Influence of pH on Antimicrobial Activity of Organic Acids against Rabbit Enteropathogenic Strain of *Escherichia coli* *

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ABSTRACT. Susceptibility of the rabbit enteropathogenic strain *Escherichia coli* C6 (O128 serogroup) to C_6–C_{14} fatty acids, oleic, citric, lactic and fumaric acid at 5 mg/mL was determined by the plating technique in the near-neutral pH region (pH ≈ 6.5), and in a weakly acid and acid environment (pH 5.4 ± 0.1 and 2.2–2.5, respectively). In the near-neutral pH region caproic and caprylic acid reduced the concentration of viable cells by 3 and 6 orders, respectively. At lower pH the bactericidal effect of caproic acid remained similar, but caprylic acid decreased the concentration of viable cells to <100/mL. The bactericidal activity of capric acid was low at pH 6.5 but increased at pH 5.3. High environmental acidity was intrinsically bactericidal and at very low pH the effects of fatty acids were thus less pronounced. Citric acid reduced the counts of viable cells to 1/10. Antimicrobial activity of other acids examined was marginal or absent. Medium-chain fatty acids, caprylic and, to a lesser extent, also caproic and capric acid were better antimicrobials than other organic acids examined; the antimicrobial activity of fatty acids toward the C6 strain was pH-dependent. Beneficial effects of citric, lactic and fumaric acid reported by animal nutritionists are thus probably related to factors other than their direct antimicrobial action.

Coliform bacteria (mainly *Escherichia coli*) are normal inhabitants of the intestinal tract of many animal species. In the intestine of healthy rabbits their counts are low, 10^2–10^4 per g intestinal contents. In rabbits with enteritis, however, the concentration of *E. coli* exceeds 10^8/g (Cortez *et al.* 1992). Digestive problems caused by enteropathogenic *E. coli* strains are often responsible for high morbidity and mortality of young rabbits after weaning, and consequently for important economic losses in rabbit farms (Licois 2004). Antibiotics are routinely used to prevent enteritis infections, but approvals for their routine application are disappearing. Among the candidate replacements for antibiotics are organic acids.

Organic acids have been used for decades as food and feed preservatives. Some of them (formic, fumaric, citric) positively influence the growth and feed conversion in piglets (Partanen and Mroz 1999). The growth-inhibitory activity of linoleic and oleic acid was also shown in some rumen bacteria (Koppová *et al.* 2006).

Several studies report the inhibitory effect of organic acids against *E. coli* – saturated fatty acids (Hassinen *et al.* 1951), formic and propionic acid (Cherrington *et al*. 1990), lactic acid (Dibner and Buttin 2002), and medium-chain fatty acids (Marounek *et al*. 2003); in the latter studies the authors showed that the antimicrobial effect of organic acids toward *E. coli* was pH-dependent. At low pH, more of the organic acid will be in the undissociated form. Undissociated organic acids are lipophilic and can diffuse across the cell membrane. Once in the bacterial cell they dissociate at the pH of the cytoplasm (>7) causing metabolic uncoupling (Kashket 1987).

The aim of this study was to determine the antimicrobial activity of six medium-chain fatty acids (MCFA) against enteropathogenic *E. coli* strain and compare it with that of citric, lactic and fumaric acid at different pH.

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MATERIAL AND METHODS

Strain C6 of *E. coli* serogroup O128 was a gift of Dr. A. Milon (*Ecole Nationale Vétérinaire*, Toulouse, France). The strain is rhamnose-positive, AF/R2-positive, and causes losses of body mass and mild diarrhoea in 30 d-old rabbits when given at 10^7 CFU *per os*. *E. coli* C6 was grown in Wilkins–Chalgren broth (*Oxoid*), and maintained in 20 % glycerol (*V*/*V*) at –40 °C. Caproic (C_6), caprylic (C_8), capric (C_{10}), lauric (C_{12}), myristic (C_{14}), oleic ($C_{18:1}$) acids, citric, lactic and fumaric acids were added to overnight-grown cultures at 5 mg/mL. Acids insoluble in water were added as 20 % dimethylsulfoxide solutions; controls received an equivalent amount of the solvent. The pH of control and treated cultures was adjusted to *c.* 6.5, 5.4 and 2.4 by addition of NaOH or HCl. The cultures were incubated in a water bath for 30 min at 37 °C, then samples were removed and serially diluted with the Wilkins–Chalgren broth. The number of viable bacteria was determined by streaking 0.1 mL of an appropriate dilution on three Wilkins–Chalgren agar plates using sterile glass rods. Inoculated plates were incubated for 1 d at 37 °C. Colonies were counted and means and SD calculated. Fatty acids and other compounds were tested in separate trials. The significance of differences between treated and control cultures was evaluated by the *t*-test.

RESULTS

In the near-neutral pH region (6.47 ± 0.13) caproic and caprylic acids reduced the concentration of viable cells of *E. coli* C6 by 3 and 6 orders of magnitude, respectively (Table I). A reduction of a mere 1.83 $\log_{10}$ CFU was observed in cultures treated with capric acid. At lower pH (5.36 ± 0.12) caprylic acid decreased the concentration of viable cells below the detection limit (100/mL). Caproic and capric acids decreased the concentration of viable cells by 4 and 7 orders of magnitude, respectively. In an acid environment (pH 2.35 ± 0.17) the concentration of viable cells in control cultures decreased to 10^3–10^4/mL, and in those treated with caprylic, capric and lauric acids below the detection limit. The bactericidal effects of myristic and oleic acids were significant, but weak, at pH < 6. Myristic acid reduced the number of viable cells by 0.6–0.7 $\log_{10}$ CFU ($p < 0.02$) at pH < 6.

Citric acid significantly reduced the concentration of viable cells of *E. coli* C6 to 1⁄10 ($p < 10^{-4}$), both in the near-neutral pH region (6.51 ± 0.14) and in the weakly acidic environment (pH 5.47 ± 0.05). Lactic and fumaric acid had no bactericidal effect. The concentration of viable cells decreased at low pH (2.48 ± 0.09) both in treated and control cultures to *c.* 200/mL.

DISCUSSION

Capric and lauric acid inhibited the growth of an *E. coli* strain in the study of Hassinen *et al*. (1951), and caprylic and capric acid in our previous experiment with two culture collection *E. coli* strains (Marounek *et al*. 2003). We showed that the bactericidal effect of caprylic acid was more pronounced than that of caproic and capric acid which showed that *E. coli* strains are susceptible to MCFA with 6–12 carbon atoms, but a strain-to-strain variability exists in the susceptibility to individual MCFA. The bactericidal effect of lauric, myristic, oleic, citric, lactic and fumaric acids was weak or absent (*see* Table I). Beneficial effects of citric, lactic and fumaric acids reported by animal researchers thus may be attributed to digesta acidification, increased pancreatic secretion and trophic effect on the mucosa (Dibner and Buttin 2002), rather than to a direct antibacterial activity. The antimicrobial activity of capric, caprylic and capric acids was pH-dependent, being higher at lower pH than at pH > 6. At low pH (<2.5) the survival of *E. coli* strains was negligible, both in treated and in control cultures. Neutrophiles like *E. coli* cannot grow at a low intracellular pH. Several mechanisms of acid resistance have been identified in *E. coli* (reviewed by Bearson *et al.* 1997). Synthesis of decarboxylases of arginine and glutamate enables reactions consuming protons. Synthesis of a number of acid stress proteins provides tools that repair damaged macromolecules. Acid resistance increases in bacteria grown in a complex medium, which was the case of the present experiment. In young rabbits, however, the gastric pH is high (≈5; Aboul-Ela *et al.* 2000), and the infection dose of enteropathogenic *E. coli* is rather low (Lin *et al.* 1996). Suckling rabbits are supplied with antimicrobial compounds present in the doe milk (antimicrobial lipids, immunoglobulins, oligosaccharides, lactoferrin). The supply of antimicrobials ceases after weaning. Here we showed that caprylic acid inactivated *E. coli* C6 cells in a wide range of pH. Thus, we suppose that caprylic acid, and to a lesser extent also other MCFA or oils containing MCFA, may decrease the risk of coliform infections when added to diets of weaned rabbits.

Table I. Cell concentration ($\log_{10}$ CFU/mL) of *Escherichia coli* strain C6 determined by plating technique after a 30-min incubation with caproic (C_6), caprylic (C_8), capric (C_{10}), lauric (C_{12}), myristic (C_{14}) or oleic ($C_{18:1}$) acid, or with lactic (Lct), citric (Cit) or fumaric (Fum) acid (all at concentration of 5 mg/mL)

Control cultures		Treated cultures		
pH	$\log_{10}$ CFU/mL	effector	pH	$\log_{10}$ CFU/mL
2.40	4.12 ± 0.24	C_6	2.41	3.70 ± 0.16
5.24	9.19 ± 0.13		5.54	4.94 ± 0.05
6.40	10.82 ± 0.13		6.40	5.67 ± 0.26
2.19	4.27 ± 0.17	C_8	2.11	<2
5.26	11.47 ± 0.15		5.36	<2
6.38	11.74 ± 0.11		6.54	5.83 ± 0.05
2.21	3.49 ± 0.27	C_{10}	2.22	<2
5.28	9.66 ± 0.13		5.25	2.76 ± 0.39
6.47	11.75 ± 0.18		6.51	9.92 ± 0.08
2.19	3.15 ± 0.13	C_{12}	2.23	<2
5.42	11.44 ± 0.16		5.51	10.74 ± 0.02
6.62	11.61 ± 0.03		6.59	10.83 ± 0.06
2.19	3.40 ± 0.07	C_{14}	2.23	2.69 ± 0.19
5.24	10.30 ± 0.11		5.38	9.69 ± 0.09
6.52	10.59 ± 0.09		6.54	10.52 ± 0.15
2.35	4.63 ± 0.23	$C_{18:1}$	2.52	3.12 ± 0.15
5.27	10.89 ± 0.10		5.78	10.46 ± 0.14
6.31	11.83 ± 0.09		6.57	11.57 ± 0.11
2.46	<2	Lct	2.54	< 2
5.47	9.79 ± 0.09		5.43	9.06 ± 0.52
6.33	8.95 ± 0.68		6.65	8.99 ± 0.69
2.48	<2	Cit	2.48	<2
5.56	9.53 ± 0.10		5.47	8.53 ± 0.05
6.42	9.66 ± 0.09		6.51	8.57 ± 0.04
2.68	<2	Fum	2.55	<2
5.46	9.07 ± 0.63		5.44	9.00 ± 0.66
6.68	9.38 ± 0.52		6.42	9.35 ± 0.51

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