the EU countries (Regulation EC No. 1831/2003 of the European Parliament and the Council of 22 September 2003 on additives for use in animal nutrition). As there is a potential threat of spreading antibiotic resistance genes between bacterial

Table 2. Minimum inhibitory concentrations (MIC) of tested palm oils (μg/ml)[1]

Bacterium	Strain	Modus MIC (μg/ml)				Average MIC of oils	Average MIC of penicillin G
		coconut oil	palm kernel oil	tucuma oil	penicillin G		
S. aureus	CCM 4442	2048	2048	2048	0.001953	4324	0.00138
	CCM 6188	8192	4096	4096	0.001953		
	DSM 6732	8192	4096	4096	0.000244		
Str. agalactiae	CCM 6187	1024	64	256	0.000244	331	0.00037
	DSM 6784	256	256	128	0.00049		
Str. dysgalactiae	DSM 20662	2048	2048	1024	0.000488	1707	0.00049
Str. uberis	CCM 4617	2048	1024	2048	0.000488	1045	0.00061
	DSM 20569	512	128	512	0.000977		
Total average MIC		3040	1720	1776	0.00082		

[1]modus of triplicates of three independent experiments

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strains causing mastitis in bovines, it is necessary to look for new natural antibacterial compounds acting specifically against mastitis-causing pathogens. Plant (palm) oils containing medium-chain lipids can be found in natural products such as milk and are nontoxic to the mucosa while being able to inhibit a wide variety of human and animal pathogens (Churchward et al. 2018). Moreover, all MCFAs, their triglycerides, and coconut oil have the GRAS status meaning that they are generally recognized as save for use in human nutrition (FDA 2019).

A number of biological agents are already known to possess *in vitro* antibacterial activity against both contagious and environmental bovine mastitis-causing strains. These substances include extracts of various plants, such as *Alternanthera brasiliana*, *Achillea millefolium*, *Baccharis trimera, Cedrus deodara*, *Curcuma longa*, *Eucalyptus globulus*, *Ocimum sanctum*, *Origanum vulgare*, *Solidago chilensis*, *Thymus vulgaris,* etc. (Mushtaq et al. 2018).

MCFAs are well known to possess *in vitro* antibacterial properties against various pathogens, but to the best of our knowledge, there is no evidence about the antibacterial properties of palm oils rich in MCFAs against bovine mastitis-causing staphylococci and streptococci. Batovska et al. (2009) evaluated the antibacterial activity of MCFAs against *S. aureus* 209, *S. aureus* 146 MR, and *S. aureus* ATCC 33862 USA by the agar well diffusion method and discovered that capric acid inhibits all three strains at concentrations of 250–500 µg/ml and that lauric acid inhibits *S. aureus* 209 at a concentration of 125 µg/ml. These concentrations inhibiting staphylococcal growth are approximately 10× lower than in the case of the palm oils tested in this study (2048–8192 µg ml). In addition, Schlievert and Peterson (2012) evaluated the inhibitory activity of lauric acid – in the form of glycerol monolaurate (monolaurin) – against 54 different *S. aureus* strains and found its MIC to be 300 µg/ml. Because palm oils are diverse combinations of fatty acids, not only MCFAs but also tocopherols, polyphenols, phytosterols, and phenolic acids (Srivastava et al. 2016), it can be assumed that these compounds interact due to their mechanisms of action and thus reduce the antibacterial activity of the oils themselves. Another reason for the decreased antibacterial activity of palm oils when compared to pure MCFAs can be due to their incomplete release from triglycerides during the cleavage by lipase. This fact was confirmed in a trial by Nair et al. (2005), where the growth of 15 clinical isolates of mastitis-causing pathogens including *S. aureus*, *Str. agalactiae*, *Str. dysgalactiae*, and *Str. uberis* was inhibited by caprylic acid and monocaprylin. The authors concluded that the most effective concentration inhibiting the abovementioned mastitis-causing isolates in autoclaved milk was 100 mM (6.93×10^{-4} mg/ml) of caprylic acid and 50 mM (2.29×10^{-4} mg/ml) of monocaprylin. Monolaurin is effective against the growth of *Str. agalactiae,* as shown in a study by Schlievert and Peterson (2012), where glucose monolaurate had antibacterial activity against three strains of this bacterium at MIC of 30 µg/ml. In our study, the effective concentration of oil against *Str. agalactiae* was up to 34× higher than that of glucose monolaurate in the case of coconut oil and the CCM 6178 strain, but only 2× higher in the case of palm kernel oil against the same bacterial strain. This difference could be due to the different degree of hydrolysis in both oils during the cleavage process. The susceptibility of *S. aureus* to the oils was significantly higher than the susceptibility of other species not only in the present study – average MIC 13× higher than in *Str. agalactiae* species – but also in other studies, as mentioned above. The probable reason for the profound resistance of *S. aureus* is believed to be caused by its capsule which is composed of exopolysaccharides, and slime production has also been shown to exist in mastitis-causing strains (Baselga et al. 1994).

As there is a known link between bacterial udder contamination causing mastitis and hygiene (Schreiner and Ruegg 2003), a variety of pre- and post-milking teat disinfection procedures are used on farms. The most commonly applied are iodine-based (iodophors) and chlorhexidine-based treatments, especially after milking; however, both types of disinfectants currently struggle with bacteria that are gaining resistance to these substances (Behiry et al. 2012). The antibacterial properties of plant oils rich in MCFAs against mastitis-causing pathogens are therefore desirable. The advantage of plant oils rich in MCFAs lies in their positive effect on skin conditions (Oyedeji and Okeke 2010), as well as their antibacterial activity; therefore, using plant oils rich in MCFAs as a part of teat preparation and treatment during the milking process can be beneficial.

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CONCLUSION

The potential health risk of foodborne transmission of antibiotic-resistant animal pathogenic bacterial strains has led to the need for new alternative antibacterial sources in the dairy industry. In this study, the antibacterial effect of palm oils rich in MCFAs, namely coconut, palm kernel, and tucuma oil (after cleavage with a lipase from *Mucor javanicus*), was observed on eight bovine mastitis-causing pathogens *in vitro*. The results offer an alternative approach to mastitis prevention in cattle herds since oils rich in MCFAs are valuable antibacterial remedies suitable for use in teat preparation and treatment.

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