

Sweet buttermilk intake reduces colonisation and translocation of *Listeria monocytogenes* in rats by inhibiting mucosal pathogen adherence

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Abstract

The bovine milk fat globule membrane (MFGM) contains several antimicrobial components with proven efficacy *in vitro*, but *in vivo* evidence is scarce. The present study was performed to determine the efficacy of the bovine MFGM *in vivo*. Rats were fed diets based on bovine skimmed milk powder (low in MFGM) or bovine sweet buttermilk powder (high in MFGM). After dietary adaptation, rats were orally infected with *Salmonella enteritidis* or *Listeria monocytogenes*. Whereas sweet buttermilk powder did not protect rats against infection with *S. enteritidis*, it protected against *L. monocytogenes*, as shown by a lower colonisation and translocation of this pathogen. Protection coincided with higher listericidal capacity of gastric and caecal contents. The digestion products of phosphoglycerides and sphingomyelin are bactericidal *in vitro*. To study their role, rats were fed diets containing either 0.1% phosphatidylcholine or sphingomyelin, or a control diet. After dietary adaptation, rats were infected with *L. monocytogenes*. Since *Listeria* colonisation was not affected by these diets, phosphoglycerides and sphingomyelin are not involved in the protective effect of sweet buttermilk. Additional *in vitro* experiments were performed to further explore the mechanism of the beneficial effects of sweet buttermilk. Inhibition of the adherence of *L. monocytogenes* to the intestinal mucosa is the most likely explanation, since sweet buttermilk powder inhibited the binding of *L. monocytogenes* in both a haemagglutination assay and a Caco-2 cell adherence assay. In conclusion, sweet buttermilk powder, which is rich in MFGM, protects against *L. monocytogenes* infection in rats, probably by preventing adherence of this pathogen to the intestinal mucosa.

Key words: Milk fat globule membrane: Sphingolipids: Infection: Adhesion inhibition

Food-borne gut infections are still an important problem for public health, even in developed countries. The WHO reported in 2007 that in industrialised countries, the percentage of the population suffering from food-borne diseases each year is up to 30%⁽¹⁾. This is probably an underestimation, since recent data from a Dutch study indicate that the incidence of infectious intestinal disease is 964/1000 person-years⁽²⁾. Due to the increasing resistance of pathogens to antibiotics, new strategies to prevent and control gastrointestinal infections need to be developed. Consumption of dietary compounds that are antimicrobial, either directly or indirectly after digestion, may be an attractive alternative approach to reduce gut infections.

Whole milk intake in children is associated with fewer intestinal infections than is consumption of low-fat milk⁽³⁾, suggesting that bovine milk fat may contain antimicrobial agents or precursors. We have previously shown that bovine milk fat TAG indeed protect rats against *Listeria* infection⁽⁴⁾. In addition, the bovine milk fat globule membrane contains a broad range of antimicrobial agents⁽⁵⁾. For instance, membrane glycoproteins such as mucin and lactadherin, and

glycosylated sphingolipids, may prevent adherence of pathogens as has been shown for the human milk fat globule membrane^(6,7). In addition, the milk lipid membrane contains components with bactericidal activity, such as xanthine oxidase⁽⁵⁾. Moreover, we have previously shown that digestion products of milk fat globule phospholipids and sphingolipids possess bactericidal activity *in vitro*⁽⁸⁾. Most research has been performed *in vitro*, or in animal studies using isolated milk fat globule membrane components such as mucin and lactadherin⁽⁹⁾. In the present study, we investigated the anti-infective effect of the milk fat globule membrane in a rat model. Because sweet buttermilk is a rich source of milk fat globule membrane components compared with skimmed milk, we used these dairy products to test the antimicrobial activity of milk fat globule membrane components *in vivo* and performed exploratory mechanistic studies *in vitro*. Based on previous *in vitro* and *in vivo* studies with milk components^(4,8), *Salmonella enteritidis* was chosen as a human-relevant Gram-negative pathogen with moderate sensitivity to bactericidal digestion products of milk components, whereas *Listeria monocytogenes* was chosen as

Abbreviations: CFU, colony-forming units; HA, haemagglutination.

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a Gram-positive pathogen with high sensitivity to these antimicrobial agents.

Experimental methods

Animals

The experimental protocol was approved by the Ethical Committee on Animal Testing of Wageningen University and was in accordance with national law. Male specific pathogen-free Wistar rats (Hsd/Cpb:WU; Harlan), 9 weeks old, were housed in metabolism cages to ensure quantitative measurement of food intake and adequate collection of faeces. Rats were fed a standard rat diet (18 g/100 g protein; Harlan Teklad TRM Rat/Mouse Diet) until they received test diets. Temperature (22–24°C), relative humidity (50–60%) and dark–light cycle (light 06.00–18.00 hours) were kept constant.

Infection Expt 1: pathogen colonisation

Since rats are intolerant to lactose, skimmed milk powder and sweet buttermilk powder were treated with lactase (Maxilact LX500; DSM), which converts lactose to glucose and galactose. Lactase-treated skimmed milk powder (containing (per 100 g) 30.5 g protein, 2.2 g residual lactose, <1 g fat, 24 mg cholesterol and 37.8 mmol Ca) and sweet buttermilk powder (containing (per 100 g) 23.7 g protein, 2.9 g residual lactose, 5.3 g fat, 87 mg cholesterol and 29.8 mmol Ca) were manufactured from the same batch of bovine milk by the pilot plant of our institute. Skimmed milk and sweet buttermilk were treated with lactase before spray drying. Table 1 summarises the composition of the diets. The final composition of the diets was 20% protein, 20% fat, 2% cellulose, 4.5% vitamin and mineral mix and 53.5% carbohydrates. The phosphate content of phospholipids was determined to quantify the enrichment of

Table 1. Composition of the experimental diets in infection Expts 1 and 2

	Sweet buttermilk diet (g/kg)	Skimmed milk diet (g/kg)
Milk powder*	650	500
Lactose†	–	8
Cholesterol‡	–	0.36
Butterfat§	–	34
Constant components	277	277
Dextrose¶	73	180.64

* Powders were added to the diet as 65 and 50% to keep the protein (23.7% for buttermilk powder and 30.5% for skimmed milk powder) and Ca contents (298 µmol/g buttermilk powder and 378 µmol/g skimmed milk powder) constant. The final protein content of the diets derived from milk powder was 15%, whereas the final Ca content was 190 mmol/kg diet.

† Lactase (BDH Chemicals Limited) was added to correct for the higher residual lactose content of sweet buttermilk powder. The final lactose content of the diets was 18 g/kg diet.

‡ Cholesterol (Sigma) was added to skimmed milk diets to correct for the higher cholesterol content of buttermilk powder. Final cholesterol content of the diets was 0.57 g/kg.

§ Membrane-lipid free butterfat (manufactured by the pilot plant of our institute) was added to skimmed milk diets to correct for the fat contents of buttermilk powder. The final butterfat content of both diets was 3.4%.

|| Constant components of the diets (per kg): 46 g acid casein (Nestlé), 20 g cellulose (Arbocel®; Internatio B.V.), 45 g vitamin and mineral mix (according to AIN-93 recommendations⁽¹¹⁾) without Ca and 166 g maize oil (Albert Hein).

¶ Obtained from Avébé.

the sweet buttermilk diet with milk fat globule membrane components. To this end, membrane lipids were extracted from the diets according to Bligh & Dyer⁽¹⁰⁾. Evaporated lipid extracts were destructed (15 min at 180°C) in 5.8 M-perchloric acid and 4.9 M-H₂O₂. Phosphate was subsequently measured spectrophotometrically according to Chen *et al.*⁽¹¹⁾. The sweet buttermilk diet contained (per 100 g) 1034 µmol phosphorus-containing membrane lipids, whereas the skimmed milk diet contained 173 µmol/100 g diet. Diets were supplied as a porridge with 68% dry weight (dry diets mixed with double-distilled water). Rats were given free access to food and demineralised water. Food intake was measured every 2 d. Body weight was measured every 2–4 d.

After 14 d of habituation of rats to housing conditions and diets, rats (*n* 8 per diet group, with a mean body weight of 300 g) were orally dosed by gastric gavage with 9.6 log₁₀ colony-forming units (CFU) of *L. monocytogenes* 4b (NIZO B1242) or 9.9 log₁₀ CFU of *S. enterica* serovar *enteritidis* phage type 4 (NIZO B1241) in 1 ml saline containing 3 g/100 ml NaHCO₃. Both strains are clinical isolates. Bacteria were cultured and stored as described previously⁽⁸⁾. The inoculum was enumerated by plating on PALCAM (Merck) for *Listeria* and on modified brilliant green agar (Oxoid) supplemented with sulphomandelate (Oxoid) for *Salmonella*. Excretion of viable pathogens was measured in fresh faecal samples collected at days 1, 3 and 5 for *Listeria* and days 1, 3 and 5 for *Salmonella*. Samples were homogenised in 1 ml saline. Then, tenfold dilutions were plated on PALCAM or modified brilliant green agar for the determination of *Listeria* and *Salmonella*, respectively. Plates were cultured aerobically at 37°C as described previously⁽⁸⁾. Detection limits were 2.6 log₁₀ CFU/g faeces.

Complete 24 h faecal samples were collected during 3 d pre-infection and 3 d post-infection. Faeces were lyophilised for dry weight determination. Diarrhoea was determined by calculating the water content of the faeces using measurements of cations, as described previously⁽⁴⁾.

Infection Expt 2: *Listeria* translocation

The sweet buttermilk and skimmed milk diets in this experiment were comparable with those in infection Expt 1. Rats (*n* 8 per diet group with a mean body weight of 335 (SEM 4) g) were acclimatised to the diets and housing conditions for 2 weeks, after which they were orally inoculated with 1 ml of 3% sodium bicarbonate in saline containing 9.7 log₁₀ CFU of *Listeria*. At 24 h after inoculation, rats were killed by inhalation of CO₂. The abdominal cavity was opened, and the spleen and a liver lobule were removed. Subsequently, the stomach was clamped at the cardiac and pyloric junctions. The intestine was ligated at the pyloric, ileocaecal and rectal side. Subsequently, the stomach was removed and its contents collected. Next, the small intestine was excised and divided into a proximal and a distal part of equal length, followed by collection of its contents in pre-weighed test-tubes containing 1 ml sterile saline. The intestines were longitudinally opened and washed in sterile saline to remove remaining debris. Subsequently, the mucosa of the

small intestine was scraped off using a spatula, followed by collection in a tube containing 1 ml sterile saline. The caecal and colonic contents were collected in vials, weighed, vigorously mixed, and a sample was transferred to pre-weighed test-tubes containing 1 ml sterile saline. Intestinal samples (mucosa and contents) were weighed and then homogenised (Ultraturrax Pro200; Pro Scientific, Inc.). Spleen and the liver lobule were homogenised in 1 ml sterile saline. The number of *Listeria* was enumerated as described for Expt 1. The remaining gastrointestinal contents were frozen at -20°C until use.

In vitro studies with gastric and intestinal contents

Gastric and intestinal samples were thawed, and pH was measured at 37°C using a Methrom 632 pH meter (Applikon). Bactericidal activity was measured as described previously⁽⁸⁾. In short, 40 μl gastric, caecal or colonic chyme were mixed with 60 μl sterile saline inoculated with $7 \log_{10}$ CFU of *L. monocytogenes*. The content weight of the small intestine incubated with *L. monocytogenes in vitro* was corrected for the dilution made by collecting the contents in 1 ml saline. Samples were taken at the start and after several time intervals during aerobic incubation at 37°C in a shaking water-bath. Control incubations with saline were used to assess spontaneous killing of *L. monocytogenes*. Viable pathogens were enumerated by plating tenfold dilutions on PALCAM agar. The detection limit was $5 \log_{10}$ CFU/l.

To investigate whether sphingolipid or phosphoglyceride digestion is responsible for the observed listericidal activity, 40 μl saline supplied with bovine buttermilk sphingomyelin (Matreya, Inc.), egg-yolk phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine or lysophosphatidylethanolamine (all obtained from Sigma) were added to 40 mg gastrointestinal contents. Because of the limited amount of gastrointestinal contents available, samples from all rats belonging to the same group were pooled. The final concentration of sphingomyelin was 150 $\mu\text{mol/l}$, whereas the final concentration of phosphoglycerides was 500 $\mu\text{mol/l}$. After an incubation period of 2, 6 or 18 h in a shaking water-bath at 37°C , 20 μl saline containing $7 \log_{10}$ CFU of *L. monocytogenes* were added. Bactericidal activity was determined in triplicate at several time intervals as described previously.

To determine whether the xanthine oxidase–lactoperoxidase system is responsible for the observed listericidal effects, the xanthine oxidase substrate hypoxanthine (500 $\mu\text{mol/l}$) and the lactoperoxidase substrate NaSCN (1 mM) were added to reconstituted milks (10 g powder/100 ml water) adjusted to pH 5 and pH 7. *Listeria* was added at a concentration of $5.7 \log_{10}$ CFU/ml.

The concentration of NEFA in gastric contents was measured using GC as described previously⁽⁴⁾.

Expt 3: feeding experiment with supplemented sphingomyelin or phosphatidylcholine

To further explore the role of membrane lipids in preventing *Listeria* infection, rats (n 8 per diet group) were fed a diet

containing no supplemental sphingomyelin or phosphatidylcholine or a diet supplemented with either 0.1% bovine buttermilk sphingomyelin (Matreya, Inc.) or 0.1% egg-yolk phosphatidylcholine (Sigma). The diets contained (per 100 g) 20 g casein, 50.4 g dextrose, 20 g maize oil, 2 g cellulose and 45 g vitamin and mineral mix. Vitamins and minerals were in accordance with AIN-93⁽¹²⁾, except for Ca, which was added to the diet at a concentration of 20 mmol/kg. Rats were acclimatised to the diets and housing conditions for 2 weeks, after which they were orally inoculated with 1 ml of 3% sodium bicarbonate in saline containing $9.8 \log_{10}$ CFU of *L. monocytogenes*. At 24 h after inoculation, rats were killed by inhalation of CO_2 . Gastrointestinal contents and mucosa were sampled and stored as described for Expt 2. In addition, the number of *Listeria* was quantified as described for infection Expt 1.

Pathogen adhesion studies in vitro

The capacity of sweet buttermilk powder to inhibit pathogen adherence was studied using a haemagglutination (HA) study and with Caco-2 cells. All *in vitro* studies were performed in triplicate. For the HA study, *L. monocytogenes* was cultured as described previously⁽⁸⁾. In brief, twofold dilutions of *L. monocytogenes* were incubated with 1% guinea pig erythrocytes (bioTRADING) for 1 h at room temperature to determine the highest dilution that induced HA. This concentration is defined as 1 HA unit. Sweet buttermilk powder and skimmed milk powder were dissolved in PBS at a concentration of 10 g/l. For HA inhibition, twofold dilutions of these milk powder solutions were incubated with an equal volume of 4 HA units of *Listeria*. After incubation for 1 h at room temperature, 1% guinea pig erythrocytes were added. The mixture was incubated for another 1 h at room temperature. The concentration of the milk powder solution that inhibited the HA activity of 4 HA units of *L. monocytogenes* was recorded as the minimal inhibitory concentration. *S. enteritidis* did not induce HA of guinea pig, horse, cow or human erythrocytes.

To study the inhibition of pathogen binding to Caco-2 cells, these cells were grown to near confluence in a twenty-four-well plate in minimal essential medium (Sigma) with 20% fetal bovine serum and without antibiotics. Sweet buttermilk powder and skimmed milk powder were dissolved in PBS at a concentration of 10 g/l. Thereafter, $7 \log_{10}$ CFU of *L. monocytogenes* or *S. enteritidis* were added to the wells simultaneously with 1 ml of the test solutions. After incubation at 37°C for 1 h, cells were washed three times with warm PBS. Caco-2 cells were lysed in MilliQ water. Lysates were plated on brain heart infusion (Difco) agar in order to count cell-associated bacteria.

Statistics

Results are presented as means with their standard errors. The studies are single-factor models with the type of diet as the only source of variation. Data were tested for normality with Shapiro–Wilk's test and checked for homogeneity with Levene's test. Normally distributed data were tested with

Student's *t* test, using Welch's estimate in the case of unequal variances (Expt 1 and HA and Caco-2 cell assay). Otherwise, data were tested with the Mann-Whitney *U* test (Expts 2 and 3). Listericidal activity over time was tested with ANOVA, with time as a covariant. The effect of diets on pathogen colonisation and translocation was tested one-sided; the effect of diets on food intake and growth was tested two-sided. Differences were regarded as significant if $P < 0.05$. Statistics were performed with a commercially available statistical package (Statistica '99 edition; StatSoft, Inc.).

Results

Infection Expt 1: effect of sweet buttermilk on the feed intake, growth and colonisation of Salmonella enteritidis and Listeria monocytogenes

The sweet buttermilk diet contained (per 100 g) 1034 μmol phosphorus-containing membrane lipids, whereas the skimmed milk diet contained 173 $\mu\text{mol}/100\text{ g}$ diet. This indicates that sweet buttermilk powder is indeed rich in the milk fat globule membrane compared with skimmed milk powder.

In the first infection study, the effect of sweet buttermilk on feed intake, growth and faecal pathogen excretion over time as a marker of colonisation was determined. Before the oral administration of *S. enteritidis*, rats fed the diet containing sweet buttermilk powder consumed less than rats fed the skimmed milk diet (14.7 (SEM 0.4) *v.* 17.1 (SEM 0.6) g/d, for sweet buttermilk and skimmed milk, respectively; $P < 0.05$). In addition, food consumption after infection was lower in rats fed the sweet buttermilk diet (13.5 (SEM 0.3) *v.* 15.2 (SEM 0.05) g/d for sweet buttermilk and skimmed milk, respectively; $P < 0.05$). Though food intake differed, body-weight gain was not affected by the diet (before infection 3.5 (SEM 0.3) g/d; after inoculation 2.2 (SEM 0.3) g/d). Inoculation with *S. enteritidis* did not result in diarrhoea (not shown). Faecal excretion of *S. enteritidis* was not significantly affected by the diet (Fig. 1).

Food intake was also affected by the diet before oral inoculation with *L. monocytogenes* (15.4 (SEM 0.6) and 17.0 (SEM 0.4) g/d for sweet buttermilk and skimmed milk, respectively; $P < 0.05$), as well as after infection (13.1 (SEM 0.7) *v.* 15.2 (SEM 0.3) for sweet buttermilk and skimmed milk, respectively; $P < 0.05$). Again, growth was not affected by the diet (before infection 3.9 (SEM 0.2) g/d; after inoculation 1.8 (SEM 0.3) g/d). Inoculation with *L. monocytogenes* did not induce diarrhoea (not shown). Faecal excretion of *L. monocytogenes* was significantly decreased in rats fed buttermilk powder compared with skimmed milk powder, indicating that the intestinal colonisation or growth of this pathogen was diminished (Fig. 1).

Infection Expt 2: effect of sweet buttermilk on the feed intake, growth, colonisation and translocation of Listeria monocytogenes

In the second infection study, rats were killed 24 h after oral inoculation with *L. monocytogenes* to determine the amount of pathogens in the gastrointestinal tract as a marker of

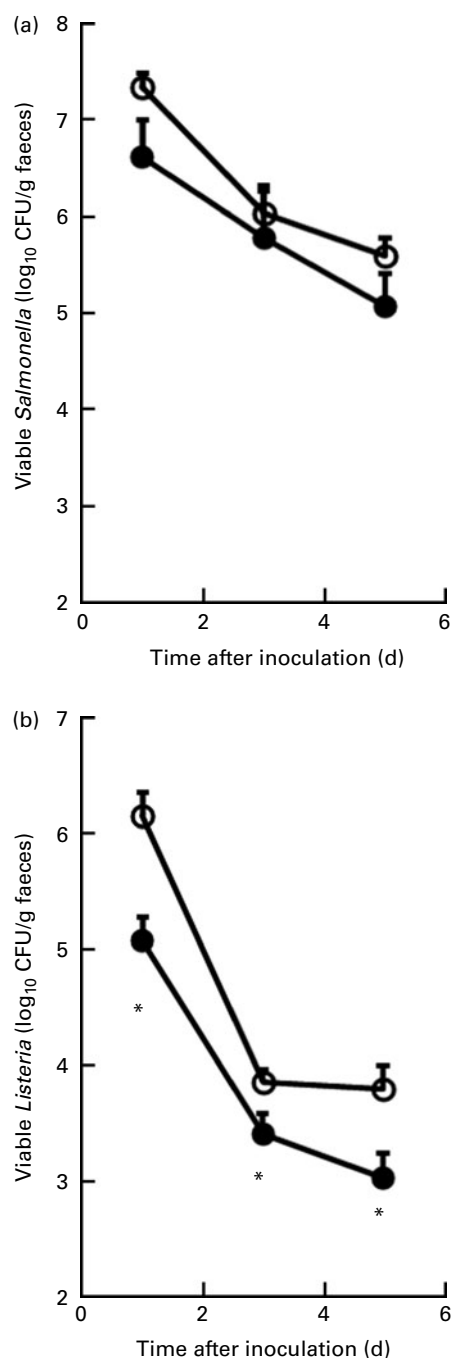


Fig. 1. Faecal excretion of pathogens after oral administration of (a) 8×10^9 colony-forming units (CFU) of *Salmonella enteritidis* or (b) 4×10^9 CFU of *Listeria monocytogenes* in rats fed a skimmed milk (—○—) or sweet buttermilk (—●—) diet. Faecal pathogens were measured by plating techniques. Values are means, with their standard errors represented by vertical bars (n 8). * Mean values were significantly different from those of the skimmed milk group ($P < 0.05$; Student's *t* test).

colonisation and the number of pathogens in extra-intestinal organs as a marker of translocation. In this experiment, neither food intake (mean 14.6 g/d) nor body-weight gain (mean 2 g/d) was affected by the diet. As shown in Table 2, relatively low numbers of *L. monocytogenes* were detected in the lumen of the small intestine of rats fed the skimmed milk diet,

Table 2. *Listeria* counts in organs 24 h after infection†
(Mean values with their standard errors, *n* 8)

	Skimmed milk		Buttermilk	
	Mean	SEM	Mean	SEM
Gastrointestinal contents				
Proximal small intestine	3.05	0.21	2.62*	0.18
Distal small intestine	3.89	0.14	3.96	0.08
Caecum	6.91	0.35	5.47*	0.5
Colon	6.98	0.36	5.60*	0.45
Faeces	7.41	0.21	5.93*	0.44
Mucosa				
Proximal small intestine	3.75	0.10	3.45*	0.16
Distal small intestine	3.88	0.19	3.29*	0.24
Organ				
Spleen	3.03	0.26	2.50*	0.09
Liver	3.04	0.20	2.57*	0.05

* Mean values were significantly different from those of the skimmed milk group ($P < 0.05$; Mann–Whitney *U* test).

† Data for faeces and liver are given as \log_{10} colony-forming units (CFU)/g wet weight, and for all other gastrointestinal contents and organs as \log_{10} CFU/total contents.

whereas the lumen of the caecum and colon contained high amounts. No viable *Listeria* could be detected in the stomach (not shown). The sweet buttermilk diet significantly decreased the number of viable luminal *L. monocytogenes* in the proximal small intestine, caecum and colon. Faecal numbers of *Listeria* were also lower (Table 2), which is in line with infection Expt 1. In addition, the number of viable *L. monocytogenes* colonising the mucosa tended to decrease in the proximal small intestine ($P = 0.06$) and was significantly decreased in the distal small intestine (Table 2). Moreover, translocation of *L. monocytogenes* to the spleen and liver was significantly lower in rats fed the sweet buttermilk diet (Table 2).

Listericidal capacity of gastrointestinal contents

The stomach possessed high listericidal capacity (Fig. 2). Listericidal activity was significantly enhanced in rats fed the sweet buttermilk diet compared with the skimmed milk diet. The higher listericidal activity could not be explained by gastric acidity, since gastric pH was significantly higher ($P < 0.05$) in rats fed the sweet buttermilk diet (5.21 (SEM 0.10)) compared with the skimmed milk diet (4.62 (SEM 0.21)). Analysis of NEFA patterns showed that butyric acid, medium-chain and long-chain fatty acids were liberated in the stomach. However, the concentration of individual gastric fatty acids was not higher in rats fed the buttermilk diet (not shown), indicating that the enhanced listericidal capacity of the stomach cannot be explained by bactericidal fatty acids.

The addition of hypoxanthine and NaSCN to the solutions of milk powders did not kill *L. monocytogenes* (not shown). When the same experiment was performed in the presence of gastric contents, again, no enhanced killing of *L. monocytogenes* was observed. This indicates that the xanthine oxidase–lactoperoxidase system is not active in the milk powders.

Pre-incubation of pooled stomach contents with phosphatidylcholine, phosphatidylethanolamine and lysophosphoglycerides for 2, 6 or 18 h did not enhance the bactericidal activity of gastric contents during the 2 h incubation period with *L. monocytogenes* (not shown), showing that digestion products of these phospholipids are not involved in the observed differential bactericidal activity of gastric contents. In addition, pre-incubation of pooled gastric samples with sphingomyelin before the addition of *Listeria* did not enhance listericidal capacity (not shown). This implies that digestion products of sphingolipids are also not responsible for the enhanced bactericidal activity of gastric contents of rats fed the sweet buttermilk powder.

Hardly any listericidal activity was observed in the contents of the small intestine, caecum and colon of rats fed the skimmed milk diet. Table 3 shows the number of viable *L. monocytogenes* after an incubation period of 6 h. Listericidal activity was not enhanced in the chyme of the small intestine and colon of rats fed the sweet buttermilk diet. A small, but significant, increase in listericidal activity was observed for the caecal contents of rats fed the sweet buttermilk diet. Except for a small decrease in caecal pH (skimmed milk 6.37 (SEM 0.12) and sweet buttermilk 5.80 (SEM 0.05); $P < 0.05$), acidity of the intestinal contents was not affected by the diet (proximal small intestine: pH 6.08 (SEM 0.04) *v.* 6.06 (SEM 0.03); distal small intestine: pH 6.87 (SEM 0.08) *v.* 6.77 (SEM 0.07); colon: pH 6.18 (SEM 0.07) *v.* 6.12 (SEM 0.05), for skimmed milk and sweet buttermilk, respectively). Pre-incubation of pooled caecal contents with 150 $\mu\text{mol/l}$ sphingomyelin before the administration of *L. monocytogenes* did not enhance bactericidal activity during the 6 h incubation period with *L. monocytogenes* (not shown).

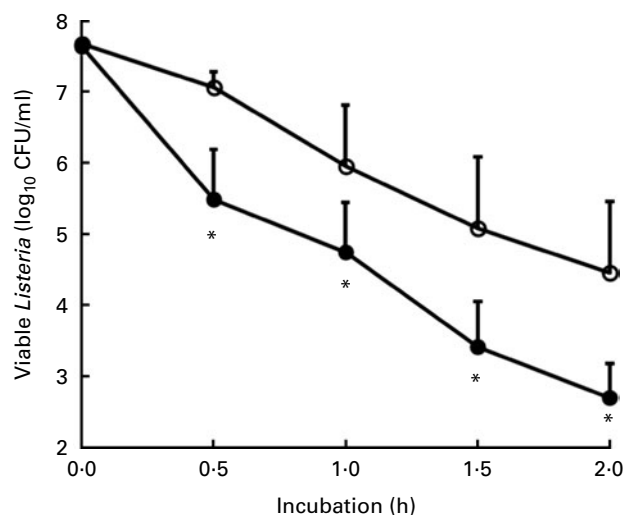


Fig. 2. Listericidal activity of the gastric contents of rats fed a skimmed milk (—○—) or sweet buttermilk (—●—) diet. Killing was measured by incubating saline-diluted chyme (1:1, w/v) with 10^7 colony-forming units (CFU)/ml of *Listeria monocytogenes* at 37°C. Viable *Listeria* were determined by plating on PALCAM agar. Values are means, with their standard errors represented by vertical bars (*n* 8). Listericidal activity was significantly greater in the sweet buttermilk group as determined by ANOVA with time as a covariant ($P < 0.05$). * Mean value was significantly different from that of the skimmed milk group ($P < 0.05$; Student's *t* test).

Table 3. Listericidal activities of the intestinal contents of rats fed either a skimmed milk or sweet buttermilk diet (Mean values with their standard errors, *n* 8)

Intestinal contents	Skimmed milk				Sweet buttermilk			
	<i>t</i> = 0 h		<i>t</i> = 6 h		<i>t</i> = 0 h		<i>t</i> = 6 h	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Log ₁₀ CFU/ml								
Proximal small intestine	7.54	0.02	7.14	0.15	7.57	0.01	7.28	0.03
Distal small intestine	7.61	0.01	7.47	0.03	7.60	0.01	7.49	0.02
Caecum	7.60	0.01	7.23	0.07	7.60	0.01	6.85*	0.07
Colon	7.53	0.04	7.62	0.09	7.61	0.01	7.48	0.09

CFU, colony-forming units.

*Mean values were significantly different from those of the skimmed milk group (*P* < 0.05; Mann–Whitney *U* test).

Infection Expt 3: feeding experiment with supplemented sphingomyelin or phosphatidylcholine

Since sphingomyelin-degrading enzymes are located in the mucosa^(13–16), their activity in collected luminal contents may not be sufficient to convert sphingolipids to bactericidal sphingosine *in vitro*. Therefore, an infection experiment was performed in rats fed a diet exclusively enriched in sphingomyelin. However, dietary supplementation of 0.1% sphingomyelin did not affect *Listeria* counts in intestinal contents, intestinal mucosa, mesenteric lymph nodes and liver (not shown), indicating that sphingomyelin does not affect the colonisation and translocation of *Listeria*. In addition, 0.1% phosphatidylcholine in the diet did not influence *Listeria* colonisation and translocation in the gastrointestinal tract (not shown).

Inhibition of pathogen adherence *in vitro*

Both skimmed milk powder and sweet buttermilk powder showed the inhibition of *L. monocytogenes*-induced HA (Fig. 3) and also decreased the number of Caco-2 cell-associated *L. monocytogenes*. However, sweet buttermilk showed a higher inhibition of pathogen adherence, since the minimal inhibitory concentration in the HA assay was about fourfold lower than that of skimmed milk and there were about threefold lower numbers of adhered *L. monocytogenes* in the Caco-2 cell assay (Fig. 3). The number of Caco-2 cell-associated *S. enteritidis* was not reduced by the presence of sweet buttermilk powder (Fig. 3).

Discussion

The present study shows that the intake of sweet buttermilk powder (rich in milk fat globule membrane) in rats increases the resistance to *L. monocytogenes* infection compared with skimmed milk consumption (low amount of milk fat globule membrane compounds). The enhanced resistance to *Listeria* infection coincided with an enhanced listericidal capacity of gastric and caecal contents. Several components of the milk fat globular membrane could be responsible for this protection. First, digestion products of phosphoglycerides, i.e. lysophosphoglycerides, showed antimicrobial activity *in vitro*⁽⁸⁾. Approximately 60–90% of ingested and biliary

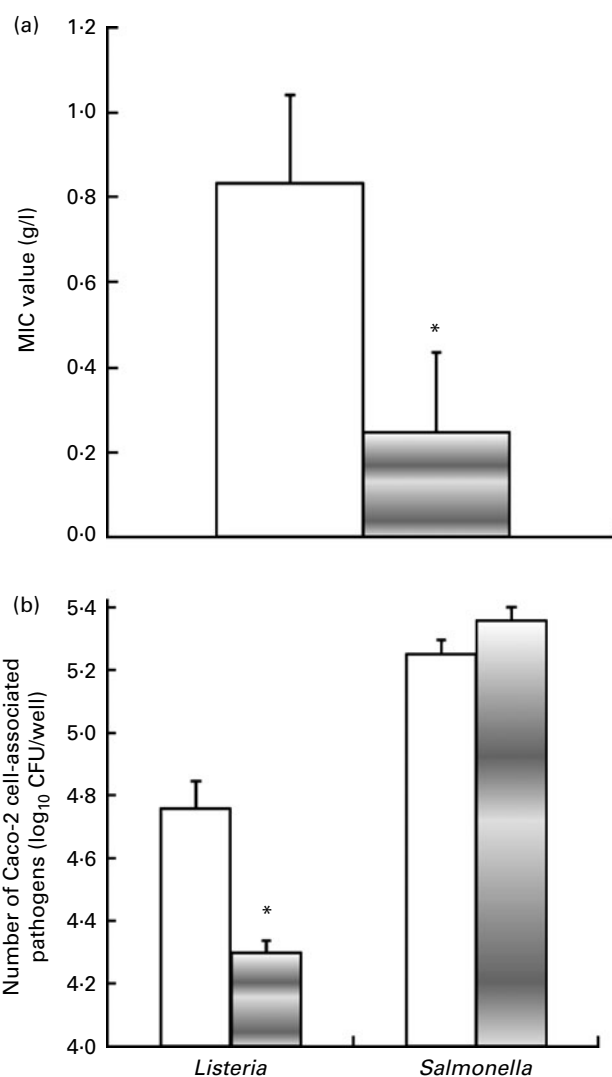


Fig. 3. Inhibition of pathogen binding to cellular receptors. (a) The minimal inhibitory concentration (MIC) of skimmed milk (□) and sweet buttermilk (■) in the *Listeria*-induced haemagglutination assay. (b) Binding of *Listeria monocytogenes* or *Salmonella enteritidis* to Caco-2 cells in the presence of either the skimmed milk or sweet buttermilk powder (10 g/l). CFU, colony-forming units. Values are means, with their standard errors represented by vertical bars. *Mean values were significantly different from those of the skimmed milk group (*P* < 0.05; Student's *t* test).

phosphoglycerides are hydrolysed^(17,18) in the gastrointestinal tract. Pancreatic phospholipase A₂ (PLA₂) plays an important role in the digestion of phosphoglycerides. In addition, PLA₂ is present in the gastric glands of rats⁽¹⁹⁾, and PLA₂ activity is observed in the gastric juice of guinea pigs⁽²⁰⁾. However, the possibility of antimicrobial compounds being endogenously formed from phosphoglycerides in the lumen of the gastrointestinal tract is refuted by the following observations: (1) the lack of antimicrobial activity of the intestinal contents of rats fed the sweet buttermilk diet; (2) the lack of enhanced antimicrobial activity after the addition of (lyso)phosphoglycerides to the gastrointestinal contents of rats fed the sweet buttermilk diet; (3) the lack of anti-infective effect of dietary phosphatidylcholine. Thus, another mechanism must be responsible for the protective effect of sweet buttermilk powder.

Digestion products of sphingolipids are bactericidal *in vitro*⁽⁸⁾. It can thus be speculated that endogenously formed antimicrobial sphingolipid derivatives accounted for the observed protective effect of sweet buttermilk powder. Alkaline sphingomyelinase is responsible for the release of ceramide from sphingomyelin, whereas ceramidase is responsible for the hydrolysis of ceramide to sphingosine and a fatty acid^(13,14). Ceramide and sphingosine are indeed identified as intestinal degradation products^(21,22). Sphingosine, in contrast to ceramide, acts as a very powerful antimicrobial agent *in vitro*⁽⁸⁾. However, this explanatory mechanism is refuted by the following observations. (1) The pattern of the listericidal activity of gastrointestinal contents does not follow the reported activity of sphingomyelin-degrading enzymes in rats (high in the proximal jejunum and lower in the other parts of the rat gastrointestinal tract) and does not match with the corresponding concentrations of sphingolipid metabolites in these gastrointestinal compartments^(13–16). (2) Addition of sphingomyelin to gastrointestinal contents did not enhance listericidal activity. (3) Dietary sphingomyelin did not protect against *Listeria* infection in rats. This is in line with the experiments performed by Possemiers *et al.*⁽²³⁾, who reported that sphingosine did not show antibacterial effects in the Simulator of the Human Intestinal Microbial Ecosystem, an *in vitro* simulation of the human intestinal environment.

An alternative explanation could be that membrane lipids act as emulsifiers lowering the fat droplet size, and thereby facilitate gastric fat digestion⁽²⁴⁾. Recently, we showed that C_{10:0} and C_{12:0} liberated from milk fat TAG during milk fat digestion protected rats against *L. monocytogenes* infection⁽⁴⁾. Therefore, enhanced gastric fat digestion could lead to higher concentrations of listericidal fatty acids. However, the fatty acid concentration in the stomach was not increased in rats fed the sweet buttermilk diet, indicating that the enhanced resistance towards *L. monocytogenes* cannot be explained by an increased gastric release of listericidal fatty acids.

Xanthine oxidase is another proposed antimicrobial compound of the milk fat globule membrane⁽⁵⁾. This enzyme is still active at gastric pH⁽²⁵⁾. However, addition of the xanthine oxidase substrate hypoxanthine (500 µM) with or without the lactoperoxidase substrate NaSCN (1 mM) to solutions of the milk powders and to gastric contents did not enhance the

gastric *Listeria*-killing capacity, indicating that the observed listericidal activity of gastric contents of rats fed sweet buttermilk is not caused by the xanthine oxidase–lactoperoxidase system. Which compound in sweet buttermilk is responsible for the enhanced listericidal activity of gastric contents of rats fed the sweet buttermilk powder is not known and needs further investigation.

The small increase in listericidal activity and lower pH in caecal contents suggest bacterial fermentation of non-digestible sweet buttermilk components, such as glycoconjugates. This was further evidenced by the visual observation that caeca of rats fed sweet buttermilk were more swollen and filled with gas than rats fed the diets with skimmed milk powder (not shown). This requires further investigation.

Several compounds of the milk fat globule membrane can prevent pathogen binding to mammalian cell surface receptors⁽⁵⁾. In the present study, we showed that sweet buttermilk powder had a lower minimal inhibitory concentration value for inhibiting *Listeria*-induced HA and resulted in a lower number of Caco-2 cell-associated *L. monocytogenes* than skimmed milk powder, indicating that components of the milk fat globule membrane in sweet buttermilk prevent the binding of *Listeria* to cellular receptors. This suggests that the mechanism of protection against *Listeria* infection may at least be partly due to the inhibition of pathogen binding. Interestingly, sweet buttermilk powder did not reduce the number of Caco-2 cell-associated *S. enteritidis*. This agrees with the lack of the effect of sweet buttermilk powder on *S. enteritidis* infection *in vivo*. The differential effect of sweet buttermilk on *L. monocytogenes* *v.* *S. enteritidis* adherence *in vitro* and infection *in vivo* might be explained by their different adhesion factors. Several mannose-sensitive adhesion factors (SEF 21, type 1 and type 3 fimbriae) have been described for *S. enteritidis*^(26,27). The presence of mannose in sugar chains of bovine milk fat globule membrane glycoproteins has been shown^(28–30). However, the present study indicates that these mannose residues are not accessible to *S. enteritidis* adhesion factors. For *L. monocytogenes*, internalin A binding to gut cell E-cadherin is an important step in the colonisation and translocation of *L. monocytogenes*⁽³¹⁾. The binding of *L. monocytogenes* to E-cadherin can be blocked by a 26 kDa N-terminal E-cadherin peptide⁽³²⁾. In addition, *L. monocytogenes* binding to enterocytes can be blocked by heparan sulphate⁽³³⁾. More research is necessary to unravel the milk fat globule membrane compound(s) responsible for the inhibition of the adhesion and infection of *L. monocytogenes*.

In conclusion, the present study shows that dietary sweet buttermilk protects against *Listeria* colonisation and translocation, but not against *Salmonella* infection. Which sweet buttermilk milk fat globular membrane-specific compounds induce resistance to *Listeria* infection is at present not known, but inhibition of pathogen adhesion to mucosal receptors is the most likely preventative mechanism. Further research is necessary to establish the contribution of individual milk fat globule membrane compounds to the reduced *Listeria* infection and to determine the precise mechanism of prevention.

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