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1 Inhibitory activity of rabbit milk and medium-chain fatty
2 acids against enteropathogenic *Escherichia coli* O128

3 Eva Skřivanová, Zuzana Molatová, Věra Skřivanová, Milan Marounek*

4 *Institute of Animal Science, Prague, Přátelství 815, CZ-104 00, Czech Republic*

5 * Corresponding author. Tel.: +420 776053360; fax: +420 267090500.

6 *E-mail address:* marounek.milan@vuzv.cz (M. Marounek)

7

8 Abstract

9 Colibacillosis represents a major cause of diarrhea in young rabbits. In order to elucidate
10 protective effect of milk, *in vitro* and *in vivo* experiments were carried out. In the *in vitro* experiment,
11 rabbit milk treated with lipase significantly decreased the number of viable cells in cultures of
12 *Escherichia coli*, O128 serotype, from 10.3 log₁₀ cfu/mL to 6.2 - 7.3 log₁₀ cfu/mL. The lipase effect
13 was the same with heat-treated (100°C/10min) and raw milk. Raw milk without lipase decreased the
14 number of *E. coli* only marginally. In the *in vivo* experiment, weaned rabbits received feed
15 contaminated with the same bacterium. The course of the infection was moderate, only 2 out of 36
16 infected rabbits died. Seven days after inoculation, caprylic acid at 5g/kg feed and triacylglycerols of
17 caprylic and capric acid at 10 g/kg feed decreased faecal output of *E. coli* from 10.2 log₁₀ cfu/g to 5.8
18 and 6.1 log₁₀ cfu/g, respectively. The number of *E. coli* in faeces of non-infected rabbits averaged 4.0
19 log₁₀ cfu/g. The growth of infected rabbits was slow for two weeks after infection. In the third week a
20 compensatory growth was apparent. At the end of the experiment, average body weights of rabbits
21 receiving caprylic acid and those of non-infected rabbits were not significantly different. It can be
22 concluded that (i) lipids rather than proteins seem to be responsible for the antimicrobial activity of
23 rabbit milk; and (ii) this activity was lipase-dependent. Caprylic acid or oils with high a concentration
24 of it may be used as feed supplements for weanlings.

25 *Keywords:* Rabbit milk; Milk fat; Antimicrobial activity; *Escherichia coli*; EPEC

26 1. Introduction

27 Milk contains several antimicrobial factors which contribute to the protection of neonates
28 against infectious diseases. This activity has been attributed both to proteins
29 (immunoglobulins, lactoferrin, lactoperoxidase, lysozyme) and non-protein compounds
30 (fucosylated oligosaccharides and antimicrobial lipids). Mammalian species differ in the
31 manner of protection of their young against microbial pathogens. In some mammals newborns

32 receive immunoglobulins mainly via colostrum (ungulates), whereas others mainly in utero
33 (humans, rabbits, guinea-pigs). Concentration of immunoglobulins in mature milk, however,
34 is much lower than in the colostrum (Cowie et al., 1980), thus other antimicrobials have to
35 fulfill the need for protection of offspring against infection. Rabbit milk contains
36 triacylglycerols (TAG) of medium-chain fatty acids (MCFA), which represent about one half
37 of the TAG in milk (Lebas et al., 1996). The principal MCFA in rabbit milk is caprylic (C8:0)
38 and capric (C10:0) acid. The antimicrobial activity of MCFA has long been known (Hassinen
39 et al., 1951; Nieman, 1954). Saturated MCFA dissipate the electrochemical proton gradient
40 and deplete the energy reserves of bacterial cells (Galbraith and Miller, 1973). Several studies
41 showed the bactericidal activity of digestion products released from rabbit and bovine milk fat
42 (Cañas-Rodríguez and Smith, 1966; Sprong et al., 1999, 2001, 2002; Sun et al., 2002, 2007).

43 The purpose of this study was to test the hypothesis that (i) a significant part of the
44 antibacterial activity of rabbit milk is derived from the MCFA in its lipid fraction; and (ii)
45 caprylic acid and TAG of caprylic and capric acid added to the feed may improve the
46 resistance of weaned rabbits to colibacillosis. As far as we know, the *in vitro* experiments
47 described below (effect of lipase and heat treatment of rabbit milk) were not carried out
48 before. The EPEC strain of the O128 serotype was used for *in vitro* and *in vivo* experiments.

49 2. Materials and methods

50 2.1. *E. coli* strain

51 The strain C6 of *E. coli* O128 is rhamnase and AF/R2 positive, only weakly pathogenic.
52 Use of 10^7 CFU *per os* produces weight losses and some cases of mild diarrhea in 30 days old
53 rabbits. This strain was isolated from the caecal contents of diarrheic rabbits (Canguilhem
54 and Milon, 1989) and maintained in 20% glycerol (v/v) at -70°C . The strain E22 of *E. coli*
55 O103, used in our previous study (Skřivanová et al., 2008), is rhamnase negative, LEE (eae)

56 and CIF positive, and highly pathogenic for rabbits. Its LD₅₀ is about 10⁴ CFU when given *per*
57 *os* to 30 days old rabbits.

58 2.2. *In vitro* antibacterial activity of rabbit milk

59 Five does (Hyplus breed), 7 days after parturition, were milked after intramuscular
60 injection with 1.5 IU of oxytocin. After milking, the samples were transported to the
61 laboratory on ice and pooled. A portion of milk was frozen for analyses. The remaining milk
62 was divided into two parts: control (A), and heat-treated at 100°C for 10 min (B). Milk A and
63 milk B were added to a glucose medium (Skřivanová et al., 2004) at a 1 : 9 or 1 : 4 ratio, with
64 final pH 7.0. Lipase from *Candida rugosa* (Sigma, cat. no. L 1754) was added at 5 mg to 10
65 ml samples. The product activity was 901 U/mg. The medium with milk and lipase was
66 incubated for 2 h at 37°C to release fatty acids from the milk lipids. Afterwards the *E. coli*
67 O128 cultured overnight was added at 0.5 ml. The overnight culture *E. coli* O128 was also
68 added to the same medium without milk and lipase, to the medium without milk, but with
69 lipase, and to the medium with milk, but without lipase. Samples were incubated aerobically
70 18 h at 37°C, then serially diluted by sterile saline and plated on TBX agar (Oxoid) plates.
71 The plates were incubated at 37°C for 24 h, and after that the colonies were counted.

72 2.3. *Experimental infection*

73 In this experiment 48 Hyplus rabbits of both sexes, weaned at 35 days of age, were used.
74 The rabbits were housed individually and randomly divided into four groups of 12 animals.
75 Rabbits of the 1st and 2nd group were fed a granulated feed, free of antimicrobials, containing
76 dry matter, crude protein, starch and acid-detergent fibre at 911, 177, 159 and 180 g/kg,
77 respectively. The feed was supplemented with rapeseed oil at 15 g/kg. The rabbits of the 3rd
78 group were fed the same diet supplemented with caprylic acid (Fluka, cat. no. 21650) at 5
79 g/kg. Those of the 4th group received the basal diet supplemented with Akomed R

80 (AarhusKarlshamn AB, Malmö, SE-21119 Sweden; local supplier AarhusKarlshamn Czech
81 Republic, Prague, CZ-111 21) at 10 g/kg. Akomed R is commercially available oil, containing
82 99% of TAG of caprylic and capric acid. Supplements were added at the expense of rapeseed
83 oil to the basal diet. Cages of the rabbits of the 1st group (negative control) were separated by
84 a plastic barrier from the other cages. The environmental temperature was maintained at $18 \pm$
85 2°C , with a lighting schedule 7:00 - 19:00. The experiment was performed in accordance with
86 the Czech law no. 207/2004 Sb. and approved by the Ethical Committee of the Institute of
87 Animal Science.

88 On the third day of the experiment, the feed of the rabbits of the 2nd, 3rd and 4th groups
89 was contaminated with 0.5 ml of an overnight culture of *E. coli* O128 strain C6 (ca 10^9
90 bacteria per rabbit). The rabbits were checked daily for morbidity and mortality, and their
91 weights were recorded once a week. Morbidity control was performed according to
92 Bennegadi et al. (2001). Feed consumption was recorded daily. Two, 5, 7, 9, 12 and 14 days
93 after infection, faecal samples of randomly selected rabbits (5 rabbits from each group) were
94 taken for microbiological analyses.

95 2.4. Analyses and statistics

96 Milk fat was extracted with 1: 1 diethyl ether-petrol ether according to ISO 1211 (2002)
97 and the extract was weighed. Alkaline trans-methylation of extracted fatty acids was carried
98 out according to ISO 5509 (2001). Gas chromatography of methyl esters was done as
99 described previously (Kudrna and Marounek, 2008). Ash was determined after burning at
100 550°C . Crude protein and lactose were determined in a commercial laboratory according to
101 ISO 1871 (1994) and AOAC 984.15 (1990).

102 The number of *E. coli* in faecal samples was determined by plating technique on TBX
103 plates. After incubation (aerobically, at 37°C for 24 h), the blue colonies of *E. coli* were

104 counted. Samples of the faeces were examined microscopically for the presence of coccidia in
105 accordance to Pavlasek (1991).

106 Two rabbits that died during the experiment were examined using standard pathological,
107 bacteriological and parasitological methods at the State Veterinary Institute (Prague). Other
108 rabbits were slaughtered at 55 days of age. Immediately after slaughter, the gastric and caecal
109 contents were removed and samples taken to determine the count of *E. coli* and of total
110 anaerobic bacteria by plating on TBX and Wilkins-Chalgren agar (Oxoid), respectively. The
111 inoculated plates were incubated at 37°C for 24h (TBX agar plates) or 48 h (Wilkins-
112 Chalgren agar plates). Those plates with Wilkins-Chalgren agar were incubated anaerobically.

113 Data were statistically analyzed using the GLM procedure of SAS, version 8.2 (SAS
114 Institute, Cary, NC, U.S.A.). The data on morbidity were analyzed using the Fisher's exact
115 test. To compare the average body weights, feed intake and numbers of total anaerobic and
116 coliform bacteria in the control and infected rabbits, one-way analysis of variance was
117 employed followed by the Tukey's test .

118 **3. Results**

119 *3.1. In vitro antibacterial activity of rabbit milk*

120 The rabbit milk contained fat, protein, ash and lactose at 120, 117, 20.4 and 18.3 mg per
121 g, respectively. Table 1 presents fatty acid composition of milk fat and MCFA oil used in *in*
122 *vivo* experiment. Rabbit milk added to a glucose medium significantly decreased the number
123 of *E. coli* O128 from 10.3 log₁₀ cfu/g by three to four orders of magnitude in samples with
124 added lipase. The lipase effect was the same with heat-treated and raw milk. When lipase was
125 not added, no significant change in *E. coli* number was observed (Table 2). Lipase itself had
126 no effect on *E. coli* numbers.

127

128 3.2. *In vivo experiment*

129 The course of the infection with the *E. coli* O128 strain C6 was moderate. The addition of
130 the pathogen to the feed caused diarrhea in six rabbits from the 2nd group, and two of these
131 animals died. Also one rabbit from the group receiving caprylic acid, and one rabbit from the
132 group receiving Akomed R, suffered from mild diarrhea (Fig. 1). The diarrhea lasted for two
133 or three days. No rabbit from the 1st, 3rd or the 4th group died. The differences in mortality and
134 morbidity between the experimental groups were not statistically significant. Necropsy of
135 both dead rabbits revealed colibacillosis. Sporadic occurrence of *Eimeria* sp. oocysts in faeces
136 was observed.

137 The growth of infected rabbits was slow for two weeks after infection. In the third week a
138 compensatory growth was apparent (Table 3). At the end of the experiment, body weights of
139 rabbits of the 2nd and the 4th group were significantly lower than body weights of non-infected
140 rabbits of the 1st group. Average body weights of rabbits receiving caprylic acid and those of
141 non-infected rabbits were not significantly different, except the first week after infection.
142 Infected rabbits consumed less feed than non-infected ones, the difference, however was
143 small and in rabbits receiving caprylic acid statistically nonsignificant.

144 In the non-infected rabbits the number of *E. coli* in the faeces was maintained at $10^3 - 10^4$
145 per g, whereas in the infected rabbits reached a maximum of 10^{10} per g in the 7th and the 9th
146 day post-infection. In both treated groups *E. coli* shedding decreased significantly, but
147 remained higher than in the non-infected rabbits until day 12 post-infection (Table 4).
148 Fourteen days after infection the number of *E. coli* in the caecal content of the rabbits of the
149 2nd group was significantly higher than in the non-infected rabbits (Table 5). Dietary
150 supplementation with caprylic acid and Akomed R reduced *E. coli* count in the caecal content
151 by three orders of magnitude. Counts of total anaerobic bacteria in the gastric and caecal
152 content, and the counts of *E. coli* in the gastric content were not significantly influenced.

153 **4. Discussion**

154 Gallois et al. (2007) reported that suckling temporarily reduced faecal *E. coli* excretion
155 and delayed the development of diarrhea in young rabbits infected with a highly virulent
156 EPEC strain E22 of the O103 serotype. In the next study Gallois et al. (2008) found a clear
157 antimicrobial effect of caprylic acid *in vitro*, however, TAG of MCFA, did not reduce the
158 mortality and faecal excretion of EPEC in young rabbits challenged with this strain. TAG are
159 not efficient antimicrobials (Kabara et al., 1972), and therefore lipolysis is necessary to
160 release MCFA from their ester bonds. The activity of the gastric lipase in rabbits is high
161 (Moreau et al., 1988), thus we assume that the TAG of MCFA are hydrolyzed in the first part
162 of the digestive tract. In piglets, exogenous lipase addition to TAG of MCFA was necessary
163 to obtain a significant suppression (>10-fold) of *E. coli* in the gastric contents (Dierick et al.,
164 2002). Insufficient gastric lipolytic activity may be the reason for variable results of MCFA-
165 containing oils in different experiments (Gallois et al., 2008 vs Skřivanová et al., 2008). The
166 results of the present *in vitro* experiment demonstrated that the antimicrobial activity of rabbit
167 milk was thermostable and lipase-dependent. Thus, TAG rather than proteins seem to be
168 responsible for a significant portion of the bactericidal activity of the rabbit milk. Fat of milk
169 used in the present study contained less MCFA than reported by Lebas et al. (1996), the fat
170 concentration in milk, however, was higher (0.12 vs 0.09 - 0.10 g per g). The amount of
171 MCFA reaching the caecum might be very low, thus we assume that the stomach is the site of
172 the antimicrobial action of MCFA against pathogenic bacteria entering the rabbit *per os*. The
173 antimicrobial activity of organic acids is pH-dependent, and a greater bactericidal effect of
174 MCFA against *E. coli* was observed in an acid environment than in the near-neutral pH area
175 (Skřivanová and Marounek, 2007). The growth rate was non-significantly higher and *E.coli*
176 faecal shedding lower in rabbits treated with caprylic acid than in those treated with Akomed
177 R. Our findings are in line with the fact that organic acids are the candidate replacements for

178 antibiotics (Ricke, 2003). In chickens experimentally infected with *Campylobacter jejuni*
179 caprylic acid supplemented in feed at 0.7% consistently reduced enteric *C. jejuni* counts (de
180 los Santos et al., 2008).

181 **5. Conclusion**

182 It can be concluded that caprylic acid or oils with a high concentration of it may improve
183 the resistance of weaned rabbits to EPEC.

184 **Conflict of interest statement**

185 None of the authors of this paper has a financial or personal relationship with other people
186 or organizations that could inappropriately influence or bias the contents of this paper.

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- 269

Table 1

Fatty acid profile (mg per 1 g fatty acids determined) of rabbit milk and MCFA oil

Fatty acid		Rabbit milk	Akomed R
Caproic	C 6:0	2.6	0.4
Caprylic	C 8:0	140	539
Capric	C 10:0	171	453
Lauric	C 12:0	35	1.3
Myristic	C 14:0	17	0.2
Palmitic	C 16:0	151	1.7
Palmitoleic	C 16:1	7.1	0
Stearic	C 18:0	25	1.1
Oleic	C 18:1n9	153	2.2
Linoleic	C 18:2	231	0.8
α -Linolenic	C 18:3	38	0
Arachidonic	C 20:4	2.3	0
Other acids		27	0.3

Table 2

Effect of rabbit milk, heat treatment of milk (100°C for 10 min) and lipase on the number (\log_{10} cfu/g) of *E. coli* O128, strain C6, surviving after an 18 h incubation in glucose medium

Heat treatment of milk	Milk : medium ratio	Lipase (mg/10ml)	\log_{10} cfu/ml
–	–	–	10.33 ± 0.61^a
–	–	5	9.85 ± 0.60^a
No	1 : 4	–	9.62 ± 0.54^a
No	1 : 4	5	6.77 ± 1.26^b
No	1 : 9	5	7.29 ± 0.94^b
Yes	1 : 4	5	6.19 ± 0.65^b
Yes	1 : 9	5	6.88 ± 0.95^b

Means of quadruplicates \pm SD.

Values (a, b) in the same column with a different superscript differ significantly ($P < 0.05$).

Table 3

Average body weight and feed intake of rabbits in the course of *in vivo* experiment

Treatment group	1	2	3	4
Diet	Basal	Basal	Caprylic acid	Akomed R
Infection	No	Yes	Yes	Yes
Average body weight (g)				
35 th day	988 ± 52	942 ± 65	924 ± 75	927 ± 72
42 nd day	1328 ± 82 ^a	1119 ± 96 ^b	1182 ± 73 ^b	1167 ± 132 ^b
49 th day	1684 ± 147 ^a	1202 ± 246 ^b	1489 ± 161 ^{ac}	1354 ± 309 ^{bc}
55 th day	2046 ± 105 ^a	1811 ± 79 ^b	1919 ± 161 ^{ab}	1880 ± 199 ^b
Average weight gain (g)	1058 ± 117 ^a	869 ± 108 ^b	995 ± 165 ^{ab}	953 ± 181 ^{ab}
Feed intake (g/day)				
35 th – 41 st day	98 ± 17	73 ± 31	85 ± 27	75 ± 31
42 nd – 48 th day	141 ± 35 ^a	83 ± 35 ^b	114 ± 38 ^{ab}	103 ± 35 ^{ab}
49 th – 55 th day	177 ± 12 ^a	135 ± 35 ^b	152 ± 25 ^{ab}	142 ± 39 ^b

Mean values for 12 rabbits per treatment ± SD. Rabbits were infected at 38 days of age.

Values (a, b, c) in the same row with different letters differ ($P < 0.05$).

Table 4

Mean number (\log_{10} cfu/g) of *E. coli* in the faeces of rabbits before and after infection with *E. coli* O128, strain C6

Treatment group	1	2	3	4
Diet	Basal	Basal	Caprylic acid	Akomed R
Infection	No	Yes	Yes	Yes
Before infection	4.01 ± 0.59	4.10 ± 0.52	3.77 ± 0.35	4.00 ± 0.99
After infection				
2 days	4.32 ± 0.37 ^a	6.36 ± 0.77 ^b	4.90 ± 1.09 ^{ab}	5.57 ± 0.71 ^{ab}
5 days	4.23 ± 0.59 ^a	9.62 ± 0.92 ^b	6.10 ± 0.88 ^c	7.33 ± 0.72 ^c
7 days	4.14 ± 0.69 ^a	10.22 ± 0.36 ^b	5.78 ± 0.67 ^c	6.06 ± 0.95 ^c
9 days	4.31 ± 0.82 ^a	10.49 ± 0.35 ^b	5.99 ± 0.50 ^c	6.90 ± 0.76 ^c
12 days	3.81 ± 0.49 ^a	9.38 ± 0.86 ^b	5.78 ± 0.66 ^c	6.69 ± 0.77 ^c
14 days	3.91 ± 0.54 ^a	7.97 ± 1.08 ^b	4.91 ± 0.92 ^a	5.12 ± 0.77 ^a

Mean values for 12 rabbits per treatment ± SD. Rabbits were infected at 38 days of age.

Values (a, b, c) in the same row with different letters differ ($P < 0.05$).

Table 5

Mean number (\log_{10} cfu/g) of *E. coli* and anaerobic bacteria in the contents of the stomach and caecum of rabbits infected with *E. coli* O128, strain C6

Treatment group	1	2	3	4
Diet	Basal	Basal	Caprylic acid	Akomed R
Infection	No	Yes	Yes	Yes
<i>E. coli</i>				
Stomach	2.05 ± 0.12	2.08 ± 0.16	2.03 ± 0.07	2.19 ± 0.43
Caecum	3.82 ± 0.60 ^a	7.53 ± 0.96 ^b	4.13 ± 0.79 ^a	4.93 ± 0.81 ^a
Anaerobic bacteria				
Stomach	3.72 ± 0.57	4.85 ± 0.73	3.53 ± 0.84	3.77 ± 0.92
Caecum	3.72 ± 0.81	4.85 ± 0.51	3.53 ± 0.32	3.77 ± 0.29

Mean values for 12 rabbits per treatment ± SD. Rabbits were infected at 38 days of age and slaughtered at 55 days of age. Values (a, b) in the same row with different letters differ ($P < 0.05$)

Fig. 1. Diarrhea incidence in infected rabbits from the 2nd, 3rd and 4th groups: (●) positive control, (□) rabbits receiving the diet with 5 g/kg caprylic acid, and (x) rabbits receiving the diet with 10 g/kg Akomed R.

