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1 **Testing the efficacy of medium chain fatty acids against rabbit colibacillosis**

2

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19

20 **Short title:** Dietary MCFA against rabbit colibacillosis

21

22 **Abbreviations:** MCFA: Medium Chain Fatty Acids; SO: Sunflower Oil; MCT: Medium
23 Chain Triglycerides; LB: L-Broth.

24

25 **Abstract**

26 Enteropathogenic *Escherichia coli* (EPEC) represents a major cause of lethal diarrhea in
27 young mammals. Although the pathogenicity mechanisms of EPEC are now well understood,
28 the intrinsic and environmental factors that control the expression of EPEC virulence remain
29 largely unknown. In the rabbit, suckling reduces pups' sensitivity to EPEC infection. Hence,
30 we have hypothesized that uncharacterized factors present in doe milk may mediate this
31 protection. Medium chain fatty acids (MCFA), known to possess antimicrobial properties, are
32 highly abundant in doe milk. We demonstrate that caprylic acid exhibits a clear bacteriostatic
33 effect *in vitro* against the rabbit EPEC strain E22 (O103:H2:K-), in a dose-dependent manner.
34 *In vivo*, the dietary inclusion of triglycerides of MCFA did not however reduce the sensitivity
35 of young rabbits challenged with this EPEC strain. The mortality and fecal excretion of EPEC
36 were not reduced, and the bacterial adhesion to ileum was not inhibited. Amount of MCFA
37 reaching the ileal level might have been too low and/or their association to other milk
38 antimicrobials may have been required to observe a positive effect on disease evolution in a
39 context of a highly virulent challenge.

40

41 **Keywords:** Enteropathogenic *E. coli* (EPEC), milk, rabbit, medium chain fatty acids
42 (MCFA), diarrhea

43

44

45 1. Introduction

46 Maternal milk is very efficient in preventing colibacillosis in young rabbits infected with a
47 highly pathogenic strain of enteropathogenic *E. coli* (EPEC) belonging to the O103 serogroup
48 (Gallois et al., 2007), a dominant serogroup in French fattening rabbit units (Camguilhem and
49 Milon, 1989). Milk Igs are not responsible for this protection (Gallois et al., 2007) and a cross
50 protection with Abs directed towards other strains of *E. coli* is unlikely, as all trials using
51 heterologous challenges (Donnenberg et al., 1998) or vaccines (Milon et al., 1992) failed to
52 demonstrate cross protection between different EPEC strains. Less specific milk components
53 are considered as good candidates for this protection, through antimicrobial or
54 immunomodulating properties, or by interfering with the expression of virulence factors
55 (Baranyi et al., 2003; Jia et al., 2001; Recio and Visser, 1999, Cravioto et al., 1991, Sun et al.,
56 2002). Doe milk composition is very unusual among domestic mammals. Triglycerides are
57 mainly composed of medium chain fatty acids (MCFA), with caprylic (C8:0) and capric
58 (C10:0) acids representing up to 65% (molar proportion) of total fatty acids (Demarne et al.,
59 1978, Pascual et al., 1999). This content is very high as compared to other species such as
60 cows (5%), ewes (17%), goats (18%) or humans (2.5%) (Freeman et al., 1965). MCFA are
61 very active antimicrobials *in vitro*, including against coliforms (Sun et al., 2002, Marounek et
62 al., 2002). *In vivo*, MCFA have been implicated in the control of the stomacal microflora in
63 suckling rabbits (Canas-Rodriguez and Smith, 1966), and provide health benefits during the
64 post-weaning period (Skrivanova et al., 2004). The known antimicrobial properties of MCFA
65 and their particularly high content in doe milk led us to determine whether MCFA could
66 protect pups against colibacillosis during suckling. In this study, the bacteriostatic activity of
67 MCFA against EPEC were demonstrated *in vitro*. The disease evolution was studied *in vivo* in
68 rabbits inoculated with a non-pathogenic (BM21) or a pathogenic strain of *E. coli* (strain E22,
69 O103:H2:K-), and fed with a diet supplemented or not with triglycerides of MCFA.

70

71 **2. Material and methods**72 2.1. Bacterial strains

73 An enteropathogenic *E. coli* strain belonging to O103:H2:K- serotype (E22 strain), which
74 does not metabolize the rhamnose and is fimbriae AF/R2 positive (Camguilhem and Milon,
75 1989), and the non-pathogenic BM21 strain of *E. coli* (Boullier et al., 2003a) were used in this
76 study. For the inhibition of adhesion assay, a genetically-modified pEGFP-E22 strain was
77 used (Boullier et al., 2003b).

78

79 2.2. In vitro experiments

80 Antimicrobial properties of MCFA were evaluated *in vitro* as follows. Solutions of C8:0,
81 C10:0 (Sigma) and of a mix of both, ranging from 0-40 mM, were prepared as previously
82 described (Sun et al., 2002), and adjusted to a pH of 6.5 with NaOH. 10^3 CFU of E22 were
83 cultured in 5ml of MCFA + LB media for 18h with rotary shaking or spread on LB agar plate
84 to determine the initial exact amount of bacteria added to each media. CFU were counted for
85 each concentration of MCFA after ON culture on LB agar plates. The inhibition growth rate
86 was calculated as: (number of CFU/ml in LB medium) / (number of CFU/ml in MCFA
87 medium).

88 Ability of MCFA to inhibit EPEC adhesion to epithelial cells was measured on HeLa cells,
89 cultivated as previously described (Boullier et al., 2003b). 20 μ l of pEGFP-E22 bacteria
90 grown ON (10^9 CFU.mL⁻¹) were pre-incubated with different amounts of C8:0 and C10:0
91 media for one hour at RT prior to their incubation for 30 min with HeLa cells (Boullier et al.,
92 2003b). After washing, 10^4 HeLa cells per sample were immediately acquired with a
93 Facscalibur (Becton Dickinson). Quantification of adhesion was monitored using Cellquest
94 software (Becton Dickinson). The percentage of inhibition was calculated using the following

95 formula: ((Percentage of pEGFP-E22 + HeLa cells without C8:0-C10:0 media) – (Percentage
96 of pEGFP-E22 + HeLa cells pre-incubated with C8:0-C10:0 media))/(Percentage of pEGFP-
97 E22 + HeLa cells without C8:0-C10:0 media)).

98

99 2.3. In vivo experiment

100 This experiment was approved by a regional ethical committee. 32 litters of 9 rabbits were
101 equally divided in 4 groups, according to their diet and to the *E. coli* strain inoculated at 28
102 days of age. Pelleted diets, without antibiotics, were formulated to cover the nutritional
103 requirements of the young rabbit (Gidenne, 2000) and were supplied with either 2% of
104 sunflower oil (SO) or 2% of medium chain triglycerides (MCT), a synthesized oil containing
105 55% of C8:0 and 44% of C10:0 (Radia[®]7104, Oléon France). Chemical composition of diets
106 was determined according to European recommendations (EGRAN, 2001), and fatty acids
107 composition was assayed by the ISTE (*In Situ* TransEsterification) method described by Park
108 and Goins (Park and Goins, 1994). From 14 to 35 days of age (the day of weaning), does and
109 their pups were reared in separated cages, to allow the feeding of pups with experimental diets
110 provided *ad libitum*. Suckling was controlled every morning by maintaining does with their
111 pups for about 5 minutes. Rabbits were orally inoculated at 28 days of age with 10⁴ CFU of
112 BM21 or E22 strains, 3 hours after suckling and were weaned at day 35. Mortality controls
113 were done twice a day and clinical examination every morning. Euthanasia was practiced in
114 dying rabbits (intramuscular injection of Imalgène[®] 1000 followed by an intracardiac injection
115 of T61[®]). Necropsy and a bacterial analysis of cecal content were systematically carried out to
116 identify rabbits which died from colibacillosis. Moreover, bacterial analyses were performed
117 on 10g of feces per cage twice a week. Cecal and fecal enumeration of *E. coli* was determined
118 as previously described (Gallois et al., 2007). At 36 days of age, one representative rabbit per
119 litter was sacrificed, in order to analyze composition of stomacal and ileal contents in fatty

120 acids according to the ISTE method (Park and Goins, 1994). At 63 days of age, all remaining
121 rabbits were sacrificed. Survival rates were submitted to a χ^2 test (procedure CATMOD, SAS
122 Online[®]). Bacterial shedding and MCFA composition of digestive contents were analyzed
123 with the GLM procedure (SAS Online[®]), depending on diet, the strain of *E. coli* and their
124 interaction as main effects. On day 28, two additional litters of nine MCT or SO-fed suckling
125 rabbits were inoculated with 10^4 CFU of E22 strain, and sacrificed 3 days later. Distal ileum
126 was fixed in 10% formaldehyde and embedded in paraffin. Bacteria fixed on the epithelia
127 were revealed with an anti-E22 serum prepared in the laboratory (Boullier et al., 2003b).
128 Tissue samples were scored blindly for bacterial adhesion.

129

130 **3. Results**

131 **3.1 MCFA inhibit bacterial growth *in vitro***

132 The possible bacteriostatic or bactericidal effects of MCFA were first tested against E22
133 bacteria *in vitro*. At any tested concentrations, C8:0 did not display any detectable bactericidal
134 or bacteriostatic effect, with an inhibition rate inferior to 1 log at 40 mM (5.76 g.kg^{-1} , Figure
135 1). In contrast, C10:0 exhibited a strong dose-dependent bacteriostatic activity. The mean
136 inhibition rate reaches values of up to 91 at 40 mM (6.88 g.L^{-1}). Interestingly, no synergistic
137 effect was observed between the two MCFA on the inhibition of bacterial growth. The dose-
138 response curve obtained was similar to the one obtained when using C10:0 alone. There was
139 no detectable bactericidal activity with any of the MCFA concentrations tested (data not
140 shown). These *in vitro* findings prompted us to test whether MCFA could modify bacterial
141 growth *in vivo* and protect rabbits against colibacillosis when added in pup's diet.

142

143 3.2 MCT supplementation is inefficient to protect young rabbits from colibacillosis

144 The chemical composition of diets was similar among groups (CP: 17.2%, NDF: 35.9%,
145 starch: 9.3% on an as-fed basis), with major differences restricted to their composition in
146 MCFA, MCT diet containing 19 percent units more C8:0 and 15 percent units more C10:0
147 than SO diet. In E22 groups, the first deaths due to EPEC occurred on the day of weaning and
148 the mortality rate reached 50% at 48-49 days of age, whatever the oil added in feed (Figure 2).
149 On day 63, 46% and 44% of rabbits had survived to colibacillosis in SO-E22 and MCT-E22
150 groups, respectively ($P = 0.85$). None of BM21-inoculated rabbits died from E22
151 colibacillosis, and their mortality rate was unaffected by the diet ($P = 0.19$). Morbidity,
152 characterized by anorexia, weight loss, liquid and sometimes hemorrhagic diarrhea, and
153 dehydration was similar in both E22 groups, and never exceeded 31% on a daily basis
154 whatever the diet (data not shown). Only 12 and 8 rabbits in MCT-E22 and SO-E22 groups,
155 respectively, did not show any signs of diarrhea all the experiment through. In BM21 groups,
156 excretion of *E. coli* remained stable and no colony belonging to the O103 serogroup was
157 identified (Figure 2). In infected rabbits, fecal excretion of *E. coli* increased as soon as 31
158 days of age, and most colonies were rhamnose negative. The level of excretion was between
159 10^6 and 10^8 CFU per g of feces from 31 to 45 days of age, then decreased to less than 10^4
160 CFU per g of feces at the end of the experiment independently from the diet.

161

162 3.3. MCFA does not inhibit bacterial adhesion *in vivo* nor *in vitro*

163 The addition of MCT in pup's diet could have modified the level of ileal bacterial attachment
164 and the severity of tissue lesions induced by E22. *In vivo*, the profile of E22 adhesion was
165 similar among MCT and SO rabbits, with very rare adhesion spots of bacteria on the ileum (a
166 mean of 2 to 3 colonies per slide) without any intestinal inflammation lesion (Figure 3). *In*

167 *vitro*, we showed that incubation of E22 bacteria with C8:0 and C10:0 (10 to 40 mM), alone
168 or combined, did not reduce their capacity to adhere on HeLa cells (Figure 3).

169

170 3.4. Transit of fatty acids along the stomach and the ileum

171 As MCT showed no *in vivo* beneficial effect against colibacillosis, we wondered whether the
172 available amount of MCFA along the digestive tract was sufficient. As expected,
173 concentrations of C8:0 and C10:0 were higher in the stomach of MCT-fed rabbits compared
174 to SO rabbits, both in infected and in control groups (5.5-times higher for C8:0, $P < 0.01$, and
175 4.1-times higher for C10:0, $P < 0.05$). These values reached 18-19 mM for both fatty acids in
176 MCT-E22 group. In the ileum, the concentrations of C8:0 and C10:0 also remained higher in
177 MCT compared to SO rabbits, both in infected and control groups ($P < 0.001$). However,
178 these values were low and did not exceed 0.1 g.kg^{-1} ($<0.7 \text{ mM}$) of fresh ileal content both for
179 C8:0 and C10:0 and were much lower than the active concentrations tested *in vitro*.

180

181 4. Discussion

182 In the rabbit, the disease caused by EPEC is delayed by suckling, which is in agreement with
183 our previous study (Gallois et al., 2007). Indeed, after inoculation of 32 to 42-days-old rabbits
184 with similar doses of EPEC strains belonging to the O103 serogroup, deaths are classically
185 observed 3 to 14 days post-infection (d.p.i.) (Camguilhem and Milon, 1989, Boullier et al.,
186 2003a). In our study, rabbits mainly died in a period ranging from 13 to 17 d.p.i., which
187 corresponds to 6 to 10 days after weaning. This protection during the suckling period is not
188 conferred by specific milk Igs, as all does were seronegative towards the E22 strain.

189 Antimicrobial properties of MCFA against bacteria including coliforms were shown *in vitro*
190 (Canas-Rodriguez and Smith, 1966, Marounek et al., 2002, Sun et al., 2002). In this study, we
191 show that only C10:0 inhibits the growth of E22 strain at neutral pH. Despite this clear

192 bacteriostatic effect *in vitro*, the dietary inclusion of triglycerides of MCFA does not reduce
193 the sensitivity of young rabbits to colibacillosis. Rates of morbidity and mortality, as well as
194 fecal *E. coli* excretion, were similar in all groups of rabbits regardless of their diet. This could
195 be partly due to an insufficient MCFA intake. The MCT incorporation level in diet (2%) was
196 chosen according to the MCFA content in doe milk and in reference to ingestion curves of
197 milk/solid feed (Gidenne and Lebas, 2006), to reach dietary MCFA concentrations similar to
198 the ones found in milk at the time of weaning. In rabbits fed with MCT, intake of C8:0 and
199 C10:0 (*via* feed) was similar to the intake before weaning (thus *via* the milk) (data not
200 shown). Alternatively, intestinal concentrations of MCFA were probably too low to produce a
201 bacteriostatic effect. For MCFA not to be absorbed too quickly and thus remain available in
202 EPEC sensitive tissues (ileum and cecum), they had to be included in the diet in the form of
203 MCT. In addition, because medium chain monoglycerides exert a higher antimicrobial
204 activity than their corresponding free fatty acids (Isaacs et al., 1995, Petschow et al., 1996),
205 MCFA absorption needed to be delayed. Feeding rabbits with MCT should have allowed a
206 slower absorption of MCFA (Perret, 1980). Their monoesterified derivatives would have thus
207 reached ileal and cecal segments. Even if the stomacal and ileal MCFA contents were higher
208 in MCT-fed rabbits, they remained lower than 0.2 mg.g⁻¹ of fresh matter for both C8:0 and
209 C10:0 in the ileum. It was shown that C10:0 can inhibit the growth of *E. coli* at concentrations
210 lower than 0.3 g.L⁻¹ (2mM) at a pH of 4.6 (Sun et al., 2002), but this minimal inhibiting
211 concentration increases quickly when the pH nears neutrality (Hsiao and Siebert, 1999).
212 Accordingly, in the present study, no bacteriostatic activity of C8:0 even at a dose of 5.76 g.L⁻¹
213 (40mM) was observed at neutral pH (close to conditions found in rabbit ileum and cecum),
214 whereas a 2log bacteriostatic effect was obtained with C10:0, but at a concentration of 6.88
215 g.L⁻¹ (40mM) which is much higher than the concentrations of MCFA found in the ileum of
216 infected MCT-fed rabbits (0.16 g.kg⁻¹). MCT are in part digested and absorbed at stomacal

217 level (Perret, 1980). Thus, MCFA and their derivatives are likely to be active mainly in the
218 stomach where high MCFA concentrations are observed, and limit the crossing of pylorus by
219 pathogenic *E. coli*. Moreover, acidic pH conditions in the stomach would be more adapted to
220 the expression of the antimicrobial activity of MCFA. If this hypothesis is true, our
221 experimental conditions (infectious dose/unit of time, highly pathogenic strain of EPEC) may
222 have been too drastic to allow the visualization of such phenomenon.

223 The protection conferred by suckling (Gallois et al., 2007) is probably the result of a
224 combination of different milk antimicrobial factors that may act to limit the virulence of
225 pathogens along the digestive tract through additive and synergistic effects (Isaacs et al.,
226 1995). Some components inhibit bacterial growth while others are more involved in the
227 control of the colonization of epithelial cells by microorganisms. It was shown that MCFA
228 can limit the invasion of intestinal epithelial cells by *Salmonella enterica* in chicken (Van
229 Immerseel et al., 2004). However, no inhibition of adhesion of E22 to HeLa cells in presence
230 of MCFA was observed although an inhibiting adhesion capacity of milk both *in vivo* and *in*
231 *vitro* was previously demonstrated (Gallois et al., 2007).

232 In conclusion, our results show that, despite an *in vitro* effect, MCFA alone do not protect
233 rabbits against colibacillosis. Since the pathogenicity of EPEC is related to their ability to
234 colonize epithelial cells, it would be interesting to characterize milk factors able to inhibit
235 bacterial adhesion. Once characterized, their use in association with bacteriostatic substances
236 like MCFA may lead to synergistic effects aiming at protecting rabbits against colibacillosis.

237

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244

245 **6. References**

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- 315

316 **Legends**

317 **Figure 1.** C10:0 has a bacteriostatic effect on E22 *in vitro*

318 After 18 hours of E22 culture, mean CFU of each type of culture was determined by plating
319 on LB agar plates in duplicate. Results are expressed as the rate of growth inhibition for each
320 fatty acid concentration calculated from the LB control medium. Results represent the mean
321 (\pm SD) of 6 independent experiments.

322

323 **Figure 2.** MCT diet does not confer protection against lethal challenge with O103 EPEC.

324 A: MCT diet does not limit mortality induced by E22 strain. 28-days-old rabbits were orally
325 inoculated with 10^4 CFU of a non-pathogenic strain BM21 or a pathogenic strain E22 of *E.*
326 *coli*, and fed either with a diet containing 2% of SO or 2% of MCT. In E22 groups, only
327 rabbits that died from colibacillosis are represented in the graph. In SO group, none of the
328 rabbits died from E22 colibacillosis. Final survival rates were 45.5%, 43.9%, 87% and 78.6%
329 in SO-E22, MCT-E22, SO-BM21 and MCT-BM21 groups, respectively.

330 B: MCT diet does not limit fecal excretion of E22 strain by infected rabbits. Total excretion
331 of *E. coli* is represented by triangles in BM21 group, and by the height of bars in E22 group.
332 Full bars indicate the proportion of Rh- strains (E22) among *E. coli* excreted, hatched bars
333 represent Rh+. SO-fed rabbits are represented in black and MCT-fed rabbits in grey. Data
334 show arithmetic means \pm SD.

335

336 **Figure 3.** C8:0 and C10:0 do not prevent bacterial adhesion *in vivo* and *in vitro*.

337 A: MCT diet does not modify ileal bacterial attachment and intestinal inflammation. Ileal
338 tissue sections of MCT-fed (a) or SO-fed (b) rabbits infected at day 28 and sacrificed at day
339 31 were stained with anti-E22 sera and visualized at X400 magnification. Rabbits from each

340 group presented only scarce bacterial adhesion (arrow). Villi were intact and no sign of
341 inflammation was visible.

342 B: MCFA do not inhibit bacterial adhesion on HeLa cells. Different concentrations of C8:0
343 and C10:0 were tested for their ability to inhibit bacterial adhesion on HeLa cells. Percentage
344 of pEGFP-E22 positive HeLa cells was determined by flow cytometry, by detecting the GFP
345 expression of pEGFP-E22. Data are means \pm SD of 3 independent experiments.

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