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Bactericidal Activities of Milk Lipids

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The bactericidal capacity of digestion products of bovine milk triglycerides and membrane lipids was tested in vitro using *Escherichia coli* O157:H7, *Salmonella enteritidis, Campylobacter jejuni, Listeria monocytogenes*, and *Clostridium perfringens*. $C_{10:0}$ and $C_{12:0}$ fatty acids and digestion products of sphingolipids appeared to be effective bactericidal agents, whereas digestion products of phosphoglycerides were moderately bactericidal. Thus, milk fat sphingolipids and triglycerides, particularly those containing $C_{10:0}$ and $C_{12:0}$ fatty acids, may protect against food-borne gastroenteritis.

Diet may affect resistance to gastrointestinal infections by changing luminal concentrations of bactericidal agents. For example, bovine milk fat may prevent gastrointestinal infections because whole-milk consumption is associated with fewer gastrointestinal infections than is consumption of low-fat milk (14). Table 1 summarizes the lipid composition of milk. Triglycerides are digested to fatty acids and monoglycerides that are highly bactericidal in vitro (11, 13). 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 (12). Phosphoglycerides are digested by pancreatic phospholipase A_2, yielding free fatty acids and lysophosphoglycerides. Lysophosphatidylcholine (lysoPC) displays bacteriostatic and bactericidal capacity (8, 22). Although the metabolism of sphingolipids in the gastrointestinal tract has not been completely elucidated, sphingosine (SPH) and ceramide have been identified as products of sphingolipid metabolism (16, 19). Schmelz et al. (19) also suggested that lysophingomyelin (lysoSM) could be formed in the gastrointestinal tract from sphingomyelin (SM). SPH is toxic for skin pathogens (3, 4).

The bactericidal activity of lipids depends on their nature, e.g., chain length, and on the bacterial strain involved (3, 8, 13). Generally, gram-positive bacteria are lipid sensitive whereas gram-negative bacteria are not (13). Recently, however, lipid-sensitive gram-negative bacteria have been described (17, 18). Distinct experimental conditions, such as the test medium used, pH, lipid concentrations, and probably also the bacterial strains tested, may be responsible for the observed differences in lipid sensitivity of gram-negative bacteria.

To (re-)evaluate the efficacy of bovine milk lipids in preventing gastroenteritis, we tested the bactericidal activity of digestion products on a molar basis at physiologically relevant pH. The pathogens studied were *Campylobacter jejuni* (clinical isolate), *Salmonella enteritidis* phage type 4 (clinical isolate; NIZO B1241 from the collection of our institute), *Escherichia coli* O157:H7 (clinical isolate), *Clostridium perfringens* (isolated from surface water; NIZO B542), and *Listeria monocytogenes* (clinical isolate); all are food-borne pathogens highly prevalent in The Netherlands (15). Stock solutions of bacteria were routinely stored at −80°C in culture media (listed in Table 2) containing 20% (vol/vol) glycerol. Bacterial cells were cultured as described before (20) using the agar plates, broth, and culture conditions described in Table 2. Bactericidal activity of fatty acids (all obtained from Fluka, Buchs, Switzerland) was tested in citrate buffer at pH 5 (100 mmol of potassium citrate per liter) and 37°C as described before (20). Because the strictly anaerobic bacterium *C. perfringens* did not survive the washing procedure in an aerobic environment, overnight cultures were directly diluted in sterile saline and immediately placed in an anaerobic cabinet (Coy Laboratory Products Inc., Ann Harbor, Mich.). Membrane lipids were purchased from Sigma (St. Louis, Mo.). Stock solutions (4 mmol/liter) of L-α-phosphatidylcholine (PC; type XVI from egg yolk), L-α-phosphatidylethanolamine (PE; type III from egg yolk), L-α-lysophosphatidylethanolamine (lysoPE; type I from egg yolk), SM (from bovine brain), SPH (from bovine brain SM), and ceramide (type III, from bovine brain SM) were prepared in absolute ethanol and stored at −20°C. In addition, stock solutions (4 mmol/liter) of L-α-lysoPC (type I from egg yolk), lysoSM, and galactosylsphingosine (galSPH; psychosine from bovine brain) were made in water and stored at −20°C. Bactericidal activity of membrane lipids was tested in saline at pH 7. Incubates contained 2.5 or 4% (vol/vol) ethanol for comparative and dose-dependent studies, respectively. Ethanol did not affect the bactericidal activity of lipids. The conditions of the bactericidal assay are described in Table 2. The detection limit of this assay was 2 $\log_{10}$ CFU/ml, i.e., 1 CFU in a 10-μl sample. Bactericidal activity was considered biologically significant when a reduction of ≥0.5 $\log_{10}$ CFU/ml was observed compared with control incubates.

We have shown previously that gastric triglyceride digestion, rather than intestinal digestion, was important in protection against *L. monocytogenes* (20). Therefore, the bactericidal assay was performed at pH 5 to mimic gastric acidity. This is important since protonation increases the bactericidal activity of fatty acids (17). In humans, gastric digestion yields predominantly fatty acids. Therefore, we tested only fatty acids. *C. perfringens* was completely killed by the use of buffer at pH 5 only, whereas all of the other pathogens tolerated this pH (Table 3).

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TABLE 1. Reported lipid concentrations in bovine whole milk (4% fat)

Lipid	Concn (μmol/liter)[a]	Reference(s)
Triglycerides	57,143	12
Phosphoglycerides		
PC	70–150	6, 24, 25
PE	101	6
Phosphatidylinositol	17	6
Phosphatidylserine	9	6
Sphingolipids		
SM	70–125	6, 23–25
Glucosylceramide	8	12
Lactosylceramide	17	12
Gangliosides	14	23

[a] When amounts were published in grams, moles were calculated using average molar weights of 700 g/mol for triglycerides, 787 g/mol for PC, 767 g/mol for PE, 835 g/mol for phosphatidylinositol, 710 g/mol for phosphatidylserine, 751 g/mol for SM, and 747 g/mol for glycosphingolipids.

The bactericidal activity of fatty acids depended on chain length ($P < 0.0001$) and the bacterial strain ($P < 0.0001$), as tested by two-way analysis of variance (SPSS/PC+, version 2.0; SPSS, Chicago, Ill.). $C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{16:0}$, and $C_{18:0}$ fatty acids were not bactericidal at 500 μmol/liter. $C_{14:0}$, $C_{18:1}$, and $C_{18:2}$ fatty acids only killed *C. jejuni* and *L. monocytogenes*, whereas $C_{10:0}$ and $C_{12:0}$ fatty acids were toxic to all of the test pathogens. The finding that predominantly medium-chain and unsaturated C_{18} fatty acids were bactericidal is comparable to those of other studies (11, 13). The observation that $C_{10:0}$ and $C_{12:0}$ fatty acids are toxic to gram-negative species is supported by others, who showed lipid sensitivity of several *E. coli* strains, *Salmonella typhi, Vibrio cholerae, Shigella sonnei*, and *Helicobacter pylori* (17, 18). Besides bactericidal activity, these agents show antiviral activity (21). Assuming 10 to 20% gastric release of fatty acids (1, 5, 7) and a dilution factor of 1.1 to 1.3 by saliva and gastric juice (1, 5), the calculated concentration of gastric free fatty acids in adult humans after consumption of 200 ml of bovine whole milk is approximately 4.5 to 11 mmol/liter. In rats consuming milk fat, $C_{10:0}$ and $C_{12:0}$ fatty acids constitute 8 and 7% of the gastric free fatty acids, respectively (20). Assuming a ratio of fatty acid release similar to that in humans, the calculated gastric concentration ranges from 0.4 to 0.9 and from 0.3 to 0.7 mmol/liter for $C_{10:0}$ and $C_{12:0}$ fatty acids, respectively. Both agents were highly bactericidal at 0.5 mmol/liter. Theoretically, $C_{10:0}$ and $C_{12:0}$ fatty acids liberated during gastric digestion of milk fat may prevent gastrointestinal infections. Recently, we showed that high milk fat intake indeed improves resistance to *L. monocytogenes* infection, but not to *S. enteritidis* infection, in rats (20). Therefore, further research is necessary to study the in vivo relevance of digestion products of milk fat triglycerides to the prevention of gastrointestinal infections.

Because phosphoglycerides are digested in the small intestine, bactericidal capacity was tested at neutral pH. Bactericidal activity of phosphoglycerides depended on the lipid ($P < 0.0001$) and the bacterial strain ($P < 0.0001$). Neither PC nor PE affected survival at a concentration of 500 μmol/liter. We observed moderate bactericidal activity of lysoPC, which killed only *L. monocytogenes* and *C. perfringens*, and lysoPE, which killed *C. perfringens* (Table 3). Consistent with earlier reports, gram-negative bacteria were not affected (8, 22). Because it has been suggested that gastric juice of healthy humans contains phospholipase A_2 activity (10), listericidal activity of lysophospholipids was also tested at pH 5. The listericidal activity of lysoPC was independent of pH: killing at pH 5 (viable listeriae, 4.94 ± 0.09 $\log_{10}$ CFU/ml versus 7.31 ± 0.02 $\log_{10}$ CFU/ml in control incubates) was comparable to that at pH 7 (viable listeriae, 5.00 ± 0.03 $\log_{10}$ CFU/ml versus 7.25 ± 0.03 $\log_{10}$ CFU/ml in control incubates). Assuming 60 to 90% hydrolysis (1, 2) and a dilution factor of 2 to 3 due to gastrointestinal juices (1), the concentration of lysophospholipids in the small intestine after consumption of 200 ml of whole milk probably varies between 40 and 95 μmol/liter. The reported human postprandial duodenal lysoPC concentration of 2 to 5 mmol/liter (2, 9) is predominantly derived from bile PC. Thus, the contribution of milk phosphoglycerides to the prevention of gastrointestinal infections is of minor importance compared with that of bile PC.

The bactericidal activity of 100 μmol of sphingolipids per liter depended on the nature of the lipid ($P < 0.0001$) and the bacterial strain ($P < 0.0001$). SM did not affect the survival of bacteria, and neither did ceramide, which is the major digestion product of sphingolipids (16, 19). LysoSM and galSPH showed bactericidal activity (Table 3) against all of the pathogens tested. Although formation of lysosphingolipids in the

TABLE 2. Storage media, agar plates, liquid culture media, and culture conditions of bacterial strains used in this study

Strain	Storage medium	Culturing on agar plate		Liquid culture		Bactericidal conditions
		Agar plate used	Culture conditions	Broth	Conditions	
L. monocytogenes	BHI[a,b]	PALCAM[c]	36 h, 37°C, aerobic	BHI	18 h, 37°C, aerobic	2 h, 37°C, aerobic[f]
C. perfringens	AC broth[b]	AC agar	18 h, 37°C, anaerobic[d]	AC	18 h, 37°C, anaerobic	2 h, 37°C, anaerobic[g]
S. enteritidis	BHI	Brilliant green agar[e]	18 h, 37°C, aerobic	BHI	18 h, 37°C, aerobic	2 h, 37°C, aerobic
C. jejuni	BHI	Charcoal cefoperazone, deoxycholate agar[e]	24 h, 40°C, CO_2 enriched with Anaerocult C[c]	BHI	24 h, 37°C, CO_2 enriched	2 h, 37°C, aerobic
E. coli O157:H7	BHI	BHI agar	18 h, 37°C, aerobic	BHI	18 h, 37°C, aerobic	2 h, 37°C, aerobic

[a] BHI, brain heart infusion broth.
[b] Obtained from Difco (Detroit, Mich.).
[c] Purchased from Merck (Darmstadt, Germany).
[d] Using Anoxomat (Mart by Microbiology Automation, Lichtenvoorde, The Netherlands).
[e] Obtained from Oxoid (Basingstoke, United Kingdom).
[f] Using a shaking water bath.
[g] Using an anaerobic cabinet.

TABLE 3. Bactericidal activity of digestion products of milk lipids

Lipid	Viability[a] of:				
	E. coli	*S. enteritidis*	*C. jejuni*	*L. monocytogenes*	*C. perfringens*
Fatty acids[b,c]					
None	7.30 ± 0.00*	7.30 ± 0.00*	6.46 ± 0.02*	7.48 ± 0.00*	2.00 ± 0.00
$C_{10:0}$	3.90 ± 0.11†	4.68 ± 0.05†	2.00 ± 0.00†	2.00 ± 0.00†	2.00 ± 0.00
$C_{12:0}$	5.99 ± 0.00‡	6.41 ± 0.03‡	2.00 ± 0.00†	2.00 ± 0.00†	2.00 ± 0.00
$C_{14:0}$	7.30 ± 0.00*	7.30 ± 0.00*	3.29 ± 0.00‡	4.84 ± 0.05‡	2.00 ± 0.00
$C_{18:1}$	7.30 ± 0.00*	7.30 ± 0.00*	4.45 ± 0.01§	5.97 ± 0.03§	2.00 ± 0.00
$C_{18:2}$	7.30 ± 0.00*	7.30 ± 0.00*	3.41 ± 0.12‡	2.39 ± 0.21†	2.00 ± 0.00
Phosphoglycerides[b,d]					
None	7.48 ± 0.00	7.48 ± 0.00	7.48 ± 0.00*	7.48 ± 0.00*	7.03 ± 0.03*
LysoPC	7.48 ± 0.00	7.48 ± 0.00	7.01 ± 0.01†[f]	6.00 ± 0.02†	4.54 ± 0.19†
LysoPE	7.48 ± 0.00	7.48 ± 0.00	7.22 ± 0.01‡[f]	7.28 ± 0.01*	5.25 ± 0.11‡
Sphingolipids[d,e]					
None	7.25 ± 0.03*	7.24 ± 0.01*	7.28 ± 0.00*	7.33 ± 0.02*	6.91 ± 0.02*
SPH	2.00 ± 0.00†	3.31 ± 0.03†	2.00 ± 0.00†	2.00 ± 0.00†	3.73 ± 0.06†
LysoSM	6.22 ± 0.02‡	6.43 ± 0.00‡	2.00 ± 0.00†	2.00 ± 0.00†	2.00 ± 0.00‡
GalSPH	2.36 ± 0.23†	4.09 ± 0.32§	2.00 ± 0.00†	2.00 ± 0.00†	2.00 ± 0.00‡

[a] Viability of bacteria was determined by plating after incubation for 2 h at 37°C. Data are expressed as mean ± standard deviation $\log_{10}$ CFU per milliliter of triplicate incubations. Different superscript symbols for a pathogen and a lipid class reflect significant differences ($P < 0.05$) determined by the Student-Newman-Keuls test for multiple comparisons.
[b] At 500 μmol/liter.
[c] Tested at pH 5.
[d] Tested at pH 7.
[e] At 100 μmol/liter.
[f] Though statistically significant, this value is not considered biologically significant because the reduction in viable bacteria is less then 0.5 $\log_{10}$ CFU/ml.

intestine has been suggested (19), its actual existence has not been proven. Thus, the in vivo relevance of lysosphingolipids to the prevention of gastrointestinal infections is unclear. SPH, which is also formed in the intestine from dietary sphingolipids (16, 19), was the most potent bactericidal sphingolipid (Table 3). Even a concentration of 25 μmol/liter was highly toxic for *C. jejuni* and *L. monocytogenes* and, to a lesser extent, for *E. coli* and *S. enteritidis* (Table 4). SPH also kills fungi (3, 4). Thus, SPH acts as a broad-spectrum antimicrobial agent in vitro. Its in vivo relevance is difficult to interpret, since gastrointestinal sphingolipid digestion has not been completely elucidated. Given the observation that 2% of ingested SM is present in the rat small intestine lumen as SPH (16) and using an SM concentration of 100 μmol/liter in bovine whole milk (mean of the values listed in Table 1), the luminal concentration of SPH will be less than 2 μmol/liter. Extracellular mucosal enzymes, however, seem to play an important role in SM digestion (16). It has been suggested that SPH, rather than ceramide, is taken up by enterocytes (19). Therefore, it can be speculated that SPH formed at the apical site of mucosal cells may protect the mucosa from invading pathogens. Luminal concentrations of SPH may therefore not be representative of local mucosal events. Further research is required to test this hypothesis and the in vivo relevance of dietary sphingolipids to protection against gut infections.

TABLE 4. Dose-dependent killing of pathogens by SPH at pH 7.0

Concn (μmol/liter)	Viability[a] of:			
	E. coli	*S. enteritidis*	*C. jejuni*	*L. monocytogenes*
0	7.48 ± 0.01	7.52 ± 0.01	7.56 ± 0.01	7.49 ± 0.01
25	4.62 ± 0.02	5.41 ± 0.03	2.00 ± 0.00	3.01 ± 0.14
50	3.48 ± 0.07	3.86 ± 0.02	2.00 ± 0.00	2.00 ± 0.00
100	2.00 ± 0.00	3.35 ± 0.03	2.00 ± 0.00	2.00 ± 0.00
150	2.00 ± 0.00	2.96 ± 0.06	2.00 ± 0.00	2.00 ± 0.00

[a] Viability of bacteria was determined by plating after incubation for 2 h at 37°C. Data represent mean ± standard deviation $\log_{10}$ CFU per milliliter of triplicate incubations.

In conclusion, $C_{10:0}$ and $C_{12:0}$ fatty acids, SPH, and lysosphingolipids are powerful bactericidal agents in vitro. Therefore, milk fat sphingolipids and triglycerides containing $C_{10:0}$ and $C_{12:0}$ fatty acids might enhance resistance against intestinal pathogens. Further research is required to establish the contribution of these dietary components to host defenses in the gastrointestinal tract.

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