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Bovine milk fat components inhibit food-borne pathogens

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Abstract

Bovine milk fat may protect against gastrointestinal infections by means of its antimicrobial constituents. This paper summarises our studies performed to test the bactericidal activities of milk fat. In vitro, the fatty acids $C_{10:0}$, $C_{12:0}$ and unsaturated C18 fatty acids together with digestion products of sphingolipids were effective bactericidal agents. *Listeria monocytogenes* and *Campylobacter jejuni* were very sensitive, whereas *E. coli* O157:H7 and *Salmonella enteritidis* were less vulnerable. In rats, high intake of milk fat triglycerides protected against orally administered *L. monocytogenes* but not against *S. enteritidis*. The enhanced resistance to *L. monocytogenes* was related to an increased release of gastric bactericidal saturated fatty acids. Since gastric bactericidal activity is predictive for butter fat-mediated resistance to *L. monocytogenes* in vivo, *C. jejuni* and *E. coli* O157:H7 were incubated with gastric contents of rats fed high or low butter fat diets. Ex vivo killing of *E. coli* was not affected whereas bactericidal activity towards *C. jejuni* was enhanced in gastric chyme of rats fed high butter fat diets, implying that milk fat triglycerides may also protect against campylobacter infections. Since unsaturated C_{18} fatty acids were listericidal in vitro, the efficacy of these agents was tested in rats. High corn oil intake did not protect against listeria infection, suggesting that the protective effect of fatty acids in vivo may be limited to $C_{10:0}$ and $C_{12:0}$. The effectiveness of membrane lipids was also tested in rats. Colonisation of *L. monocytogenes* was decreased in rats fed diets based on sweet butter milk powder (membrane lipid rich) compared with rats fed skim milk-based diets (low content of membrane lipids). In conclusion, bovine milk fat triglycerides containing $C_{10:0}$ and $C_{12:0}$, and sphingolipids may enhance the resistance to certain types of food-borne gastrointestinal infections.

Keywords: Fatty acids; Monoglycerides; Sphingolipids; Gastrointestinal infections

1. Introduction

Gastrointestinal infections caused by food-borne pathogens are still an enormous public health problem, even in Western societies. For example, the estimated yearly incidence of food-borne gastro-enteritis in the Netherlands is more than 10 per 100 persons (Notermans & van de Giessen, 1993). Most food-borne diseases are self-limiting and most adults experience a limited infection. However, food-borne gastrointestinal infections can be dangerous to neonates, elderly and immunocompromised subjects. Diet may be useful in increasing the resistance to gastrointestinal infections. The host immune system consists of constitutive defences and inducible antibody-mediated and cellular defences. Because the inducible humoral and cellular immune response needs more than one week to be fully expressed, dietary manipulation of this part of the defence system will probably not be successful in fighting primary intestinal infections. In contrast, dietary improvement of the constitutive non-specific gastrointestinal defence systems could be a promising tool in preventing food-borne infections. In addition to the indigenous flora, intestinal motility, and mucin secretion, luminal bactericidal agents such as gastric acid, pancreatic enzymes, and bile acids contribute to the gastrointestinal non-specific defence. We hypothesise that changing the composition of the diet may affect the concentration of luminal antimicrobial agents and could therefore affect survival and colonisation of pathogens. For example, bovine milk fat may protect against gastrointestinal infections because whole milk consumption in children is associated with fewer gastrointestinal infections than is consumption of low fat milk (Koopman, Turkish, Monto, Thompson, & Isaacson, 1984). Milk fat contains triglycerides and membrane lipids that

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may exert antimicrobial effects either directly or upon digestion. This paper summarises our studies performed to evaluate the effectiveness of these milk fat constituents in preventing food-borne infections. Part of these studies has been published before (Sprong, Hulstein, & Van der Meer, 1999, 2001), whereas other parts are new.

2. Materials and methods

2.1. In vitro bactericidal activity of milk lipids

In vitro assays to test the bactericidal activity of fatty acids, sphingosine, lysosphingomyelin, ceramide and lysophospholipids towards *Escherichia coli* O157:H7, *Salmonella enteritidis*, *Campylobacter jejuni* and *Listeria monocytogenes* were published earlier (Sprong et al., 2001).

2.2. In vivo experiments

The in vivo relevance of milk fat triglycerides was tested in male Wistar rats fed diets containing high or low amounts of butter fat as published earlier (Sprong et al., 1999). To test the hypothesis that edible oils rich in unsaturated fatty acids also protect against food-borne infections, we performed a strictly controlled experiment using corn oil as dietary fat. The protocol for this experiment was identical to that for butter fat (Sprong et al., 1999). Briefly, specific-pathogen-free male Wistar rats ($n = 8$ per diet group) were fed semi-purified diets containing either low (10 energy%) or high (40 energy%) amounts of corn oil. After adaptation to the diets for 14 days, rats were deprived of food for 14 h and subsequently fed for 2 h to ensure a filled stomach. Immediately after the feeding procedure, 5×10^9 CFU *L. monocytogenes* (NIZO B1242, from the collection of our institute) as determined by counting on listeria-selective PALCAM-plates (Merck, Darmstadt, Germany) was orally administered to the rats. The course of infection was followed 3 days. Colonisation was determined by plating 10-fold dilutions of fresh faecal samples on PALCAM. Translocation of *L. monocytogenes* was determined using urinary nitrate and nitrite (NO_x), which is a quantitative, non-invasive biomarker for translocation of bacteria (Oudenhoven, Klaassen, Lapré, Weerkamp, & Van der Meer, 1994; Sprong, Hulstein, & Van der Meer, 2000). Urinary NO_x was measured as described before (Sprong et al., 2000) Translocation was expressed as the total listeria-mediated increase in NO_x excretion. Faeces were collected 4 days before infection and 3 days after infection. Determining diarrhoea by direct measurement of the faecal water content by weighing faeces before and after lyophilisation underestimates the real water content because of evaporation of water during the 24-h collection period. Assuming that the cations sodium, potassium and ammonium and their counter anions are the mean electrolytes in faeces and that osmolarity of the intestinal contents is always 300 mOsmol L^{-1} even in diarrhoeal states (Fine, Kreijs, & Fordtran, 1993), the water content of faeces can be calculated. Cations were determined as described previously (Sprong et al., 1999). Diarrhoea was expressed as the pathogen-mediated increase in calculated faecal water content.

To check whether fatty acids were substantially liberated in the stomach, control non-infected rats ($n = 4$ per diet) fed either low or high corn oil diets were subjected to the fasting/feeding procedure described above. Rats were killed with CO_2 immediately after the feeding period and the gastric contents were collected. Fatty acids were analysed as described before (Sprong et al., 1999).

2.3. Ex vivo bactericidal activity of gastric contents

Gastric chyme of rats fed low butter fat or high butter fat diets were used. Animals, diets, and collection of gastric contents were described earlier (Sprong et al., 1999). Chyme was diluted 1:1 with saline and incubated with 2×10^7 CFU mL^{-1} *C. jejuni* (NIZO B1246) or *E. coli* O157:H7 (NIZO B1245) at 37°C for 2 h in a shaking water bath. Samples were drawn directly after incubation of bacteria and every 30 min thereafter. Viable pathogens were determined by plating on charcoal cefoperazone deoxycholate agar (Oxoid, Basingstoke, UK) and brain heart infusion agar (Difco, Detroit, MI, USA), for *C. jejuni* and *E. coli*, respectively.

3. Results and discussion

3.1. Fatty acids

Triglycerides, which constitute the major milk fat fraction, are digested to fatty acids and monoglycerides by gastric and pancreatic lipases. Unlike triglycerides and diglycerides, fatty acids and monoglycerides are highly bactericidal in vitro (Kabara, Swieckowski, Conley, & Truant, 1972; Conley & Kabara, 1973). Bactericidal activity depends on the bacterial strain involved. Generally, Gram positive bacteria are considered lipid-sensitive whereas Gram negatives are not (Kabara et al., 1972). This is because Gram positives lack the protective lipopolysaccharide-rich outer membrane of Gram negatives (Sheu & Freese, 1973). However, some exceptions have been described. For example, *Vibrio cholerae* (Petschow, Batema, & Ford, 1996), and *Helicobacter pylori* (Petschow, Batema, Talbott, & Ford, 1998) are lipid-sensitive. Bactericidal activity also depends on the nature of the fatty acids such as chain length and degree of unsaturation (Kabara

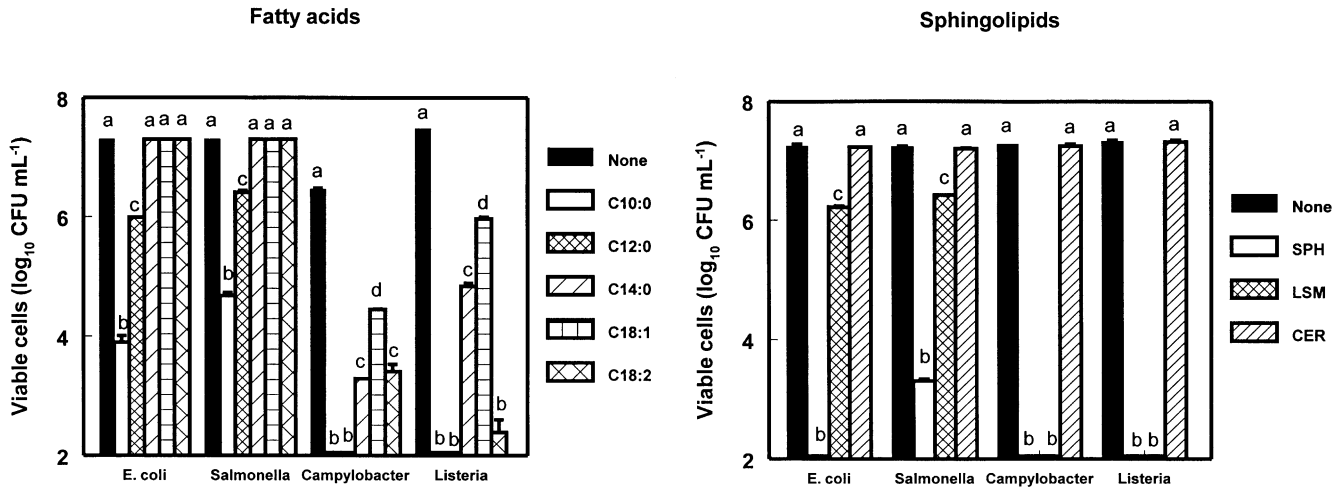

Fig. 1. Bactericidal activity of fatty acids (500 μM, pH 5) and sphingolipids (100 μM, pH 7) in vitro. Abbreviations used are SPH, sphingosine; LSM, lysosphingomyelin, and CER, ceramide. Viability of pathogens was determined by plating techniques after an incubation period of 2 h at 37°C. Data represent mean ± SD of triplicate incubations. Different letters for a pathogen and a lipid class reflect significant differences ($P < 0.05$) as tested by the Student–Newman–Keuls test for multiple comparisons. Data from Sprong et al. (2001) with permission.

Table 1
Effect of amount of dietary butter fat on faecal pathogen excretion and pathogen-induced water content of faeces of rats orally administered with 5×10^9 CFU *Listeria monocytogenes* or 2×10^9 CFU *Salmonella enteritidis*[a]

	L. monocytogenes		*S. enteritidis*	
	Low fat	High fat	Low fat	High fat
Faecal pathogen (log_{10} CFU/g wet faeces)				
Day 1	6.23 ± 0.22	5.33 ± 0.33[c]	6.77 ± 0.18	7.01 ± 0.18
Day 3	4.17 ± 0.32	3.35 ± 0.23[c]	5.25 ± 0.30	5.82 ± 0.20
Increase faecal water content[b] (g/100 g wet faeces)	20 ± 3	12 ± 2[c]	12 ± 3	15 ± 3

[a] Values are mean ± SEM, $n = 8$.
[b] The faecal water content after pathogen administration was subtracted by the faecal water content before administration of the pathogen.
[c] Significantly different ($P < 0.05$) from the low milk fat group as tested with the Student's *t*-test for independent data.
Data from Sprong et al. (1999) with permission.

et al., 1972). Bovine milk fat contains a broad spectrum of fatty acids, having saturated fatty acids varying in chain length from C_4 to C_{18}, and unsaturated $C_{18:1}$ and $C_{18:2}$ fatty acids (Jensen & Newburg, 1995). Recently, we tested the bactericidal activity of these fatty acids in vitro, using four food-borne pathogens, i.e. *C. jejuni*, *S. enteritidis*, *E. coli* O157:H7 all Gram negatives, and the Gram positive *L. monocytogenes* (Sprong et al., 2001). $C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{16:0}$, and $C_{18:0}$ were not bactericidal at the tested concentration of 500 μmol L^{-1}. $C_{14:0}$, $C_{18:1}$ and $C_{18:2}$ only decreased viable counts of *C. jejuni* and *L. monocytogenes*, whereas both $C_{10:0}$ and $C_{12:0}$ lowered viable counts of all test pathogens (Fig. 1). Others have reported in vitro bactericidal activities of $C_{10:0}$ and $C_{12:0}$ fatty acids and monoglycerides for other important gastrointestinal Gram negative bacterial pathogens such as *Salmonella typhi*, *Vibrio cholerea*, *Shigella sonnei*, *Helicobacter pylori* (Petschow et al., 1996, 1998). In addition, enveloped viruses are also lipid-sensitive (Thormar, Isaacs, Brown, Barshatzky, & Pessolano, 1987). These in vitro results suggest that bovine milk fat triglycerides may protect against gastrointestinal infections.

Recently, we described the in vivo relevance of milk fat triglycerides in preventing infections provoked by listeria and salmonella in a strictly controlled rat experiment fed low or high butter fat diets (Sprong et al., 1999). On day 1 and 3 after oral administration of the pathogen, rats fed the high butter fat diet excreted 10-fold less *L. monocytogenes* in faeces than rats fed the low butter fat diet (Table 1). Thus, butter fat significantly decreased the survival of listeria in the gastrointestinal tract. In addition, diarrhoea, defined by an increase in faecal water content, was decreased on the

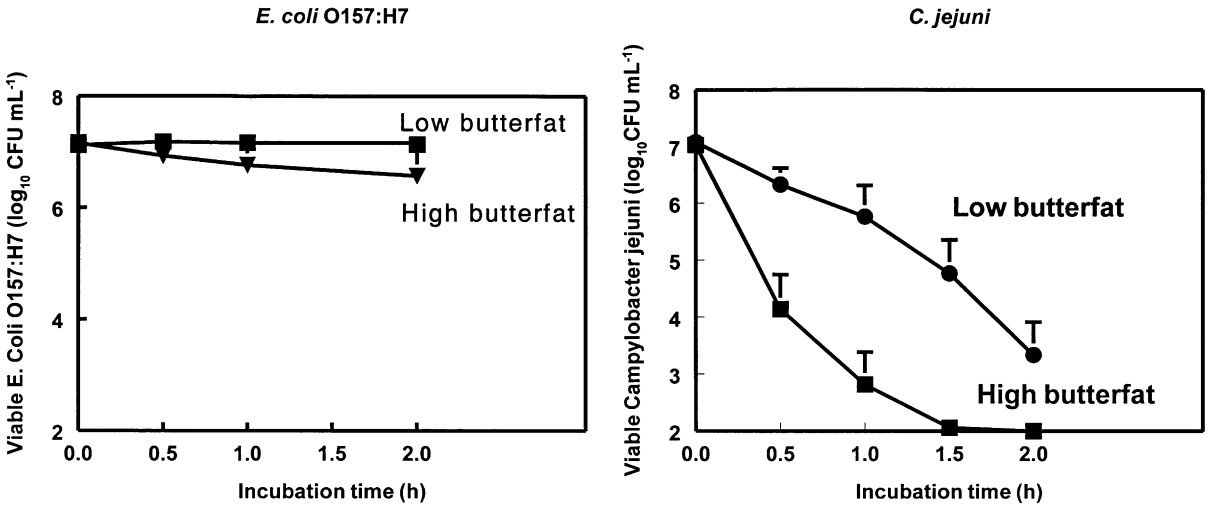

Fig. 2. Ex vivo killing capacity of gastric contents of rats fed high or low butter fat diets. Killing was measured by incubating saline-diluted chyme (1:1, wt/v) with approximately 10^7 CFU/mL *Escherichia coli* O157:H7 or *Campylobacter jejuni* at 37°C. Viable pathogens were determined by plating techniques. Data represent mean ± SEM ($n = 8$).

high butter fat diet (Table 1). In contrast, faecal excretion of *S. enteritidis* and the concomitant diarrhoea was not affected by butter fat consumption (Table 1). To gain further inside in the location and mechanism of high butter fat-mediated reduction of *L. monocytogenes* colonisation, the bactericidal activity of gastrointestinal contents was determined ex vivo (Sprong et al., 1999). Bactericidal activity was predominantly observed in gastric contents and a 10-fold increase in listericidal activity was observed in rats on high butter fat diets. The increased bactericidal activity coincided with an increase in the gastric concentrations of saturated fatty acids but not $C_{18:1}$ and $C_{18:2}$. Though the concentration of listericidal fatty acids was also increased in the small intestine, almost no bactericidal activity was observed in the small intestine. This may be explained by the lower pH of the gastric contents (approximately pH 4.5) compared with that in the proximal small intestine (about pH 6), since protonation of fatty acids improves their listericidal effects (Wang & Johnson, 1992). Taken together, these results indicate that gastric digestion of milk triglycerides enhances the resistance to *L. monocytogenes* but not *S. enteritidis* infection. In vitro, *S. enteritidis* is less susceptible to fatty acids than *L. monocytogenes* (Fig. 1). Probably, the concentration of gastric bactericidal fatty acids is insufficient to decrease survival of salmonella.

In vitro, fatty acid susceptibility of *E. coli* O157:H7 is comparable to that of *S. enteritidis*, whereas *C. jejuni* resembled *L. monocytogenes* in this respect (Fig. 1). To predict the in vivo relevance of milk fat triglycerides in protecting against infection with *E. coli* or *C. jejuni*, we incubated these pathogens in vitro with gastric chyme of rats fed low butter fat or high butter fat diets. As is shown in Fig. 2, no lowering of viable counts of *E. coli* O157:H7 was observed in the gastric chyme of rats fed the low butter fat diet. *E. coli* O157:H7 is resistant to pH 5 (see Fig. 1), which was approximately the pH of the low fat chyme (pH 4.8 ± 0.1). Viability of *E. coli* was not decreased in the gastric chyme of rats fed the high butter fat diet (pH 4.6 ± 0.2). Therefore, it is unlikely that milk fat triglycerides protect against *E. coli* O157:H7. In contrast, decreased viable counts of *C. jejuni* were observed in gastric contents of rats fed the low butter fat diet (Fig. 2). A 100- to 1000-fold decrease in viability of *C jejuni* was observed in the gastric chyme of rats fed high milk fat. Therefore, it is most likely that milk fat triglycerides are useful in preventing *C. jejuni* infections.

As already stated above, unsaturated fatty acids are also active as bactericidal agents, at least for Gram positive bacteria, *C. jejuni* and *H. pylori* (Kabara et al., 1972; Sprong et al., 2001; Thompson, Cockayne, & Spiller, 1994). To test the hypothesis that edible oils rich in unsaturated fatty acids also protect against food-borne infections, we performed a strictly controlled experiment using corn oil as dietary fat. Corn oil triglycerides predominantly contain $C_{18:1}$ and $C_{18:2}$ fatty acids. Faecal excretion of *L. monocytogenes* was not affected by the amount of corn oil in the diet (Table 2). The listeria-mediated increase in urinary NO_x excretion was not influenced by high corn oil intake (Table 2), implying that translocation of *L. monocytogenes* was not affected by the amount of corn oil intake. High corn oil intake did not decrease diarrhoea (Table 2). Thus, triglycerides containing $C_{18:1}$ and $C_{18:2}$ are not protective against listeria infection. Preduodenal lipases prefer short and medium chain fatty acids as substrates rather than long chain fatty acids (Hamosh, 1984). To check

whether $C_{18:1}$ and $C_{18:2}$ were substantially liberated in the stomach, the fatty acid profile was determined in control rats subjected to the fasting/feeding procedure. In stomachs of rats fed low corn oil diets, $C_{16:0}$, $C_{18:1}$ and $C_{18:2}$ were detected in the low millimolar range (Fig. 3). The concentration of these fatty acids was significantly increased in rats on the high corn oil diet. Thus, though high amounts of listericidal fatty acids were released on high corn oil diets, no protection against *L. monocytogenes* infection was observed. In vitro, $C_{18:1}$ is less listericidal than $C_{10:0}$ and $C_{12:0}$ (Fig. 1), and may therefore not be protective in vivo. However, in vitro listericidal activity of $C_{18:2}$ was not significantly different from $C_{10:0}$ and $C_{12:0}$ (Fig. 1) and effective listericidal activity may therefore be expected in vivo. At this time, we do not have a solid explanation for the different in vivo listericidal activities of butter fat and corn oil. We think that distinct physical–chemical characteristics of fatty acids may play a role. Hernell, Staggers, and Carey (1990) reported that ultracentrifugation of duodenal contents resulted in a oil layer, interfacial layers, a water phase and a solid pellet, each with a different free fatty acid content. Little is known about the physical chemical behaviour of various dietary lipids during gastric digestion. Perhaps, distribution of fatty acids among the phases and thereby their availability depend on their physical–chemical characteristics. Further research is required to test this hypothesis.

In humans, gastric digestion of triglycerides is catalysed by gastric lipases while in rats gastric lipolysis is mediated by lingual lipases. However, the specificity of human gastric lipase is comparable to that of rat lingual lipase (Hamosh, 1984). In adult subjects, gastric lipolysis accounts for 10–20% of total fat digestion (Cohen, Morgan, & Hofmann, 1971) whereas in neonates 40–60% of dietary fat is digested in the stomach (Moreau et al., 1988). Since bactericidal activity of the lipid fraction of gastric aspirates has been shown in neonates (Isaacs, Kashyap, Heird, & Thormar, 1990), bovine milk triglycerides may indeed protect humans against lipid-sensitive gastrointestinal pathogens. Further research is necessary to investigate the human relevance.

Table 2
Effect of amount of dietary corn oil on faecal pathogen excretion, pathogen-induced cumulative urinary NO_x excretion and pathogen-induced water content of faeces of rats orally administered with 5×10^9 CFU *Listeria monocytogenes*[a]

	Low corn oil	High corn oil
Faecal listeria ($\log_{10}$ CFU/g wet faeces)		
Day 1	5.93±0.21	5.86±0.45
Day 3	3.91±0.35	3.76±0.31
Increase in urinary NO_x (μmol/3 days)[b]	20.0±4.2	19.1±4.0
Increase in faecal water content (g/100 g wet faeces)[c]	17±2	16±4

[a] Values are means±SEM ($n = 8$)
[b] The cumulative excretion of nitrite and nitrate (NO_x) over 3 days after listeria administration was subtracted by the basal NO_x excretion
[c] The faecal water content after *L. monocytogenes* administration was subtracted by the water content of faeces before pathogen administration.

3.2. Membrane lipids

The minor milk lipid fraction, i.e. approximately 1% of total fat, consists of membrane lipids. Two major classes of membrane lipids are present in milk, i.e. phosphoglycerides and sphingolipids. Phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) are the major phosphoglycerides, comprising 34% and 25%

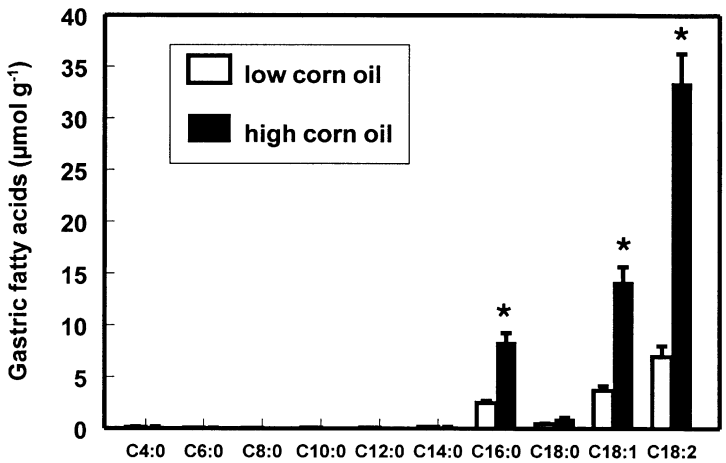

Fig. 3. Gastric free fatty acids in rats consuming diets containing low or high amounts of corn oil. Results are expressed as mean±SEM ($n = 4$). *$P < 0.05$ as tested with the Student's *t*-test for independent data.

of the membrane lipids, respectively (Christie, Noble, & Davies, 1987). Phosphatidyl inositol (6% of membrane lipids), and phosphatidyl serine (3%) are the minor ones. Sphingomyelin (SM) is the major sphingolipid in milk fat (24% of membrane lipids); smaller amounts of glycosphingolipids, i.e. glucosylceramide (5%), lactosylceramide (3%), and gangliosides (0.5%), are also present (Christie et al., 1987).

Membrane lipids of epithelial cells may serve as pathogen receptor. For example, helicobacter subspecies have been shown to bind PE, lactosyl ceramide, and the gangliosides gangliotetrosylceramide, gangliotriaosylceramide and GM3 (Bitzan et al., 1998; Angstrom et al., 1998). Membrane lipid receptors have also been shown for *E. coli* subspecies (Idota & Kawakami, 1995), campylobacter (Sylvester, Philpott, Gold, Lastovica, & Forstner, 1996; Grange, Erickson, Levery, & Francis, 1999), rotavirus (Guo et al., 1999), and bacterial toxins (Kolsto-Otnaess, Laegreid, & Ertresvag, 1983). Bovine colostrum, which is rich in membrane lipids, has been shown to prevent binding of *H. pylori* to immobilised membrane lipids (Bitzan et al., 1998). Though less active as human milk lipids, bovine milk gangliosides prevented fluid accumulation caused by cholera toxin in rabbit small bowel loops (Laegreid, Kolsto-Otnaess, & Fuglesang, 1986). Therefore, dietary membrane lipids may exert antimicrobial activity by preventing adhesion of pathogens to the mucosa and may thus protect against colonisation and translocation of pathogens. However, the human relevance still needs to be established.

Membrane lipids are also digested in the gastrointestinal tract to compounds that might possess antimicrobial activity. Phosphoglycerides are digested by pancreatic phospholipase A_2, which hydrolyses the fatty acid bound at the sn-2 position, yielding a free fatty acid and a lysophosphoglyceride. Recently, we tested the bactericidal activity of lysophosphatidyl choline and lysophosphatidyl ethanolamine in vitro, using the Gram positives *L. monocytogenes* and *Clostridium perfringens*, and the Gram negatives *C. jejuni*, *S. enteritidis* and *E. coli* O157:H7 (Sprong et al., 2001). The Gram positive bacteria were only moderately vulnerable to lysophosphoglycerides, whereas the Gram negatives were not sensitive. In contrast to triglycerides, bovine milk phosphoglycerides are probably insignificant in preventing gastro-enteritis.

Though the metabolism of sphingolipids in the gastrointestinal tract has not been completely elucidated, sphingosine and ceramide have been identified as products of sphingolipid metabolism (Nyberg, Nilsson, Lundgren, & Duan, 1997; Schmelz, Crall, Laroque, Dillehay, & Merrill, 1994). Schmelz et al. (1994) also suggested that lysosphingomyelin could be formed in the gastrointestinal tract from sphingomyelin. Recently, we tested the bactericidal activities of ceramide, sphingosine and lysosphingomyelin in vitro using *L. monocytogenes*, *C. perfringens*, *E. coli* O157:H7, *S. enteritidis* and *C. jejuni* (Sprong et al., 2001). Ceramide was not bactericidal at the tested concentration of $100\,\mu mol\,L^{-1}$ (Fig. 1). In contrast, $100\,\mu mol\,L^{-1}$ lysophingomyelin appeared highly bactericididal against *C. jejuni*, *L. monocytogenes* and *C. perfringens*, and moderately lowered viable counts of *E. coli* and *S. enteritidis*. In addition, $100\,\mu mol\,L^{-1}$ sphingosine decreased viable counts of all pathogens tested.

Though our in vitro results suggest that membrane lipids may protect against gastrointestinal pathogens, the in vivo relevance is not known. We showed that a diet containing lactase-treated sweet butter milk powder (10.4 μmol membrane lipids/g diet) protected rats against colonisation and translocation of *L. monocytogenes* compared with a diet containing lactase-treated skim milk powder (1.7 μmol membrane lipids/g diet) (Sprong, Hulstein, Van der Meer, 1998). These results suggest that milk membrane lipids may indeed protect against gastrointestinal infections. Further research is necessary to test the efficacy of milk membrane lipids in protecting against gastrointestinal infections in vivo and to determine the molecular mechanism of their protective effect.

4. Conclusion

Our results indicate that bovine milk fat triglycerides containing $C_{10:0}$ and $C_{12:0}$ protect against food-borne gastrointestinal infections caused by *L. monocytogenes*. Based on our ex vivo experiments with gastric contents, milk fat triglycerides may also protect against *C. jejuni*. Though $C_{18:1}$ and $C_{18:2}$ were bactericidal in vitro, our in vivo results with corn oil indicate that these fatty acids are not effective in vivo. Our in vitro results showed powerful bactericidal activities of digestion products of sphingolipids. Together with the observation that colonisation of *L. monocytogenes* is decreased in rats fed diets based on sweet butter milk powder (membrane lipid rich) compared with rats fed skim milk diets (low content of membrane lipids), these results suggests that cows' milk sphingolipids may also protect against gastrointestinal infections. Further research is needed to test the antimicrobial efficacy of these bovine milk fat components in humans.

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