Testing the efficacy of medium chain fatty acids against rabbit colibacillosis

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Abstract

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

Keywords: Enteropathogenic *E. coli* (EPEC); Milk; Rabbit; Medium chain fatty acids (MCFA); Diarrhea

1. Introduction

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

2. Material and methods

2.1. Bacterial strains

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

2.2. In vitro experiments

Antimicrobial properties of MCFA were evaluated *in vitro* as follows. Solutions of C8:0, C10:0 (Sigma) and of a mix of both, ranging from 0 to 40 mM, were prepared as previously described (Sun et al., 2002), and adjusted to a pH of 6.5 with NaOH. 10^3 CFU of E22 were cultured in 5 ml of MCFA + LB media for 18 h with rotary shaking or spread on LB agar plate to determine the initial exact amount of bacteria added to each media. CFU were counted for each concentration of MCFA after ON culture on LB agar plates. The inhibition growth rate was calculated as: (number of CFU ml^{-1} in LB medium)/(number of CFU ml^{-1} in MCFA medium).

Ability of MCFA to inhibit EPEC adhesion to epithelial cells was measured on HeLa cells, cultivated as previously described (Boullier et al., 2003b). 20 µl of pEGFP-E22 bacteria grown ON (10^9 CFU ml^{-1}) were pre-incubated with different amounts of C8:0 and C10:0 media for one hour at RT prior to their incubation for 30 min with HeLa cells (Boullier et al., 2003b). After washing, 10^4 HeLa cells per sample were immediately acquired with a Facscalibur (Becton Dickinson). Quantification of adhesion was monitored using Cellquest software (Becton Dickinson). The percentage of inhibition was calculated using the following formula: ((percentage of pEGFP-E22 + HeLa cells without C8:0-C10:0 media) − (percentage of pEGFP-E22 + HeLa cells pre-incubated with C8:0–C10:0 media))/(percentage of pEGFP-E22 + HeLa cells without C8:0–C10:0 media).

2.3. In vivo experiment

This experiment was approved by a regional ethical committee. 32 litters of 9 rabbits were equally divided in 4 groups, according to their diet and to the *E. coli* strain inoculated at 28 days of age. Pelleted diets, without antibiotics, were formulated to cover the nutritional requirements of the young rabbit (Gidenne, 2000) and were supplied with either 2% of sunflower oil (SO) or 2% of medium chain triglycerides (MCT), a synthesized oil containing 55% of C8:0 and 44% of C10:0 (Radia® 7104, Oléon, France). Chemical composition of diets was determined according to European recommendations (EGRAN, 2001), and fatty acids composition was assayed by the ISTE (*In Situ* TransEsterification) method (Park and Goins, 1994). From 14 to 35 days of age (the day of weaning), does and their pups were reared in separated cages, to allow the feeding of pups with experimental diets provided *ad libitum*. Suckling was controlled every morning by maintaining does with their pups for about 5 min. Rabbits were orally inoculated at 28 days of age with 10^4 CFU of BM21 or E22 strains, 3 h after suckling and were weaned at day 35. Mortality controls were done twice a day and clinical examination every morning. Euthanasia was practiced in dying rabbits (intramuscular injection of Imalgène®1000 followed by an intracardiac injection of T61®). Necropsy and a bacterial analysis of cecal content were systematically carried out to identify rabbits which died from colibacillosis. Moreover, bacterial analyses were performed on 10 g of feces per cage twice a week. Cecal and fecal enumeration of *E. coli* was determined as previously described (Gallois et al., 2007). At 36 days of age, one representative rabbit per litter was sacrificed, in order to analyze composition of stomacal and ileal contents in fatty acids according to the ISTE method (Park and Goins, 1994). At 63 days of age, all remaining rabbits were sacrificed. Survival rates were submitted to a χ^2 test (procedure CATMOD, SAS Online®). Bacterial shedding and MCFA composition of digestive contents were analyzed with the GLM procedure (SAS Online®), depending on diet, the strain of *E. coli* and their interaction as main effects. On day 28, two additional litters of nine MCT or SO-fed suckling rabbits were inoculated with 10^4 CFU of E22 strain, and sacrificed 3 days later. Distal ileum was fixed in 10% formaldehyde and embedded in paraffin.

Bacteria fixed on the epithelia were revealed with an anti-E22 serum prepared in the laboratory (Boullier et al., 2003b). Tissue samples were scored blindly for bacterial adhesion.

3. Results

3.1. MCFA inhibit bacterial growth in vitro

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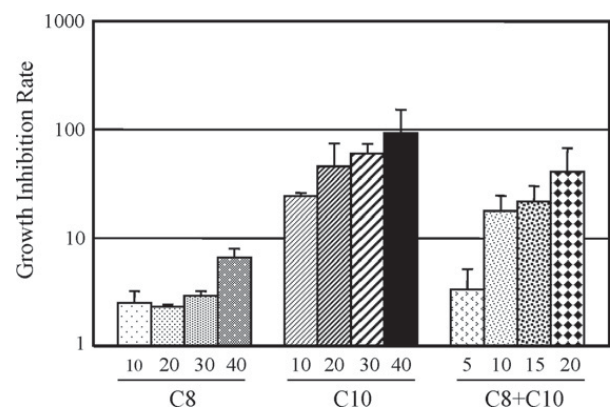

Fig. 1. C10:0 has a bacteriostatic effect on E22 *in vitro*. After 18 h of E22 culture, mean CFU of each type of culture was determined by plating on LB agar plates in duplicate. Results are expressed as the rate of growth inhibition for each fatty acid concentration calculated from the LB control medium. Results represent the mean (±S.D.) of six independent experiments.

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

The chemical composition of diets was similar among groups (CP: 17.2%, NDF: 35.9%, starch: 9.3% on an as-fed basis), with major differences restricted to their composition in MCFA, MCT diet containing 19% units more C8:0 and 15% units more C10:0 than SO diet. In E22 groups, the first deaths due to EPEC occurred on the day of weaning and the mortality rate reached 50% at 48–49 days of age, whatever the oil added in feed (Fig. 2A). On day 63, 46 and 44% of rabbits had survived to colibacillosis in SO-E22 and MCT-E22 groups, respectively ($P = 0.85$). None of BM21-inoculated rabbits died from E22 colibacillo-

sis, and their mortality rate was unaffected by the diet ($P = 0.19$). Morbidity, characterized by anorexia, weight loss, liquid and sometimes hemorrhagic diarrhea, and dehydration was similar in both E22 groups, and never exceeded 31% on a daily basis whatever the diet (data not shown). Only 12 and 8 rabbits in MCT-E22 and SO-E22 groups, respectively, did not show any signs of diarrhea all through the experiment. In BM21 groups, excretion of *E. coli* remained stable and no colony belonging to the O103 serogroup was identified (Fig. 2B). In infected rabbits, fecal excretion of *E. coli* increased as soon as 31 days of age, and most colonies were rhamnose negative. The level of excretion was between 10^6 and 10^8 CFU per g of feces from 31 to 45 days of age, then decreased to less than 10^4 CFU per g of feces at the end of the experiment independently from the diet.

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

The addition of MCT in pup's diet could have modified the level of ileal bacterial attachment and the severity of tissue lesions induced by E22. *In vivo*, the profile of E22 adhesion was similar among MCT and SO rabbits, with very rare adhesion spots of bacteria on the ileum (a mean of two to three colonies per slide) without any intestinal inflammation lesion (Fig. 3A). *In vitro*, we showed that incubation of E22 bacteria with C8:0 and C10:0 (10–40 mM), alone or combined, did not reduce their capacity to adhere on HeLa cells (Fig. 3B).

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

As MCT showed no *in vivo* beneficial effect against colibacillosis, we wondered whether the available amount of MCFA along the digestive tract was sufficient. As expected, concentrations of C8:0 and C10:0 were higher in the stomach of MCT-fed rabbits compared to SO rabbits, both in infected and in control groups (5.5 times higher for C8:0, $P < 0.01$, and 4.1 times higher for C10:0, $P < 0.05$). These values reached 18–19 mM for both fatty acids in MCT-E22 group. In the ileum, the concentrations of C8:0 and C10:0 also remained higher in MCT compared to SO rabbits, both in infected and control
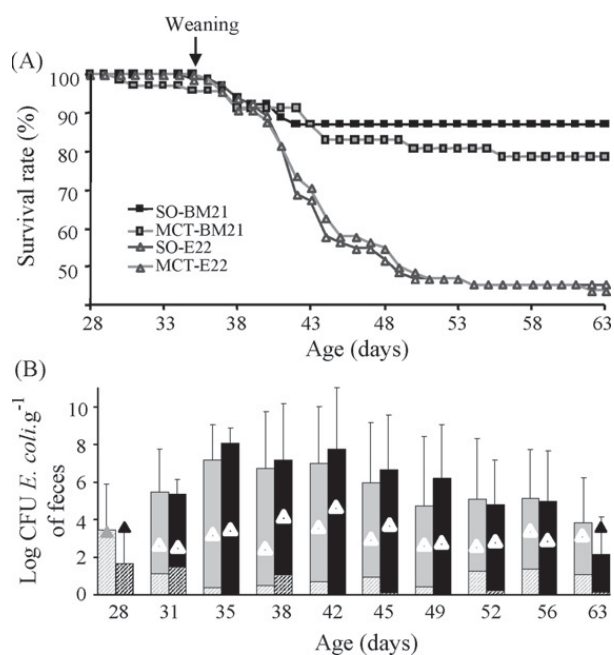

Fig. 2. MCT diet does not confer protection against lethal challenge with O103 EPEC. (A) MCT diet does not limit mortality induced by E22 strain. 28-day-old rabbits were orally inoculated with 10^4 CFU of a non-pathogenic strain BM21 or a pathogenic strain E22 of *E. coli*, and fed either with a diet containing 2% of SO or 2% of MCT. In E22 groups, only rabbits that died from colibacillosis are represented in the graph. In SO group, none of the rabbits died from E22 colibacillosis. Final survival rates were 45.5, 43.9, 87 and 78.6% in SO-E22, MCT-E22, SO-BM21 and MCT-BM21 groups, respectively. (B) MCT diet does not limit fecal excretion of E22 strain by infected rabbits. Total excretion of *E. coli* is represented by triangles in BM21 group, and by the height of bars in E22 group. Full bars indicate the proportion of Rh− strains (E22) among *E. coli* excreted, hatched bars represent Rh+. SO-fed rabbits are represented in black and MCT-fed rabbits in grey. Data show arithmetic mean ± S.D.
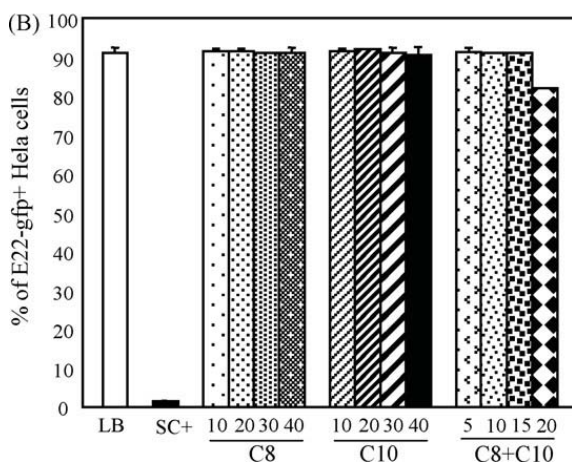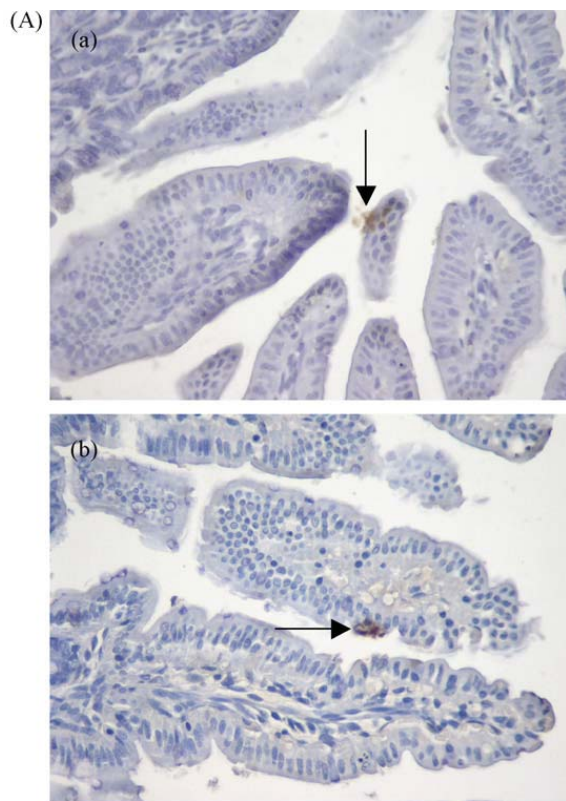
Fig. 3. C8:0 and C10:0 do not prevent bacterial adhesion *in vivo* and *in vitro*. (A) MCT diet does not modify ileal bacterial attachment and intestinal inflammation. Ileal tissue sections of MCT-fed (a) or SO-fed (b) rabbits infected at day 28 and sacrificed at day 31 were stained with anti-E22 sera and visualized at ×400 magnification. Rabbits from each group presented only scarce bacterial adhesion (arrow). Villi were intact and no sign of inflammation was visible. (B) MCFA do not inhibit bacterial adhesion on HeLa cells. Different concentrations of C8:0 and C10:0 were tested for their ability to inhibit bacterial adhesion on HeLa cells. Percentage of pEGFP-E22 positive HeLa cells was determined by flow cytometry, by detecting the GFP expression of pEGFP-E22. Data are mean ± S.D. of three independent experiments.

groups ($P < 0.001$). However, these values were low and did not exceed 0.1 g kg^{-1} (<0.7 mM) of fresh ileal content both for C8:0 and C10:0 and were much lower than the active concentrations tested *in vitro*.

4. Discussion

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

Antimicrobial properties of MCFA against bacteria including coliforms were shown *in vitro* (Canas-Rodriguez and Smith, 1966; Marounek et al., 2002; Sun et al., 2002). In this study, we show that only C10:0 inhibits the growth of E22 strain at neutral pH. Despite this clear bacteriostatic effect *in vitro*, the dietary inclusion of triglycerides of MCFA does not reduce the sensitivity of young rabbits to colibacillosis. Rates of morbidity and mortality, as well as fecal *E. coli* excretion, were similar in all groups of rabbits regardless of their diet. This could be partly due to an insufficient MCFA intake. The MCT incorporation level in diet (2%) was chosen according to the MCFA content in doe milk and in reference to ingestion curves of milk/solid feed (Gidenne and Lebas, 2006), to reach dietary MCFA concentrations similar to the ones found in milk at the time of weaning. In rabbits fed with MCT, intake of C8:0 and C10:0 (*via* feed) was similar to the intake before weaning (thus *via* the milk) (data not shown). Alternatively, intestinal concentrations of MCFA were probably too low to produce a bacteriostatic effect. For MCFA not to be absorbed too quickly and thus remain available in EPEC sensitive tissues (ileum and cecum), they had to be included in the diet in the form of MCT. In addition, because medium chain monoglycerides exert a higher antimicrobial activity than their corresponding free fatty

acids (Isaacs et al., 1995; Petschow et al., 1996), MCFA absorption needed to be delayed. Feeding rabbits with MCT should have allowed a slower absorption of MCFA (Perret, 1980). Their mono-esterified derivates would have thus reached ileal and cecal segments. Even if the stomacal and ileal MCFA contents were higher in MCT-fed rabbits, they remained lower than 0.2 mg g^{-1} of fresh matter for both C8:0 and C10:0 in the ileum. It was shown that C10:0 can inhibit the growth of *E. coli* at concentrations lower than 0.3 g l^{-1} (2 mM) at a pH of 4.6 (Sun et al., 2002), but this minimal inhibiting concentration increases quickly when the pH nears neutrality (Hsiao and Siebert, 1999). Accordingly, in the present study, no bacteriostatic activity of C8:0 even at a dose of 5.76 g l^{-1} (40 mM) was observed at neutral pH (close to conditions found in rabbit ileum and cecum), whereas a 2 log bacteriostatic effect was obtained with C10:0, but at a concentration of 6.88 g l^{-1} (40 mM) which is much higher than the concentrations of MCFA found in the ileum of infected MCT-fed rabbits (0.16 g kg^{-1}). MCT are in part digested and absorbed at stomacal level (Perret, 1980). Thus, MCFA and their derivates are likely to be active mainly in the stomach where high MCFA concentrations are observed, and limit the crossing of pylorus by pathogenic *E. coli*. Moreover, acidic pH conditions in the stomach would be more adapted to the expression of the antimicrobial activity of MCFA. If this hypothesis is true, our experimental conditions (infectious dose/unit of time, highly pathogenic strain of EPEC) may have been too drastic to allow the visualization of such phenomenon.

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

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

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