Susceptibility of *Escherichia coli*, *Salmonella* sp. and *Clostridium perfringens* to organic acids and monolaurin

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ABSTRACT: The antimicrobial activity of fatty acids, monolaurin, citric, succinic, fumaric, malic and lactic acid was determined in cultures of two strains of *Escherichia coli*, three strains of *Salmonella* sp. and two strains of *Clostridium perfringens*. Antimicrobial activity was expressed as minimum inhibitory concentration (MIC) that prevented growth and glucose utilization in treated cultures. Caprylic acid was the only acid inhibiting glucose utilization in all cultures. Its MIC varied from 1 to 3 mg/ml. Strains CCM 3954 and CCM 4225 of *E. coli* were inhibited also by capric acid at 5 mg/ml. Strains CCM 4435^T and CNCTC 5459 of *Cl. perfringens* were inhibited by medium-chain fatty acids (C_8 to C_{14}), oleic acid and one strain also by linoleic acid. The minimum MICs were those of lauric and myristic acid (between 0.1 and 0.2 mg/ml). Growth of *Cl. perfringens*, but not other bacteria, was inhibited also by monoglyceride of lauric acid (MIC = 3 mg/ml), and by citric acid (MIC = 4 mg/ml). Inhibitory effects of other acids were not observed at 5 mg/ml. Caprylic and lauric acid did not influence the K^+ permeability of the cytoplasmic membrane in cells of *E. coli* CCM 4225 and *Cl. perfringens* CCM 4435^T, respectively. In cultures of both strains of *E. coli* treated with caprylic acid at 5 mg/ml, and in those of *Cl. perfringens* CCM 4435^T treated with lauric acid at 1 mg/ml, or with its monoglyceride at 5 mg/ml, the transmission electron microscopy revealed damage of cytoplasmatic structures. In cells of *Cl. perfringens* the separation of inner and outer membranes was apparent, the integrity of the outer membrane, however, was maintained. It can be concluded that medium-chain fatty acids are more efficient antimicrobials than other, more polar organic acids tested.

Keywords: fatty acids; monolaurin; enteropathogenic bacteria; inhibition

Organic acids are among the candidate replacements for in-feed antibiotics. Some of the acids (acetic, lactic, benzoic, sorbic) have a long history of use in the food industry as food preservatives. Formic and propionic acid have been used in preservation of feeds, protecting them from microbial deterioration. Formic, fumaric and citric acid have a beneficial effect on growth and feed-to-gain ratio in weaned piglets and fattening pigs (reviewed by Partanen and Mroz, 1999). There are also studies, though less numerous, testing performance effects of feeding propionic, malic, sorbic, tartaric, lactic and formic acid in poultry (reviewed by Dibner and Buttin, 2002). In beef cattle, malic and fumaric

Supported by the Ministry of Agriculture of the Czech Republic (Project No. QF 3134) and Academy of Sciences of the Czech Republic (Projects No. S5045317 and No. AVOZ 50450515).

acid are considered as a substitute for monensin to prevent subacute acidosis in feedlots (Castillo et al., 2004). Other potential feed additives are succinic and pyruvic acid. Succinic acid, when added to lactating cows, was decarboxylated by rumen microbes to propionate, which is an important substrate for gluconeogenesis (Fisher et al., 1971). The increase of propionate production is a major effect of antibiotic feed additives in the rumen (Parker and Armstrong, 1987). In rabbits, caprylic acid had no effect on the rate of growth, but decreased mortality in the post-weaning period (Skrivanova and Marounek, 2002). Caprylic acid belongs to medium-chain fatty acids (MCFA) containing 8 to 14 carbon atoms, the antimicrobial activity of which has been known for a long time (Hassinen et al., 1951; Nieman, 1954).

The mode of action of organic acids in the animal digestive tract is not fully understood. It is known that their activity is related to the reduction of pH, as well as the ability to dissociate. The lowering of dietary pH alone, however, failed to show any nutritive efficacy. Thus, it seems that the antimicrobial properties of organic acids are of pivotal importance for their beneficial effects (Roth and Kirchgessner, 1998). It has been assumed that undissociated forms of organic acids penetrate the lipid membrane of the bacterial cell and dissociate within the cell. As bacteria maintain a neutral pH of the cytoplasm, the export of excess protons consumes cellular ATP and results in depletion of energy (Ricke, 2003). Unlike antibiotics, the antimicrobial activity of organic acids is thus pH dependent. At low pH, more of the organic acid will be in the undissociated form. Consequently, antimicrobial activity of organic acids is indisputable at low pH, but uncertain at pH above 6. In cultures of *E. coli* treated with caprylic acid at pH 5.2 the number of viable cells decreased to $\approx 10^2$ per ml. A reduction of between 0.94 and 1.96 $\log_{10}$ colony forming units was observed at pH 6.5 or 6.6 (Marounek et al., 2003). Similarly, incubation of *Salmonella* sp. with caprylic acid at pH between 5.2 and 5.3 led to a reduction in the concentration of viable cells below the detection limit, but between 2 and 6 percent of *Salmonella* sp. cells survived at pH between 6.3 and 6.6 (Skrivanova et al., 2004a). The aim of this study was to compare antimicrobial activity of C_2 to C_{18} fatty acids with that of citric, succinic, fumaric, malic and lactic at pH near to 6, i.e. at low acidity typical for sections of the animal digestive tract other than stomach. Strains of *E. coli*, *Salmonella* sp. and *Cl. perfringens* were used. Monolaurin (an ester of glycerol and lauric acid) was also included into our study. The microbicidal effects of fatty acids and their monoglycerides have been studied extensively in recent years (reviewed by Thormar and Bergsson, 2001).

MATERIAL AND METHODS

E. coli strains CCM 3954 (ATCC 25922) and CCM 4225 (ATCC 35218), and *Cl. perfringens* CCM 4435^T were obtained from the Czech Collection of Microorganisms (Brno, Czech Republic). *Cl. perfringens* CNCTC 5459 was obtained from the culture collection of the National Institute of Public Health (Prague, Czech Republic), *Salmonella enteritidis* ATCC 13076 from the American Type Culture Collection, *Salmonella infantis* K2 and *Salmonella typhimurium* K3 from the Institute of Chemical Technology (Prague, Czech Republic). Bacteria were grown in a medium containing (in g/l) glucose: 5, bactopeptone: 6, yeast extract: 3, $K_2HPO_4.3H_2O$: 5.9, KH_2PO_4: 4.5, $NaHCO_3$: 3.0, $(NH_4)_2S0_4$: 2.9, NaCl: 0.9, $MgSO_4.7H_2O$: 0.09, $CaCl_2$: 0.09. The medium was dispensed into gas-tight glass flasks containing acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic, caprylic, lauric, myristic, palmitic, stearic, oleic, linoleic, citric, succinic, fumaric, malic and lactic acid at 0, 0.1, 0.2, 0.5, 1, 2, 3, 4 and 5 mg/ml, together with an equivalent amount of NaOH. Flasks with monolaurin were prepared in the same way, except that NaOH was not added. Monolaurin was purchased from Med-Chem Labs (Galena, IL, USA), other antimicrobials from Sigma-Aldrich (St. Louis, MO). Flasks were filled with CO_2, closed with rubber stoppers and autoclaved at 110°C for 45 minutes. Inoculated cultures were incubated in triplicate at 37°C for 1 day. Then the cultures were examined for visible growth and pH was measured. The minimum inhibitory concentration (MIC) was the minimum concentration of an acid that prevented the visible growth and pH drop in treated cultures (Brander, 1982). Media with fatty acids containing more than 8 carbon atoms were turbid, thus residual glucose was determined using a commercial kit from Pliva-Lachema (Brno, Czech Republic). The MIC was the minimum concentration of fatty acids that prevented glucose utilization in treated cultures.

To assess the effect of fatty acids on inner membrane permeability, the potassium efflux from cells

of *E. coli* CCM 4225 and *Cl. perfringens* CCM 4435^T was measured by means of the K^+ ion-selective electrode 20-19+ and AgCl reference electrode 10-251 (2Theta, Ceský Tesin, Czech Republic), connected to a pH/mV meter PHI 04 (Labio, Prague, Czech Republic). A procedure of Ohmizo et al. (2004) with a few modifications was used. Bacterial cells were harvested in the exponential phase of growth by centrifugation at 3 000 g for 20 min (centrifuge K-23, Janetzki, Germany). Cells were washed three times with a buffer containing 0.1M choline chloride and 0.05M 4-morpholinepropanesulphonic acid-Tris(hydroxymethyl)aminomethane (MOPS-Tris), pH = 7.2. Washed cells were suspended in this buffer at 10 mg of cell protein per ml. The cell suspension was diluted in an assay solution containing 0.1M choline chloride, 0.01M Tris-lactate and 0.05M MOPS-Tris (pH = 7.2). The final volume was 50 ml and the final concentration of protein was 0.5 mg/ml. The cell suspension was constantly stirred by a magnetic stirrer. Fatty acids were added at 5 mg/ml (final concentration) as dimethylsulfoxide (DMSO) solutions. *E. coli* was treated with caprylic acid, *Cl. perfringens* with lauric acid. To disrupt the outer membrane of *E. coli*, EDTA at 0.1mM (final concentration) was added. Cetyltrimethylammonium bromide (CTAB) at 0.25 mg/ml was used as a positive control, to determine the 100% level of K^+ efflux from bacteria (Yasuda et al., 2003).

The effect of caprylic acid on strains of *E. coli* and that of lauric acid and its monoglyceride on *Cl. perfringens* CCM 4435^T was studied by transmission electron microscopy. Lauric acid was added to the overnight culture of *Cl. perfringens* CCM 4435^T at 1 mg/ml (as DMSO solution), and incubated at 37°C for 30 minutes. Monolaurin was added as DMSO solution at 5 mg/ml. The control received an equivalent amount of DMSO. One ml samples were removed and centrifuged at 1 800 g for 2 min (centrifuge Hawk 15/05, MSE, UK). The pellet was fixed 1 h by 0.48 ml of 0.5M glutaraldehyde in 0.2M Na-cacodylate buffer (pH 7.4) and 0.48 ml of a solution of ruthenium red (1.5 mg/ml in 0.2M Na-cacodylate buffer). After fixation the sample was centrifuged at 1 800 g for 2 min, the pellet washed three times in 0.1M Na-cacodylate buffer (pH 7.4) and fixed for 4 h by 0.4 ml of 0.15M OsO_4 in 0.2M cacodylate buffer and 0.4 ml of ruthenium red solution (1.5 mg/ml 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 2 mm size were cut by a blade after congelation, dehydrated in a graded series of ethanol, then passed through propylene oxide and embedded in PolyBed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut with an ultramicrotome and examined in a Philips CM 100 electron microscope (Royal Philips Electronics, The Netherlands), equipped with slow scan digital camera Mega View II (Sis, GmbH) at 80 kV.

RESULTS

In control cultures and those containing organic acids at sub-inhibitory concentrations the pH de-

Table 1. Minimum inhibitory concentrations (mg/ml) of C_2 to C_{18} fatty acids against *E. coli*, *Salmonella* sp. and *Cl. perfringens* grown on glucose. Inoculated cultures were incubated in triplicate for 1 day

Fatty acid	*E. coli*		*S. enteritidis*	*S. infantis*	*S. typhimurium*	*Cl. perfringes*	
	CCM 3954	CCM 4225	ATCC 13076	K2	K3	CCM 4435^T	CNCTC 5459
C_2 to C_6	r	r	r	r	r	r	r
C_8	2	2	3	3	3	2	1
C_{10}	5	5	r	r	r	1	1
C_{12}	r	r	r	r	r	0.1	0.2
C_{14}	r	r	r	r	r	0.1	0.2
C_{16},C_{18}	r	r	r	r	r	r	r
$C_{18:1}$	r	r	r	r	r	2	3
$C_{18:2}$	r	r	r	r	r	r	2

r = resistant (MIC > 5 mg/ml)

Table 2. Minimum inhibitory concentrations (mg/ml) of monolaurin, citric, succinic, fumaric, malic and lactic acid against *E. coli*, *Salmonella* sp. and *Cl. perfringens* grown on glucose. Inoculated cultures were incubated in triplicate for 1 day

Compounds tested	*E. coli* (2 strains)	*Salmonella* sp. (3 species)	*Cl. perfringens* (2 strains)
Monolaurin	r	r	3
Citric acid	r	r	4
Succinic, fumaric, malic, lactic acid	r	r	r

r = resistant (MIC > 5 mg/ml)

creased from 6.7 at the beginning of the incubation to *ca* 5.6 at its end. The MIC of fatty acids at this pH against strains of *E. coli*, *Salmonella* sp. and *Cl. perfringens* are shown in Table 1. Caprylic acid (C_8) was the only acid inhibiting glucose utilization in all strains tested. Its inhibitory concentrations varied from 1 mg/ml (*Cl. perfringens* CNCTC 5459) to 3 mg/ml (*Salmonella* sp.). Caprylic acid was the only acid inhibiting *Salmonella* sp. Strains of *E. coli* were inhibited also by capric acid (C_{10}). Both strains of *Cl. perfringens* were susceptible to caprylic, capric, lauric (C_{12}), myristic (C_{14}) and oleic acid ($C_{18:1}$), strain CNCTC 5459 also to linoleic acid ($C_{18:2}$). The minimum inhibitory concentrations were those of lauric and myristic acid (0.1 or 0.2 mg/ml). Growth of *Cl. perfringens*, but not other bacteria, was inhibited by monolaurin at 3 mg/ml (Table 2). Both strains of *Cl. perfringens* were susceptible to citric acid at 4 mg/ml, but not to succinic, fumaric, malic and lactic acid. None of these acids influenced growth of *E. coli* strains and *Salmonella* sp.

Figure 1 shows the effect of caprylic and lauric acid on efflux of K^+ ions from cells of *E. coli* CCM 4225 and *Cl. perfringens* CCM 4435 ^{T}, respectively. Potassium ions were released by action of CTAB, however, no K^+ efflux from cells treated with caprylic and capric acid occurred. Figure 2 illustrates morphological changes in *E. coli* strains CCM 3954 and CCM 4225 after 30 min incubation of cells with caprylic acid at 5 mg/ml. Control (untreated) cells are shown in Figures 2A and 2B. Figures 3A and 3B present longitudinal and diagonal cut of cells of *Cl. perfringens* CCM 4435^T, respectively. Figure 3C shows morphological changes of cells of *Cl. perfringens* following 30 min of incubation with lauric acid at 1 mg/ml. Figure 3D shows cells treated with monolaurin at 5 mg/ml. In all treated cells the cytoplasma was disorganized. In treated cells of *Cl. perfringens* CCM 4435^T inner and outer membranes were separated, the integrity of membranes, however, was maintained.

DISCUSSION

Effect of organic acids was more pronounced in cultures of *Cl. perfringens*, which is a Gram positive bacterium, than in *E. coli* and *Salmonella* sp.,

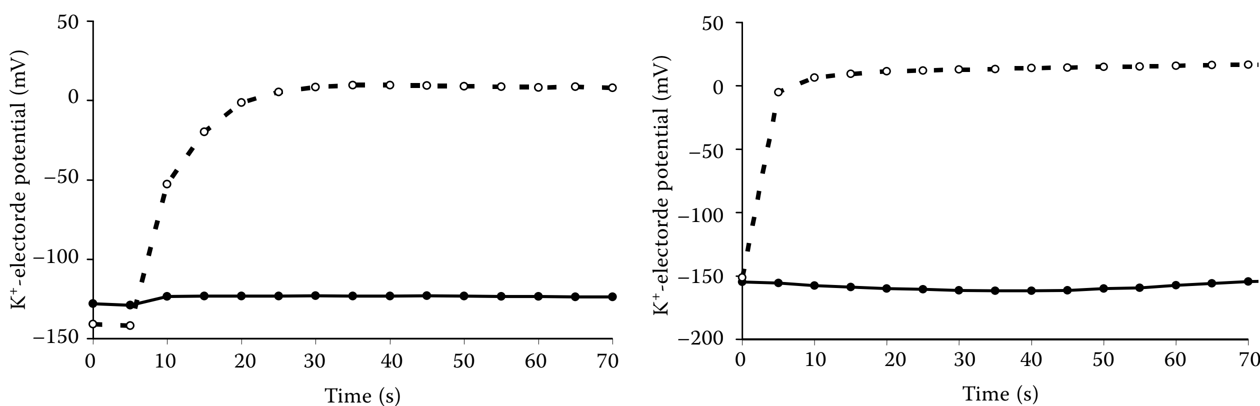

Figure 1. Efflux of K^+ from cells of *E. coli* CCM 4225 (left) and *Cl. perfringens* CCM 4435 (right) monitored by K^+ ion-selective electrode. Cells were treated with caprylic acid (strain 4225) and lauric acid (strain 4435^T). Dashed lines show electrode response in suspension of cells treated with CTAB

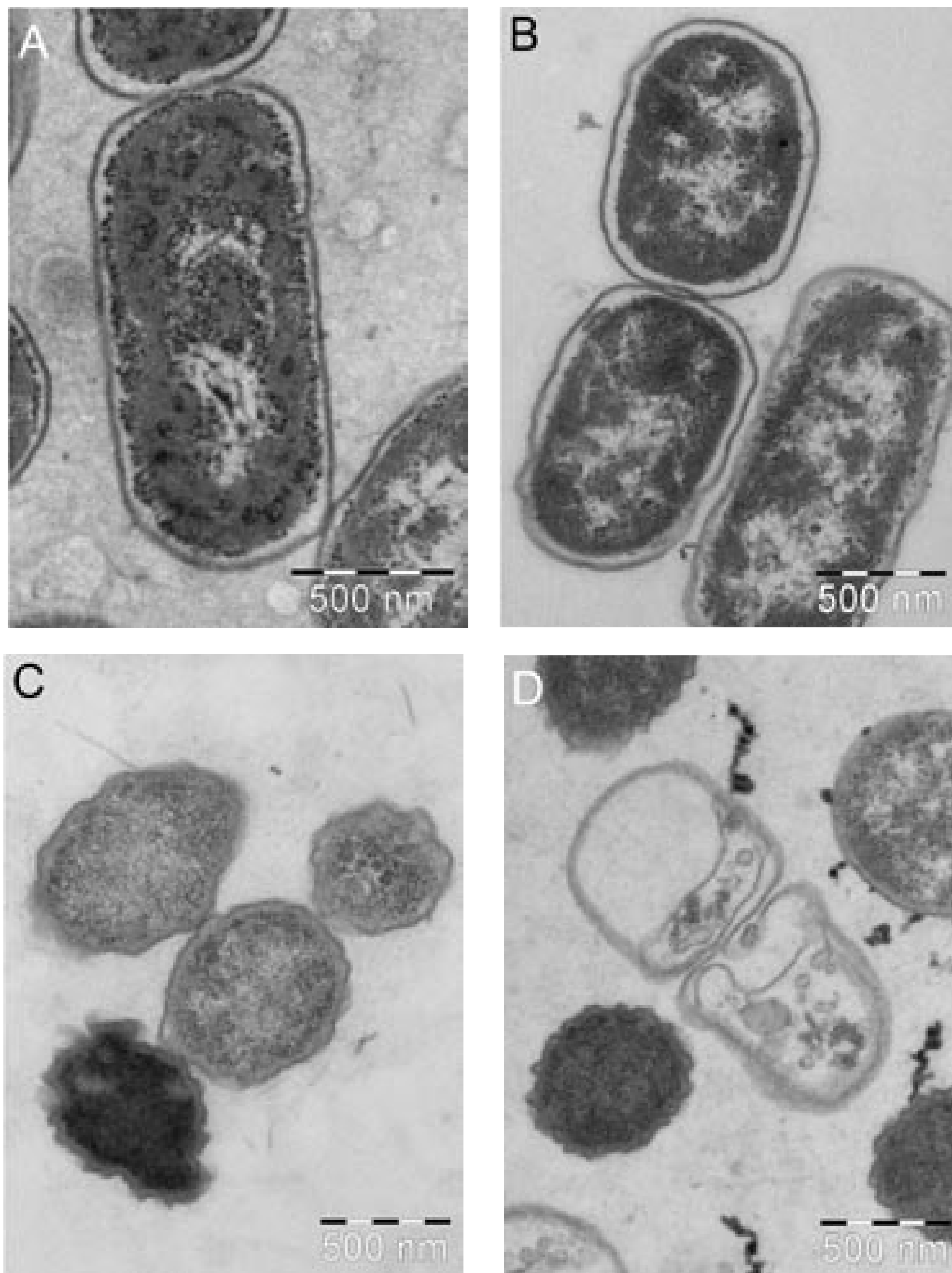

Figure 2. Effect of caprylic acid on the ultrastructure of *E. coli* CCM 3954 (A, C) and CCM 4225 (B, D) as demonstrated by transmission electron microscopy

A, B = control samples; C, D = cells treated with caprylic acid at 5 mg/ml for 30 min

which are Gram negative bacteria belonging to Enterobacteriaceae. This is not surprising as Gram positive bacteria are more susceptible to the action of compounds interfering with the transport of ions across the cell membrane (Nagaraja, 1995). It is known that most of antimicrobial feed additives are substances active against Gram positive bacteria (Brander, 1982). Lactic acid, di- and tricarboxylic acids tested did not inhibit growth of bacteria, with exception of a weak effect of citric acid (MIC of 4 mg/ml) against *Cl. perfringens*. The reason was probably a limited ability of these acids to penetrate bacterial cell wall at pH around 6. The pK_as of the acids (the pH at which the acid is half dissociated) varies between 3 and 4 (Dibner and Buttin, 2002), whereas pK_as of caprylic and capric acid are 4.88

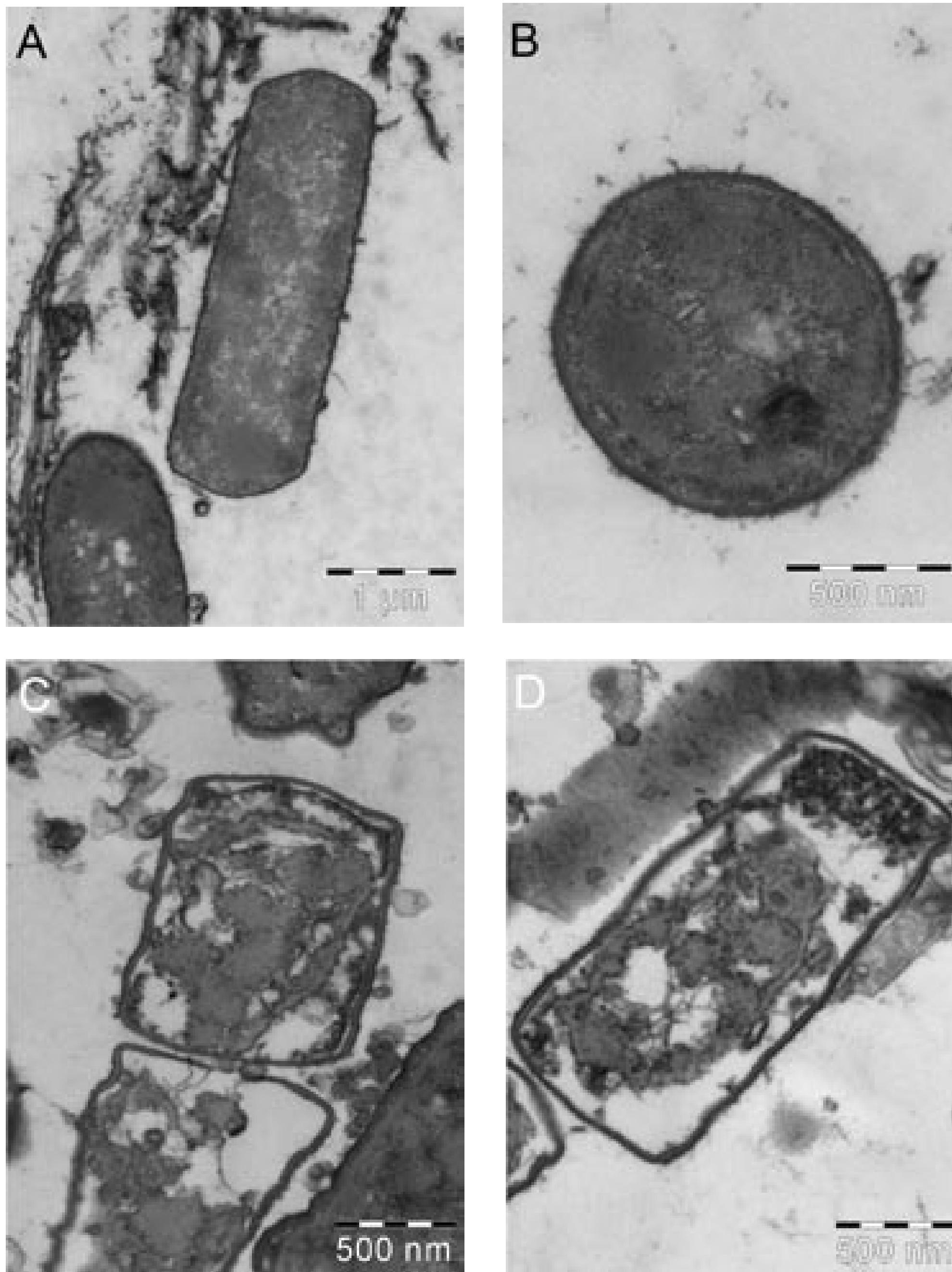

Figure 3. Effect of lauric acid and its monoglyceride on the ultrastructure of *Cl. perfringens* CCM 4435^{T} as demostrated by transmission electron microscopy

A, B = control samples; C = a cell treated with lauric acid at 1 mg/ml for 30 min; D = cells treated with monolaurin at 5 mg/ml

and 4.90, respectively (Freese et al., 1973). Contrary to MCFA, lactic acid and di- and tricarboxylic acids thus may exert antimicrobial effect in the gizzard of poultry and the stomach of pigs, but not in other parts of the digestive tract, or in the feed. In the small intestine, however, the antimicrobial effect of MCFA is restricted by their rapid absorption. Triacylglycerols of MCFA thus may be a suitable alternative to free MCFA as shown by Dierick et al. (2002) and Skrivanova et al. (2004b), supposing that the digesta lipolytic activty is high enough or exogenous lipase is added to the feed.

Traditional explanation of toxicity of organic acids (i.e. that based on export of protons and depletion of energy of microbial cells) does not explain antimicrobial activity of fatty acid derivatives. Antimicrobial activity of monolaurin, a lipophilic substance, suggests that mechanisms other than the release of protons inside cells exist. In experiments of Razavirohani and Griffiths (1994) monolaurin was effective against all Gram positive bacteria studied, but was only effective against Gram negative bacteria in the presence of EDTA. Various authors have hypothesized that organic acids may damage outer or cytoplasmic membrane, hinder macromolecular synthesis or denature proteins and DNA (reviewed by Ricke, 2003). Effect of monolaurin may be similar. The increase of 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). In our study bacteria maintained integrity of membranes in cells treated with caprylic and lauric acid or monolaurin. The increase in K^+ permeability of the cytoplasmic membrane, which usually leads to dissipation of membrane potential (Katsu et al., 1984), was not observed.

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Received: 2005–04–01
Accepted after corrections: 2006–03–03

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