

## Antimicrobial Activities of Medium-chain Fatty Acids and Monoacylglycerols on *Cronobacter sakazakii* DBM 3157<sup>T</sup> and *Cronobacter malonaticus* DBM 3148

MILAN MAROUNEK<sup>1</sup>, VANNAPHONE PUTTHANA<sup>2</sup>, OLDŘICH BENADA<sup>3,4</sup>  
and DANIELA LUKEŠOVÁ<sup>2</sup>

<sup>1</sup>Institute of Animal Physiology and Genetics and <sup>3</sup>Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic; <sup>2</sup>Institute of Tropics and Subtropics, Czech University of Life Sciences Prague, Prague, Czech Republic; <sup>4</sup>Department of Biology, Faculty of Science, J.E. Purkinje University in Usti nad Labem, Ústí nad Labem, Czech Republic

### Abstract

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*Cronobacter sakazakii* and *Cronobacter malonaticus* are pathogens causing infections in children that are primarily linked to the consumption of contaminated infant milk formula and food. Both *Cronobacter* strains examined were susceptible to caprylic acid, monocaprylin and, to a lesser extent, sorbic acid. Capric acid, lauric acid, monosorbin, monocaprin, monolaurin, and sucrose caprate exhibited no inhibitory activity. Caprylic acid and monocaprylin treatment (2 mg/ml) of *C. sakazakii* DBM 3157<sup>T</sup> reduced the number of viable cells by five orders of magnitude. In the case of *C. malonaticus* DBM 3148, both caprylic acid and monocaprylin (2 mg/ml) decreased the viable cell counts below the limits of detection. The bactericidal activity of monocaprylin increased as a function of concentration (0.5–2.0 mg/ml) and temperature (40–55°C). The exposure of each *Cronobacter* strain to monocaprylin resulted in the release of cellular proteins and nucleic acids. Electron microscopy revealed that the antimicrobial treatment damaged cytoplasmic structures and resulted in cell aggregation. The combination of monocaprylin at 0.5 mg/ml and increased temperature (50°C) appears to be a suitable treatment against *C. sakazakii* and *C. malonaticus*.

**Keywords:** *Cronobacter* sp.; fatty acids; bacterial activity; monocaprylin

*Cronobacter* is a genus of the family *Enterobacteriaceae*. The type species of the genus is *Cronobacter sakazakii* (formerly *Enterobacter sakazakii*). The genus *Cronobacter* includes opportunistic human pathogens (*C. sakazakii*, *C. malonaticus*, *C. turicensis*) that can cause infections in neonates and children, such as neonatal bacteraemia, meningitis, and necrotising enterocolitis (KUCEROVA *et al.* 2010). Case reports on *Cronobacter* infections

in adults are rarely published because of the less severe nature of the illness compared to the illness in children. GOSNEY *et al.* (2006), however, isolated *C. sakazakii* from the mouths of seven stroke patients. The authors concluded that in these patients *C. sakazakii* might be associated with the presence of clinical complications, such as pneumonia. Strains of *Cronobacter* sp. have been isolated from powdered infant milk formulae,

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infant cereals, vegetables, ready-to-eat food, dried products, spices, meat, and eggs (reviewed by FRIEDEMANN 2007). *Cronobacter* strains are resistant to dry stress and alkaline environments of up to pH 10 but do not survive acid stress (pH < 4.0) and heating (FU *et al.* 2011). An effective method is desirable for reducing *Cronobacter* in foods by using natural antimicrobial substances, such as bacteriocins, essential oils, or antimicrobial lipids. The following compounds that occur in plants have been reported to inhibit *C. sakazakii* growth: cinnamaldehyde (AMALARADJOU *et al.* 2009), carvacol, and thymol (LEE & JIN 2008). BACK *et al.* (2009) reported that acetic and propionic acids display inhibitory activities against *C. sakazakii* in liquid foods, and GURTNER and BEUCHAT (2007) observed that *C. sakazakii* growth was inhibited in reconstituted infant formulae by the lactoperoxidase system.

Several free fatty acids and their monoglycerides have been reported to possess inhibitory activity against a wide range of microorganisms (KABARA *et al.* 1972). In experiments with bacteria belonging to the *Enterobacteriaceae*, caprylic acid was more active against *Escherichia coli* and *Salmonella* than fatty acids with shorter or longer chain lengths (MAROUNEK *et al.* 2003; SKŘIVANOVÁ *et al.* 2004). Both caprylic acid and its monoglyceride monocaprylin were active against *Campylobacter jejuni* (MOLATOVÁ *et al.* 2010). In the case of *Cronobacter* sp., NAIR *et al.* (2004) reported that monocaprylin displays a significant inhibitory activity against *C. sakazakii* strains that were isolated from infant formulae or processing plants. Monocaprylin displayed antimicrobial activity both at room temperature (23°C) and at lower temperatures (8°C and 4°C). JANG and RHEE (2009) investigated the combined effect of caprylic acid and temperature on three strains of *Cronobacter*, and they observed that the numbers of bacteria in the treated cultures were reduced more rapidly at increased temperature (45–55°C) compared to 25°C. In contrast, monolaurin exhibited only a slight inhibitory effect on *Cronobacter* sp. (AL-HOLY *et al.* 2010).

The aim of the present study was to determine the antimicrobial activity of four medium-chain fatty acids (MCFAs), their monoglycerides, and sucrose caprate against *C. sakazakii* and the related bacterium *Cronobacter malonaticus*. The effect of monocaprylin on bacterial cells was examined in greater detail using transmission and scanning electron microscopy.

## MATERIAL AND METHODS

**Bacteria and culture medium.** The bacteria used in this study were obtained from the culture collection of the Department of Biochemistry and Microbiology (DBM) of the Institute of Chemical Technology Prague (Czech Republic) and maintained in 20% glycerol (v/v) at –40°C. *Cronobacter sakazakii* DBM 3157<sup>T</sup> and *Cronobacter malonaticus* DBM 3148 were originally deposited in the Czech National Collection of Type Cultures of the National Institute of Public Health (Prague, Czech Republic) as strains CNCTC 5739<sup>T</sup> (type strain ATCC 29544<sup>T</sup>) and CNCTC 6830, respectively. The bacteria were grown in a medium containing (g/l): Peptone from casein tryptic digest (8.5), Soy Peptone AX (1.5), NaCl (2.5), K<sub>2</sub>HPO<sub>4</sub> (1.3), and glucose (1.3). The medium components were supplied by Sigma-Aldrich, Ltd. (Prague, Czech Republic).

**Antimicrobial activity of fatty acids and their derivatives.** The medium (20 ml) was dispensed into gas-tight glass flasks. Sorbic, caprylic, capric, and lauric acids, monosorbin, monocaprylin, monocaprin, monolaurin, and sucrose monocaprate were added at 0, 1, 2, 3, 4, and 5 mg/ml (final concentrations) in 20% dimethylsulphoxide (DMSO) solutions. The acids were added together with a stoichiometrically equivalent amount of 5M NaOH. The control cultures received an equivalent amount of DMSO. The flasks were filled with CO<sub>2</sub>, closed with rubber stoppers and autoclaved at 110°C for 45 min to minimise the Maillard reaction. The medium was inoculated with 0.2 ml of an overnight culture (10<sup>9</sup> CFU/ml) and incubated at 37°C overnight (16 h). The pH was subsequently measured, and the residual glucose was determined enzymatically using a commercial Glucose Assay Kit (Sigma-Aldrich, Ltd., Prague, Czech Republic). The amount of residual glucose was expressed relative to the initial glucose concentration and plotted against the concentration of the acid or monoacylglycerol. The IC<sub>50</sub> represents the concentration at which the initial glucose level decreased by 50%. This experiment was performed three times. This assay avoids the problems caused by the media opalescence or turbidity as a result of poorly soluble lipid substances.

Sorbic acid, caprylic acid, and monocaprylin were added to the overnight-grown cultures of *C. sakazakii* DBM 3157<sup>T</sup> and *C. malonaticus* DBM 3148 at 2 mg/ml as DMSO solutions. The control

cultures received an equivalent amount of DMSO. The pH of both the control and the treated cultures was adjusted to 6.5–6.7, and the cultures were incubated at 37°C for 30 minutes. After the incubation, the cultures were serially diluted, and the numbers of viable bacteria were determined by streaking 0.1 ml of an appropriate dilution on Wilkins-Chalgren agar. The plates were incubated at 37°C for 1 day; then, the resulting colonies were counted, and the mean values and standard deviations (SD) were calculated. The significance of the differences between the control and the treated cultures was evaluated by one-way ANOVA followed by the Tukey's test.

To investigate the combined effect of the increased temperature and monocaprylin on the two *Cronobacter* strains, monocaprylin was added to the overnight cultures at concentrations of 0.5, 1, and 2 mg/ml as a DMSO solution. The control cultures received an equivalent amount of DMSO. The control and the treated cultures were incubated at 40, 45, 50, and 55°C for 30 minutes.

Monosorb and monocaprylin were prepared from glycidol and sorbic or caprylic acid, respectively, by Prof. R. Janiš from Tomas Bata University in Zlín (JANIŠ *et al.* 2000). Other chemicals were supplied by Sigma-Aldrich, Ltd. (Prague, Czech Republic).

#### **Measurement of the release of cellular material.**

The release of cellular protein and nucleic acids was measured spectrophotometrically at 280 and 260 nm, respectively (KURDI *et al.* 2006). The overnight-grown cultures (100 ml) of *C. sakazakii* DBM 3157<sup>T</sup> and *C. malonaticus* DBM 3148 were centrifuged at 2500 g for 20 minutes. The cell pellet was washed once with 50mM sodium phosphate buffer (pH 6.5) and resuspended in 50 ml of 150mM sodium phosphate buffer (pH 6.5). Monocaprylin was dissolved in DMSO and added at 2 mg/ml to 20 ml of the cell suspension. The control cell suspension received an equivalent amount of DMSO. The treated and control suspensions were incubated at 37°C in a water bath. The samples were harvested at 0, 1, 2, and 3 h, centrifuged at 15 000 g for 10 min, and the absorbance of the supernatants was measured at 260 and 280 nm, respectively.

**Transmission and scanning electron microscopy.** Monocaprylin was added to *C. malonaticus* DBM 3148 and *C. sakazakii* DBM 3157<sup>T</sup> cultures in the late exponential phase of growth at 0, 2, and 4 mg/ml in DMSO solutions. The bacterial cultures (50 ml) were centrifuged after 30 min of exposure,

and the cell pellet was resuspended in 10 ml of the culture medium. Fixation was performed according to HIGGINS and SHOCKMAN (1970) with minor modifications. The bacteria were pre-fixed in 3% glutaraldehyde for 1 h at room temperature. After extensive washing, the pre-fixed bacteria were transferred into 2 ml Eppendorf tubes and fixed with 2% OsO<sub>4</sub>. The fixed bacteria were dehydrated through an alcohol series followed by embedding into Vestopal resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate (REYNOLDS 1963), and the samples were viewed with a Philips CM100 electron microscope (Royal Philips Electronics, Amstelveen, The Netherlands) at 80 kV. Digital images were recorded using a Mega-ViewII slow scan camera at a magnification of 46 000× with the aid of the MIA module of Analysis 3.2 software (Olympus Soft Imaging Solutions GmbH, Münster, Germany). The bacterial cultures for scanning electron microscopy were processed in parallel with the samples for TEM but without OsO<sub>4</sub> fixation. The glutaraldehyde-fixed bacteria were extensively washed and allowed to sediment overnight onto poly-L-lysine-treated SPI Pore Filters (pore size of 0.2 µm) at 4°C. The filters with the attached bacteria were dehydrated through an alcohol series followed by absolute acetone and critical-point drying in a Balzers CPD 010 unit. The dried samples were sputter-coated with gold in a Polaron Sputter-Coater (E5100) (Quorum Technologies Ltd, Ringmer, UK). The final samples were examined with a Tescan Vega LSU scanning electron microscope (Tescan, Brno, Czech Republic) at 20 kV in secondary electron mode.

**Sensory evaluation.** Infant milk formula (Sunar; Hero Czech Ltd., Prague, Czech Republic) was bought from a local shop and reconstituted with warm drinking water (50°C) according to the manufacturer's instructions. Monocaprylin was added at concentrations of 0, 0.5, and 1 mg/ml. The flavour and odour were assessed by four employees of the Institute of Animal Physiology and Genetics, Prague, Czech Republic.

## **RESULTS AND DISCUSSION**

DBM 3157<sup>T</sup> and *C. malonaticus* DBM 3148 were susceptible to caprylic acid and monocaprylin; the IC<sub>50</sub> values varied between 1 mg/ml and 2 mg/ml (Table 1). The inhibitory effect of sorbic acid was less pronounced. Capric acid, lauric acid, monosor-

Table 1. Inhibitory concentrations<sup>a</sup> of sorbic acid, caprylic acid, capric acid, lauric acid, monoacylglycerols, and sucrose caprate against *Cronobacter sakazakii* strain DBM 3157<sup>T</sup> and *Cronobacter malonaticus* strain DBM 3148 grown on glucose

Compound	DBM 3157 <sup>T</sup>	DBM 3148
Sorbic acid	2.32 ± 0.69	2.73 ± 0.17
Caprylic acid	1.13 ± 0.41	1.20 ± 0.38
Capric acid	> 5	> 5
Lauric acid	> 5	> 5
Monosorbin	> 5	> 5
Monocaprylin	1.26 ± 0.09	1.75 ± 0.45
Monocaprin	> 5	> 5
Monolaurin	> 5	> 5
Sucrose caprate	> 5	> 5

<sup>a</sup>IC<sub>50</sub> – concentration (mg/ml) at which 50% of the initial glucose was depleted within a 16-h incubation; mean values of three measurements ± SD are listed

bin, monocaprin, monolaurin, and sucrose caprate showed no antimicrobial activity against either bacterial strain. In the control cultures and those containing lauric acid, monosorbin, monolaurin, or derivatives of capric acid, glucose was completely depleted from the medium. The exhaustion of glucose resources was accompanied by a decrease in pH from 6.85 ± 0.24 to 6.27 ± 0.10 (data not shown). In *C. sakazakii* DBM 3157<sup>T</sup> cultures, the incubation with caprylic acid and monocaprylin (2 mg/ml) reduced the number of viable cells by 5.40 and 5.35 log<sub>10</sub> CFU/ml, respectively (Table 2). In *C. malonaticus* DBM 3148 cultures, caprylic acid and monocaprylin (2 mg/ml) decreased the number of viable cells to below 10<sup>2</sup> cells/ml. In both strains, sorbic acid reduced the number of viable cells but this decrease was not significant ( $P > 0.05$ ). No effect on *C. sakazakii* DBM 3157<sup>T</sup>

Table 2. Viable cell counts<sup>a</sup> (log<sub>10</sub> CFU/ml) of *Cronobacter sakazakii* strain DBM 3157<sup>T</sup> and *Cronobacter malonaticus* strain DBM 3148 as determined by bacterial plating following a 30-min incubation with sorbic acid, caprylic acid, and monocaprylin (2 mg/ml) at 37°C

Strain	Control	Sorbic acid	Caprylic acid	Monocaprylin
DBM 3157 <sup>T</sup>	8.40 ± 0.66 <sup>b</sup>	7.05 ± 0.50 <sup>b</sup>	3.00 ± 1.11 <sup>c</sup>	3.05 ± 0.96 <sup>c</sup>
DBM 3148	8.75 ± 0.83 <sup>b</sup>	7.83 ± 0.61 <sup>b</sup>	< 2.00 <sup>c</sup>	< 2.00 <sup>c</sup>

<sup>a</sup>mean values and SD of five (strain DBM 3157<sup>T</sup>) or four (strain DBM 3148) cultures; cell concentrations < 10<sup>2</sup>/ml were considered to be 2.00 log<sub>10</sub> CFU/ml for the statistical calculations; <sup>b,c</sup>values that are listed in the same row with different superscript letters are significantly different ( $P < 0.05$ )

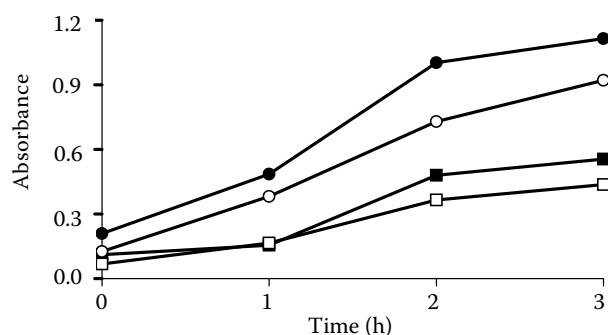


Figure 1. Leakage of protein (■,□) and nucleic acids (●,○) from *C. sakazakii* DSM 3157<sup>T</sup> (full symbols) and *C. malonaticus* DSM 3148 (empty symbols) upon exposure to monocaprylin (2 mg/ml) at 37°C

or *C. malonaticus* DBM 3148 was observed at a low monocaprylin concentration (0.5 mg/ml) at 45°C (Table 3). At 50°C, however, monocaprylin at a concentration of 0.5 mg/ml decreased the viable cell counts of strains DBM 3157<sup>T</sup> and DBM 3148 by two and three orders of magnitude, respectively, compared with the control cultures. At 55°C, the numbers of cells in the control cultures were reduced by several orders of magnitude and decreased to undetectable levels in monocaprylin-treated cultures (2 mg/ml).

The exposure of DBM 3157<sup>T</sup> and DBM 3148 cells to monocaprylin resulted in the release of cellular nucleic acids and proteins (Figure 1). Figure 2 illustrates morphological changes in *C. malonaticus* DBM 3148 cells after a 30-min incubation with monocaprylin (2 and 4 mg/ml). The exposure of bacterial cells to monocaprylin at 2 mg/ml induced changes in the cytoplasm; however, the integrity of the cell wall was maintained (Figure 2B). The exposure of cells to monocaprylin at 4 mg/ml led to the disruption of the cell wall and complete disorganisation of the cytoplasm (Figure 2C). No visible alterations in the cell wall structure of *C. sakazakii* DBM 3157<sup>T</sup> cells exposed to mono-

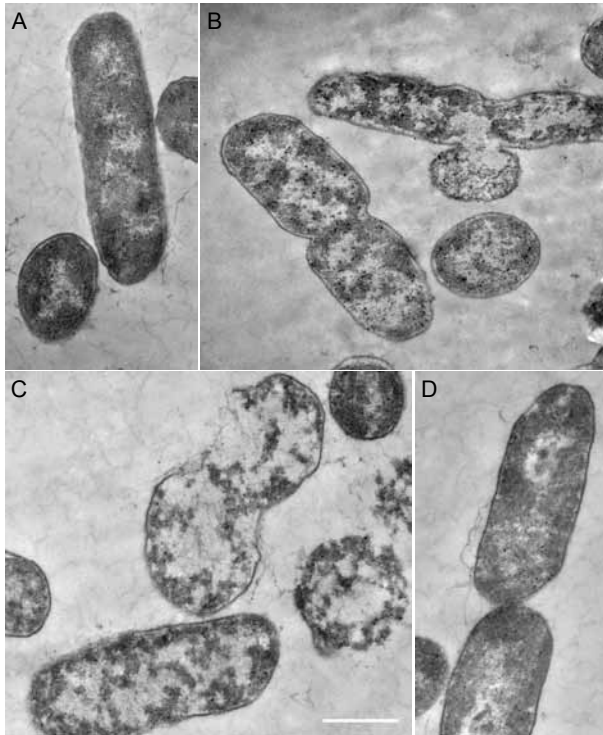


Figure 2. Effect of monocaprylin on the ultrastructure of *C. malonaticus* DSM 3148 (B,C) and *C. sakazakii* DSM 3157<sup>T</sup> (D) at 0 (A), 2 (B) and 4 (C, D) mg/ml; scale bar is 0.5  $\mu$ m

caprylin (4 mg/ml) were apparent (Figure 2D). Planktonic *C. malonaticus* DBM 3148 cells (Figure 3A) exposed to monocaprylin (4 mg/ml) aggregated into clusters (Figure 3B).

Monocaprylin itself is odourless and bitter. A weak bitter flavour persisted in the reconstituted formula supplemented with monocaprylin at a concentration of 1 mg/ml but was obscured by the sweet taste of the milk formula at a concentration of 0.5 mg/ml.

Caprylic acid and monocaprylin showed the highest antimicrobial activity against *C. saka-*

*zakii* DBM 3157<sup>T</sup> and *C. malonaticus* DBM 3148. Caprylic acid is a food-grade chemical approved by the U.S. Food and Drug Administration that is generally recognised as safe (Food and Drug Administration, HHS, § 184.1025). The inhibitory concentrations ( $IC_{50}$ ) of caprylic acid observed in this study were higher than those reported for *E. coli* (0.30–0.45 mg/ml) (MAROUNEK *et al.* 2003) and resembled those observed for *Salmonella* (0.75–1.17 mg/ml) (SKŘIVANOVÁ *et al.* 2004). The inhibitory effects of other MCFAs and their derivatives were less pronounced or absent.

The antimicrobial effects of caprylic acid and monocaprylin were similar. In the present study, we focused on the antimicrobial activity of monocaprylin for the following reasons: (i) the combined effect of caprylic acid and mild heat had been already shown by JANG and RHEE (2009), and (ii) a slightly unpleasant odour of caprylic acid had been previously described. Monoacylglycerols are normal part of the digestion of lipids and are widely used in the food industry as emulsifiers (MOONEN & BAS 2004). Monoacylglycerols containing MCFAs have been shown to be efficient antimicrobial agents in cultures of food-borne pathogens, bacteria that are associated with spoilage, and fungi (BUŇKOVÁ *et al.* 2011). Reports on the effects of capric acid, lauric acid, and monocaprin on *Cronobacter*, which is a Gram-negative bacterium and thus less susceptible to antimicrobials relative to Gram-positive bacteria, are absent from the current literature. In contrast, reports exist on the antimicrobial action of MCFAs and monoacylglycerols against several other Gram-negative food-borne pathogens that include *E. coli* (HASSINEN *et al.* 1951; PETSCHOW *et al.* 1998; MAROUNEK *et al.* 2003), *Salmonella* (SKŘIVANOVÁ *et al.* 2004; VAN IMMERSEEL *et al.* 2004) and *Campylobacter jejuni* (THORMAR *et al.*

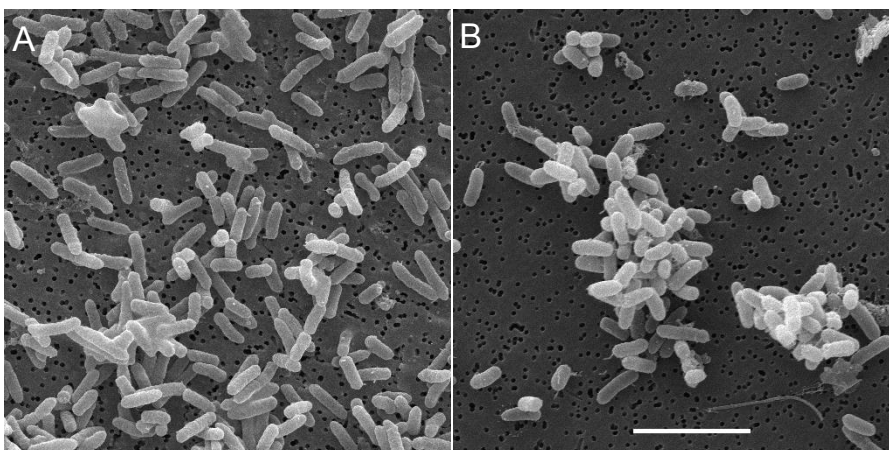


Figure 3. Scanning electron micrograph of cells of *C. malonaticus* DSM 3148 in a control sample (A) and after incubation with monocaprylin at 2 mg/ml (B); scale bar is 5  $\mu$ m

Table 3. Viable cell counts ( $\log_{10}$  CFU/ml) of *Cronobacter sakazakii* strain DBM 3157<sup>T</sup> and *Cronobacter malonaticus* strain DBM 3148 as determined by bacterial plating following a 30-min incubation with monocaprylin at 40, 45, 50, and 55°C

Strain	Temperature (°C)	Concentration of monocaprylin (mg/ml)			
		0	0.5	1	2
DBM 3157 <sup>T</sup>	40	8.75	8.37	7.90	4.95
	45	8.79	8.47	4.90	2.00
	50	8.52	6.46	3.85	< 2.00
	55	4.79	3.47	< 2.00	< 2.00
DBM 3148	40	8.40	8.38	7.67	3.00
	45	8.93	8.90	7.95	2.00
	50	7.10	5.08	2.11	< 2.00
	55	3.21	2.99	2.70	< 2.00

2006; SOLIS DE LOS SANTOS *et al.* 2008; MOLATOVÁ *et al.* 2010). The antimicrobial actions of MCFAs and monoacylglycerols in these studies were variable, and strain-to-strain variability of the susceptibility of different bacteria to these antimicrobials was apparent. In *E. coli* cultures, the greatest inhibitory effect was observed with caprylic acid (MAROUNEK *et al.* 2003). *Salmonella* species were susceptible to caproic acid (VAN IMMERSEEL *et al.* 2004) and caprylic acid (SKŘIVANOVÁ *et al.* 2004). THORMAR *et al.* (2006) found that capric acid and monocaprin were more efficient against *Campylobacter jejuni* as compared with caprylic acid and monocaprylin, this observation having been confirmed by MOLATOVÁ *et al.* (2010).

Depending on the monocaprylin concentrations and the temperatures examined, increasing temperature enhanced the bactericidal activity of monocaprylin, which can be partially explained by an increase in solubility. This effect has been similarly demonstrated for the combination of caprylic acid (5 to 30mM) and heat (45, 50, and 55°C) (JANG & RHEE 2009). At least some strains of *Cronobacter* seem to be relatively resistant to increased temperature. Decimal reduction times for *C. sakazakii* in rehydrated infant milk formula at 52, 54, 56, and 58°C were 15.3, 4.5, 2.0, and 0.53 min, respectively (SHAKER *et al.* 2008). In the present study, no effect was observed of increased temperature on the viability of *C. sakazakii* DBM 3157<sup>T</sup> cells at temperatures of up to 50°C. An increase in temperature from 45 to 50°C reduced the viable cell counts of *C. malonaticus* DBM 3148 by 1.83  $\log_{10}$  CFU/ml.

The release of the cell protein and nucleic acids suggests that the membrane damage leads to

increased cellular membrane permeability. The release of cellular material was observed for *Bifidobacterium breve* cells treated with cholic acid (KURDI *et al.* 2006). Transmission electron microscopy (TEM) revealed damage to cytoplasmic structures in the treated *C. malonaticus* cells, but no separation of the inner and outer membranes was observed for the treated *C. malonaticus* DBM 3148 and *C. sakazakii* DBM 3157<sup>T</sup> cells. TEM revealed the separation of the inner and outer membranes and complete cytoplasmic disorganisation of *Clostridium perfringens* CCM 4435<sup>T</sup> cells that had been treated with lauric acid and monolaurin (SKŘIVANOVA *et al.* 2006). JANG and RHEE (2009) reported membrane damage of *Cronobacter* spp. cells treated with caprylic acid, which was observed using confocal fluorescence microscopy. Common explanations of the antimicrobial activity of organic acids, which are based on proton export and energy depletion of cells, do not explain the antimicrobial activity of monoacylglycerols, which are non-dissociable compounds. Thus, monoacylglycerols may damage the outer or cytoplasmic membrane and increase cell permeability, resulting in a loss of cellular material and hindering the synthesis of macromolecules or denaturing proteins.

Scanning electron microscopy demonstrated that monocaprylin treatment of *C. malonaticus* cells results in their aggregation. Previous studies provided evidence that bacterial aggregation occurs in response to substrate limitation (LOGAN & HUNT 1988) or environmental stress (KLEBENSBERGER *et al.* 2006; DE PAZ *et al.* 2007). The aggregation of bacteria into clusters or biofilms most likely increases their survival in the presence of antimicrobial compounds.

NAIR *et al.* (2004) observed that monocaprylin treatment at 25 and 50mM (5.5 and 10.9 mg/ml) reduced the number of viable cells in *C. sakazakii* cultures to  $2.0 \log_{10}$  CFU/ml and to an undetectable level, respectively, following a 1-h incubation at 37°C. The authors suggested that monocaprylin could potentially be used to inactivate *C. sakazakii* in reconstituted infant formula. The addition of monocaprylin (5.5 mg/ml) or supplementation of formulas with caprylic acid, however, would decrease the flavour quality of the reconstituted formula, thus a combination of monocaprylin (0.5 mg/ml) and increased temperature (50°C) may be more acceptable. At this concentration, monocaprylin decreased the counts of viable *Cronobacter* spp. cells by two orders of magnitude, which may be sufficient to inhibit effectively *Cronobacter* growth if we consider that cronobacters are occasional contaminants present in milk powder at low concentrations (MULLANE *et al.* 2006).

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*Corresponding author:*

Prof. Ing. MILAN MAROUNEK, DrSc., Ústav živočišné fyziologie a genetiky AV ČR, v.v.i., Vídeňská 1083, 142 20 Praha 4, Česká republika  
tel. + 420 776 053 360, e-mail: marounek@iapg.cas.cz

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