Susceptibility of *Escherichia coli* and *Clostridium perfringens* to sucrose monoesters of capric and lauric acid

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ABSTRACT: The sucrose monoesters of capric and lauric acid were tested for their antibacterial activity towards two foodborne enteropathogenic bacteria – *Escherichia coli* (CCM 3954 – serotype O6 and E22 – serotype O103) and *Clostridium perfringens* (CNCTC 5459 and CIP 105178). Antibacterial activity was evaluated by the plating technique. Sucrose monocaprate significantly decreased the number of viable cells of *E. coli* at all tested concentrations (0.1–5 mg/ml). The overnight incubation of *C. perfringens* with the sucrose ester of lauric acid at 0.1–5 mg/ml reduced the number of viable cells below the detection limit (2 $\log_{10}$ CFU/ml). Incubating *E. coli* CCM 3954 and *C. perfringens* CNCTC 5459 with monoesters (0.1 and 2 mg/ml) did not influence the K^+ permeability of the cytoplasmic membrane in cells during a 2.5-minute treatment. A 30-minute incubation of *E. coli* CCM 3954 and *C. perfringens* CNCTC 5459 with esters (0.1 and 2 mg/ml) revealed damage to cytoplasmic structures, as observed by transmission electron microscopy.

Keywords: inhibition; ester; fatty acids; bacterium; antibacterial

INTRODUCTION

Escherichia coli and *Clostridium perfringens* are foodborne bacteria that are responsible for a significant percentage of intestinal and extraintestinal infectious diseases in humans and animals. The undesirable growth of these bacteria on the surface and inside animal products threatens human health; thus, it is important to develop methods to control the transmission of the bacteria from food to humans. One approach is to reduce the number of pathogens in the intestinal tract of farm animals and eliminate carcass contamination during slaughter. Another approach is to treat carcasses, processed meat and/or packaging materials with non-toxic antimicrobial agents, such as organic acids, bacteriocins, essentials oils, or antimicrobial lipids.

Mono-substituted carbohydrate fatty acid esters and ethers are non-toxic compounds that are active against a range of spoilage and pathogenic microorganisms associated with foods. These biodegradable nonionic surfactants can also be applied as emulsifiers for foodstuff, personal care products, and medical supplies (Fietcher 1992; Yan et al. 1999; Godshall 2001; Polat and Linhardt 2001). Antibacterial effects of fatty acids and their derivatives were observed in connection with *Listeria monocytogenes* (Monk et al. 1996; Nobmann et al. 2009), *Staphylococcus aureus* (Monk et al. 1996; Nobmann et al. 2010), *Bacillus cereus* (Karlova et al. 2010), *Escherichia*

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coli (Smith et al. 2008; Karlova et al. 2010), and various food spoilage microorganisms (Yang et al. 2003; Nobmann et al. 2009; Polakova et al. 2010). Contrary to the evaluation of the effect of free fatty acids or their monoglycerides studied extensively in the past (anticlostridial effect observed for example by Timbermont et al. 2010), no study on the effect of sucrose monolaurate on *Clostridium perfringens* has been conducted.

Microorganisms differ in their susceptibility to carbohydrate fatty acid derivatives, and Gram-positive bacteria were found to be more susceptible than Gram-negatives (Smith et al. 2008; Nobmann et al. 2009; Karlova et al. 2010). The antimicrobial activity of carbohydrate fatty acid derivatives depends on both the carbohydrate moiety (Smith et al. 2008; Nobmann et al. 2009) and the carbon chain length (Smith et al. 2008; Polakova et al. 2010).

The mechanism of the antibacterial action of fatty acids and their derivatives seems to be complex; however, the bacterial membrane is considered to be the prime target of their activity (Desbois and Smith 2010). At higher concentrations, free fatty acids are believed to work as detergents, and they are able to solubilize the membrane to such an extent that various membrane proteins of larger sections of the lipid bilayer are released. The key antibacterial effect of fatty acids related to bacterial membranes is most likely the interference with the electron transport chain and the disruption of oxidative phosphorylation (Sheu and Freese 1972; Galbraith and Miller 1973; Boyaval et al. 1995; Wojtczak and Więckowski 1999). Other hypotheses proposed include cell lysis, inhibition of enzyme activity, impairment of nutrient uptake, and generation of toxic peroxidation and autooxidation products (reviewed by Desbois and Smith 2010).

The aim of the present study was to determine the antimicrobial activity of sucrose monoesters of capric and lauric acid (EC_{10} and EC_{12}, respectively) against *E. coli* and *C. perfringens*. The mode of the antimicrobial action of carbohydrate fatty acid derivatives has not been fully elucidated. Thus, the effect of these compounds on potassium leakage from bacterial cells and the ultrastructure of bacterial cells, as determined by transmission electron microscopy, were investigated.

MATERIAL AND METHODS

Organisms and culture conditions. Two strains of *Escherichia coli* (CCM 3954 and E22) and two strains of *Clostridium perfringens* (CNCTC 5459 and CIP 105178) were used in all experiments. *E. coli* CCM 3954 (ATCC 25922, serotype O6) was obtained from the Czech Collection of Microogranisms (Czech Republic), and strain E22 (enteropathogenic *E. coli*, serotype O103) was kindly provided by Dr. Alain Milon from the University of Toulouse (France). The former strain is a reference strain for testing antimicrobials, and the later strain is a clinical isolate. *C. perfringens* CNCTC 5459 was obtained from the Czech National Collection of Type Cultures (National Institute of Public Health, Czech Republic), and strain CIP 105178 was obtained from Institut Pasteur (The Biological Resource Center of Institut Pasteur, France). Both strains were clinical isolates. Bacteria were grown in a medium containing (in mg/ml): glucose – 5, bactopeptone – 6, yeast extract – 3, $K_2HPO_4.3H_2O$ – 5.9, KH_2PO_4 – 4.5, $NaHCO_3$ – 3.0, $(NH_4)_2SO_4$ – 2.9, NaCl – 0.9, $MgSO_4.7H_2O$ – 0.09, and $CaCl_2$ – 0.09. For *C. perfringens*, cysteine was added to the medium (0.5 mg/ml).

Susceptibility of E. coli and C. perfringens to fatty acid sucrose esters. The growth medium was dispensed into gas-tight glass flasks containing EC_{10} or EC_{12} at 0, 0.1, 0.2, 0.5, 1, 2, 3, 4, and 5 mg/ml. Both fatty acid esters were purchased from Sigma Aldrich, spol. s r.o., Prague, Czech Republic. Flasks were filled with CO_2, closed with rubber stoppers, and autoclaved at 120°C for 10 min. Samples were inoculated with solutions (0.5 ml of the solution containing approximately 10^7 cells/ml) of aforementioned strains of *E. coli* and *C. perfringens*. Each concentration (for each bacterium) was prepared in triplicate. Inoculated cultures were incubated in a shaking incubator at 37°C for 18 h. Together with the treated samples, a triplicate of inoculated controls was incubated (inoculated medium with no EC_{10} or EC_{12}). After incubation, the samples were serially diluted (ten-fold dilution in a range of 10^2–10^8) and 0.1 ml of the dilution series was inoculated on agar plates (MacConkey or TSC agar). Both agar media were purchased in OXOID CZ s.r.o., Thermo Fisher Scientific, Brno, Czech Republic. Agar plates were incubated in an anaerobic atmosphere in the case of *C. perfringens*, using the AnaeroGen system (OXOID). After a 24-h incubation at 37°C, typical bacterial colonies were counted, and means and the standard deviation (SD) were calculated in CFU/ml. Differences between treated samples and non-treated controls were evaluated using the *t*-test.

Determination of potassium release. To assess the effect of selected fatty acids and their sucrose esters on inner membrane permeability, the potassium efflux from cells of *E. coli* CCM 3954 and *C. perfringens* CNCTC 5459 was measured with a K^+ ion-selective electrode 20-19+ and an AgCl reference electrode 10-251 (2 Theta ASE, s.r.o., Český Těšín - Mosty, Czech Republic) according to the procedure described by Ohmizo et al. (2004). Fatty acids and their corresponding esters were tested at concentrations of 0.1 and 2 mg/ml. Cetyltrimethylammonium bromide (CTAB) at 0.25 mg/ml was used as a positive control to determine the 100% level of K^+ efflux from bacteria. Potassium release was measured in real time as follows: after approximately 2 min of stabilization, the fatty acid ester was added to the solution, followed by 2.5 min of incubation. Next, CTAB was added to release the remaining K+ from bacterial cells. The suspension was incubated for another 3 min or until the values did not rise anymore. Values were automatically recorded every 3 s. The detailed description of this technique has already been used and published (Ohmizo et al. 2004; Molatova et al. 2010).

Transmission electron microscopy. Fatty acid esters at 0.1 and 2 mg/ml were added to overnight-grown cultures of *E. coli* CCM 3954 or *C. perfringens* CNCTC 5459 and incubated at 37°C for 30 min. Samples were prepared for electron microscopy according to Skrivanova et al. (2006). Briefly, 1 ml samples (both treated samples and non-treated controls) were centrifuged at 2500 rpm for 2 min. The pellet was fixed for 1 h by 0.48 ml of 5% glutaraldehyde in 0.2M Na-cacodylate buffer (pH 7.4) and 0.48 ml of a solution of ruthenium red (0.15% in 0.2M Na-cacodylate buffer). After fixation the sample was centrifuged at 2500 rpm (700 *g*-force) for 2 min, the pellet was washed three times in 0.1M Na-cacodylate buffer (pH 7.4) and fixed for 4 h by 0.4 ml of 4% OsO_4 in 0.2M cacodylate buffer and 0.4 ml of ruthenium red solution (0.15% in 0.2M Na-cacodylate buffer). The sample was centrifuged and washed three times in 0.1M Na-cacodylate buffer. The fixed cells were suspended in 1% agar at 37°C, small cubes of 1 mm size were cut with a blade after congelation, dehydrated in a graded series of ethanol, then passed through absolute acetone, and embedded in Vestopal W (Serva, Heidelberg, Germany). Ultrathin sections were cut with an ultramicrotome and examined by a Philips CM 100 transmission electron microscope (TEM) (Philips EO, Eindhoven, the Netherlands) equipped with a Mega View II slow scan digital camera (Sis GmbH, Münster, Germany) at 80 kV. TEM images were recorded as a matrix of 3 × 2 frames (1280 × 1024 pixels each) and stitched together using MIA module of AnalySis 3.2 (Version Build 788, 2003) software suit. For further image processing, shading correction and Digital Contrast Enhancement (DCE) modules were used. All chemicals for the sample preparation (if not otherwise specified), were purchased from Sigma Aldrich, spol. s r.o.

RESULTS AND DISCUSSION

Medium-chain fatty acids and their monoacylglycerols are efficient antimicrobial agents in cultures of food-borne pathogens, spoilage bacteria, and fungi (Kabara et al. 1972). Medium-chain fatty acids are natural compounds of milk and colostrum. Among livestock, goat milk is a richer source of caproic, caprylic, and capric acids than the cow milk. The names of the acids are derived from the Latin word "capric", which pertains to goats (Marounek et al. 2012). In experiments with *E. coli*, the most efficient fatty acids were caprylic and capric acids (Marounek et al. 2003), whereas *C. perfringens* strains were susceptible to lauric acid and monolaurin (Skrivanova et al. 2006). Organic acids are traditional food preservatives (acetic, lactic, benzoic, and sorbic). Fatty acids and monoacylglycerols, however, often have an unpleasant taste, which prevents their use in the treatment of carcasses and processed meat. Sucrose esters of fatty acids tend not to have an unsavoury taste; thus, they are more suitable for food preservation. As the fatty acids and their monoglycerols, sucrose esters are also known as non-toxic compounds (Habulin et al. 2008).

Susceptibility of E. coli and C. perfringens to fatty acid sucrose esters. The *in vitro* susceptibility of *E. coli* CCM 3954 and E22 to EC_{10} and EC_{12} is shown in Table 1. The incubation of *E. coli* CCM 3954 with EC_{10} led to a significant decrease in the numbers of viable bacterial cells from 9.76 to 7.91–5.13 $\log_{10}$ CFU/ml. Similar results were obtained in the case of *E. coli* E22. Even though the former strain is of a culture collection origin and the latter is a clinical isolate, their sensitivity was comparable. No inhibitory effect was observed when *E. coli* was incubated with EC_{12}. Our results are in agreement with the observation of some

Table 1. Plate counts of *E. coli* after overnight incubation with the sucrose ester of capric and lauric acid (EC_{10} and EC_{12}, respectively) compared to the control (0 mg/ml of the treatment). Average values and standard deviation in parentheses

c (mg/ml)	EC_{10}	EC_{12}
***E. coli* CCM 3954**		
0	9.76 (0.25)	9.76 (0.25)
0.1	7.91 (0.54)^{*a}	9.79 (0.28)^{b}
0.2	7.03 (0.75)^{*a}	9.50 (0.45)^{b}
0.5	6.30 (0.40)^{*a}	9.13 (0.11)^{b}
1	6.43 (0.45)^{*a}	9.32 (0.68)^{b}
2	5.89 (0.26)^{*b}	9.04 (0.17)^{b}
3	5.64 (0.23)^{*b}	9.27 (0.24)^{b}
5	5.13 (0.44)^{*b}	8.84 (0.14)^{b}
***E. coli* E22**		
0	9.76 (0.25)	9.76 (0.25)
0.1	8.86 (0.47)*	9.24 (0.21)
0.2	7.73 (0.39)^{*a}	9.42 (0.57)^{b}
0.5	7.37 (0.24)^{*a}	9.10 (0.21)^{b}
1	6.40 (0.31)^{*a}	9.81 (0.84)^{b}
2	4.99 (0.18)^{*b}	9.97 (0.20)^{b}
3	5.02 (0.33)^{*b}	9.84 (0.27)^{b}
5	4.84 (0.52)^{*b}	9.15 (0.22)^{b}

*values in the same column are significantly different from the control (0 mg/ml of the tested compound), $P < 0.05$

^{a,b}values in the same row with different superscripts are significantly different, $P < 0.05$

Table 2. Plate counts of *C. perfringens* after overnight incubation with the sucrose ester of capric and lauric acid (EC_{10} and EC_{12}, respectively) compared to the control (0 mg/ml of the treatment). Average values and standard deviations in parentheses

c (mg/ml)	EC_{10}	EC_{12}
***C. perfringens* CNCTC 5459**		
0	9.30 (0.30)	9.30 (0.30)
0.1	6.74 (0.22)^{*a}	< 2^{*b}
0.2	6.79 (0.28)^{*a}	< 2^{*b}
0.5	6.22 (0.35)^{*a}	< 2^{*b}
1	4.08 (0.51)^{*a}	< 2^{*b}
2	< 2*	< 2*
3	< 2*	< 2*
5	< 2*	< 2*
***C. perfringens* CIP 105178**		
0	9.54 (0.41)	9.54 (0.41)
0.1	7.97 (0.47)^{*a}	< 2^{*b}
0.2	6.12 (0.34)^{*a}	< 2^{*b}
0.5	5.97 (0.27)^{*a}	< 2^{*b}
1	6.12 (0.51)^{*a}	< 2^{*b}
2	3.82 (0.22)^{*a}	< 2^{*b}
3	< 2*	< 2*
5	< 2*	< 2*

*values in the same column are significantly different from the control (0 mg/ml of the tested compound), $P < 0.05$

^{a,b}values in the same row with different superscripts are significantly different, $P < 0.05$

other authors (Conley and Kabara 1973; Hathcox and Beuchat 1996; Habulin et al. 2008). Contrary to these findings, Kato and Arima (1971), or Ferrer et al. (2005) did observe an antibacterial effect of sucrose monolaurate on *E. coli* in concentrations of 4 mg/ml and 1 mg/ml, respectively. Gram-positive

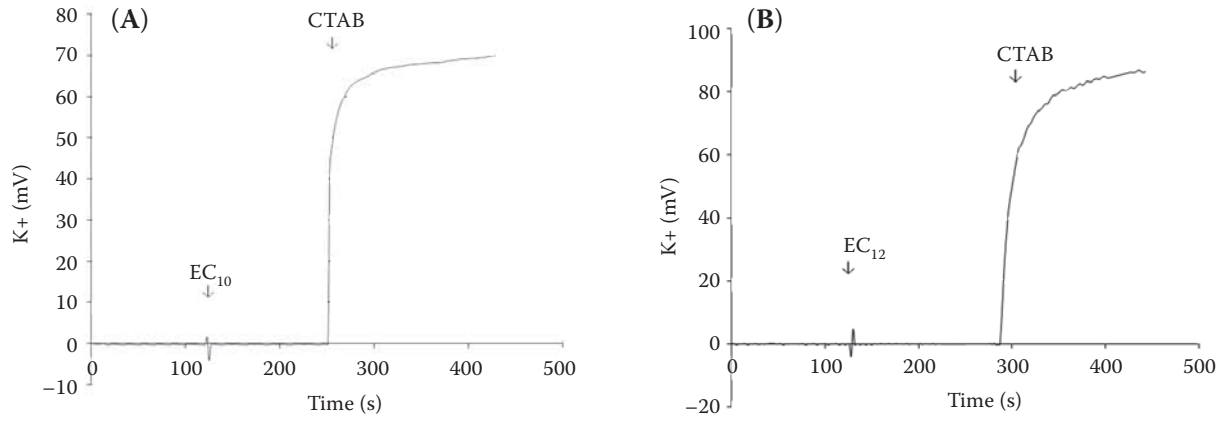

Figure 1. Efflux of K+ from *E. coli* CCM 3954 (**A**) and *C. perfringens* CNCTC 5459 (**B**) was monitored with an ion-selective electrode. Cells were treated with capric and lauric acid sucrose esters, respectively. Fatty acid ester was added at a final concentration of 2 mg/ml at the time indicated by the arrow on the left; the arrow on the right indicates the time when cetyltrimethylammonium bromide (CTAB) was added at 0.25 mg/ml

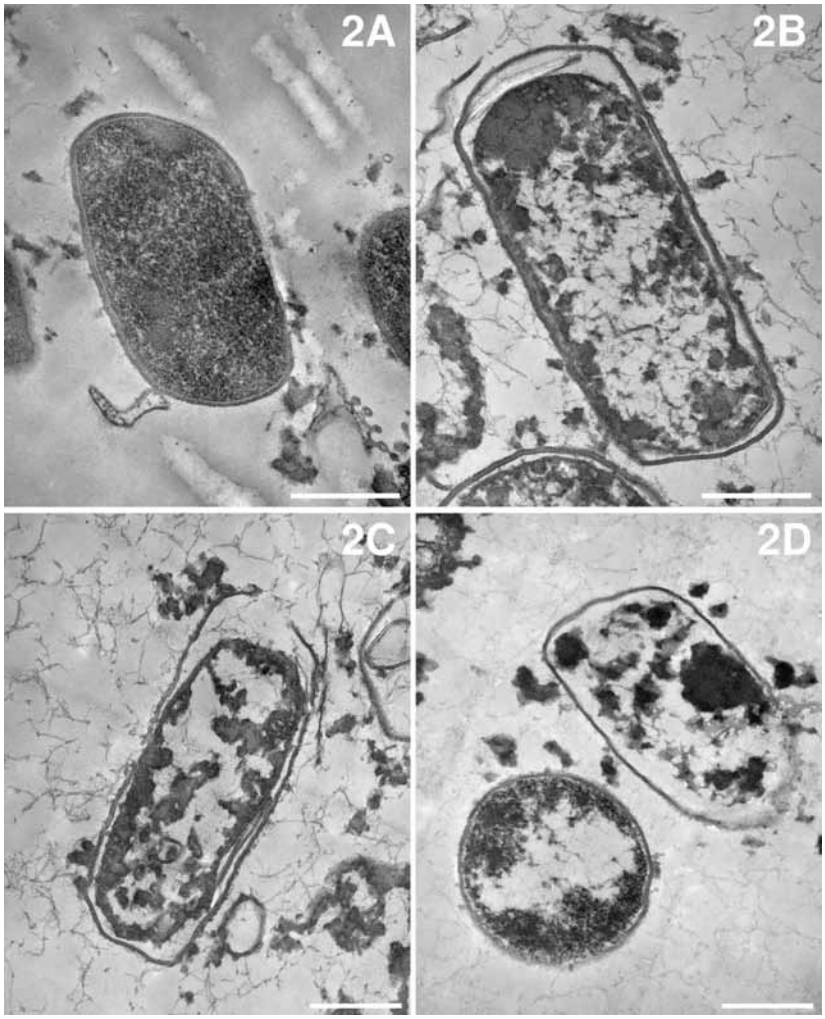

Figure 2. Effect of sucrose monolaurate (0.1 and 2 mg/ml) on the ultrastructure of *C. perfringens* CCM 4435, as demonstrated by transmission electron microscopy. Control sample (**2A**), sample treated with sucrose monoester of lauric acid at 0.1 mg/ml for 30 min (**2B**), cells treated with sucrose monoester of lauric acid at 2 mg/ml for 30 min (**2C, D**). Scale = 500 nm

C. perfringens was more sensitive to both esters (Table 2). The incubation of both *C. perfringens* CNCTC 5459 and *C. perfringens* CIP 105178 with EC_{12} (all tested concentrations) led to a reduction of viable cells below the detection limit (2 $\log_{10}$ CFU/ml). Sucrose monodecanoate showed the same effect at concentrations higher than 1 or 2 mg/ml, respectively.

Effect of fatty acids and their sucrose esters on bacterial permeability and cellular ultrastructure. Intracellular potassium ions were released by the action of CTAB. However, no K+ was released by the addition of sucrose esters of capric and lauric acid (Figure 1). In contrast, significant changes in the bacterial ultrastructure were observed after bacteria were treated for 30 min with fatty acids monoesters, as observed by transmission electron microscopy (Figure 2). The incubation of *E. coli* CCM 3954 and *C. perfringens* CNCTC 5459 with fatty acids or their esters (0.1 and 2 mg/ml) resulted in damage to cytoplasmic structures. In some clostridial cells, incubation with a higher concentration of EC_{12} led to cell wall disruption (Figure 2C).

Various authors have hypothesized that organic acids may damage outer or cytoplasmic membranes, hinder macromolecular synthesis or denature proteins and DNA (reviewed by Ricke 2003). The increase in the permeability of bacterial membranes may also potentiate the effect of other antimicrobial agents, as shown with lactic acid and sodium laurylsulphate (Alakomi et al. 2000). However, the majority of experiments were carried out on free (or organic) fatty acids. Information about the mechanism of action on fatty acids derivatives is scarce.

In our study, membrane integrity was maintained in bacteria treated with capric and lauric acid sucrose esters. An increase in the K^+ permeability of the cytoplasmic membrane, which usually leads to dissipation of the membrane potential (Katsu et al. 1984), was not observed. This result supports

our previous experiments with free fatty acids, in which no K^+ leakage or TPP^+ uptake was observed in *Clostridium perfringens* or *Campylobacter jejuni* cultures treated with caprylic, capric, and lauric acids (Skrivanova et al. 2006; Molatova et al. 2010) leading us to the conclusion that the mechanism of action of both free acids and their sucrose esters is based on a similar principle.

Contrary to the K+ permeability measurement, which showed no significant effect of fatty acids or their esters on cell permeability, electron microscopy showed obvious ultrastructural changes of the cells (Figure 2). A 30-minute incubation of the same strains of *E. coli* and *C. perfringens* with fatty acid esters (0.1 and 2 mg/ml) revealed damage to cytoplasmic structures, and in some cells of *C. perfringens*, incubation with 2 mg/l of the acid also led to the disintegration of the cell wall. However, cytoplasmic membrane appeared to remain intact. Similar changes in cytoplasmatic structures were observed in *Campylobacter jejuni, Escherichia coli,* and *C. perfringens* treated with free medium-chain fatty acids at concentrations up to 1 mg/ml (Skrivanova et al. 2006; Molatova et al. 2010). In the current set of experiments, sucrose monoesters were observed to have a similar effect on ultrastructure of treated cells as possessed the free fatty acids. Therefore, the same mechanism of action is probably present in both forms of C_{10} and C_{12}.

CONCLUSION

Sucrose esters of capric and lauric acids were found effective against *E. coli* and *C. perfringens*. Their effect on K^+ leakage and microscopic ultrastructure was comparable to the effect of their corresponding free fatty acids, as showed in our previous experiments. Sucrose esters represent another form of fatty acids that is suitable for reducing the bacterial contamination of animal products, whereas particularly in the food industry, the odourless character may be a benefit compared to free fatty acids.

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