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Dietary Milk-Fat-Globule Membrane Affects Resistance to Diarrheagenic *Escherichia coli* in Healthy Adults in a Randomized, Placebo-Controlled, Double-Blind Study[1,2]

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Abstract

Background: The milk-fat-globule membrane (MFGM) contains phospholipids and membrane glycoproteins that have been shown to affect pathogen colonization and gut barrier integrity.

Objective: In the present study, we determined whether commercial heat-treated MFGM can increase resistance to diarrheagenic *Escherichia coli*.

Methods: A randomized, placebo-controlled, double-blind, 4-wk parallel-intervention study was conducted in healthy adults. Participants were randomly assigned to a milk protein concentrate rich in MFGM [10 g Lacprodan PL-20 (Arla Foods Ingredients Group P/S), twice daily; $n = 30$; MFGM group) or a control [10 g Miprodan 30 (sodium caseinate), twice daily; $n = 28$]. After 2 wk, participants were orally challenged with live, attenuated diarrheagenic *E. coli* (10^{10} colony-forming units). Primary outcomes were infection-induced diarrhea and fecal diarrheagenic *E. coli* excretion. Secondary outcomes were gastrointestinal symptoms [Gastrointestinal Symptom Rating Scale (GSRS)], stool frequency, and stool consistency (Bristol Stool Scale).

Results: Diarrheagenic *E. coli* resulted in increased fecal output, lower relative fecal dry weight, increased fecal *E. coli* numbers, and an increase in stool frequency and gastrointestinal complaints at day 1 after challenge. MFGM significantly decreased the *E. coli*–induced changes in reported stool frequency (1.1 ± 0.1 stools/d in the control group; 1.6 ± 0.2 stools/d in the MFGM group; $P = 0.04$) and gastrointestinal complaints at day 2 (1.1 ± 0.5 and 2.5 ± 0.6 GSRS scores in the control and MFGM groups, respectively; $P = 0.05$). MFGM did not affect fecal wet weight and *E. coli* excretion at day 2 after challenge.

Conclusions: The attenuated diarrheagenic *E. coli* strain transiently induced mild symptoms of a food-borne infection, with complete recovery of reported clinical symptoms within 2 d. The present diarrheagenic *E. coli* challenge trial conducted in healthy adults indicates that a milk concentrate rich in natural, bioactive phospho- and sphingolipids from the MFGM may improve in vivo resistance to diarrheagenic *E. coli*. This trial was registered at clinicaltrials.gov as NCT01800396. *J Nutr* doi: 10.3945/jn.115.214098.

Keywords: dairy, diarrhea, *E. coli*, milk-fat-globule membrane, diet, infection, stool frequency

Introduction

Diarrhea is an important cause of morbidity and mortality in all regions of the world and among all ages (1). The annual number of enterotoxigenic *Escherichia coli* (ETEC)[6] cases in the developing world was estimated at 840 million, with another 50 million asymptomatic carriers in children aged <5 y (2). Because of the increasing resistance of bacterial pathogens to antibiotics (3), nutritional modulation of the resistance to gastrointestinal infections may form an attractive approach. The main biological functions of milk are provision of a balanced mixture of

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[6] Abbreviations used: CFAII, colonization factor antigen II; ETEC, enterotoxigenic *Escherichia coli;* GSRS, Gastrointestinal Symptom Rating Scale; LT, heat-labile; MFGM, milk-fat-globule membrane; ST, heat-stable.

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nutrients to the newborn and protecting the newborn from infectious diseases. Thus, milk contains numerous bioactive components that may promote resistance to infections. The anti-infection potential of milk can be attributed to antimicrobial activity, improvement in gut barrier function, and modulation of the immune response (4–8). The milk-fat-globule membrane (MFGM), which surrounds the lipid globules in milk, is assembled and secreted by the epithelial cells of the mammary gland. The membrane itself consists of a trilayer of phospholipids. The inner layer is derived from the endoplasmic reticulum, and the outer double layer derived from the cell membrane of the epithelial cells of the mammary gland. In addition to the phospholipids, the MFGM consists of a complex mixture of proteins, glycoproteins, enzymes, and neutral lipids. The phospholipids contribute ~40% of the lipids, and mono-, di-, and TGs contribute most of the remaining lipids; however, lactosyl and glycosyl cerebrosides as well as sphingomyelins are also important constituents of the MFGM lipid fraction (9).

Components derived from the MFGM were documented (10) to possess both antiviral and antibacterial activities in animals (6, 11) and in vitro (12–20). However, to our knowledge, evidence in humans is scarce. In children, the consumption of whole milk is associated with fewer intestinal infections than is the consumption of low-fat milk (21, 22). A recent published human clinical trial in young children showed that a supplementary intake of MFGM significantly decreased the number of febrile episodes, and thus MFGM holds the potential of being a valuable food ingredient for formulating food products with additional benefits in addition to being nutritious (22).

The present randomized, placebo-controlled, double-blind, 4-wk parallel-intervention study was designed to investigate the effect of a commercially available milk protein concentrate rich in MFGM (Arla Foods Ingredients Group P/S) on the resistance to diarrheagenic *E. coli* infection in humans. An important variable in intestinal resistance is the so-called colonization resistance. Colonization resistance is inversely related to the fecal excretion of a pathogen with time. The hypothesis is that milk protein concentrate rich in MFGM will decrease fecal diarrheagenic *E. coli* excretion and diarrhea severity.

Methods

Participants. This human intervention study was approved by the Medical Ethics Committee of Wageningen University, Netherlands, and registered at clinicaltrial.gov as NCT01800396. Healthy adults, aged 18–55 y, were recruited by posters mounted in public buildings. The 185 individuals who responded received a comprehensive brochure of the study and an invitation to visit the study's informational meeting (**Figure 1**). Individuals who gave their written informed consent (n = 128) were screened for eligibility. These individuals completed a general medical questionnaire. Those reporting the use of antibiotics, immunosuppressive drugs, antacids, laxatives, or antidiarrheal drugs in the past 3 mo before the study, current or previous underlying disease of the gastrointestinal tract, or lactose intolerance were excluded.

After passing this first screening, a nonfasting blood sample was obtained. Sera were prepared and analyzed for specific IgG against the specific immunogenic epitope of diarrheagenic *E. coli* colonization factor antigen II (CFAII), as described previously (23, 24). In addition, a fresh fecal sample was obtained and analyzed for diarrheagenic *E. coli* by qPCR, as described earlier (24). Individuals with detectable antibody titers against CFAII, induced by previous ETEC infections, or detectable fecal diarrheagenic *E. coli* counts were excluded from participation in the study because of likely resistance to the *E. coli* strain administered in the present study.

Randomization and stratification. The study had 2 primary outcomes: fecal diarrheagenic *E. coli* excretion ($\log_{10}$ copies/d) and fecal output

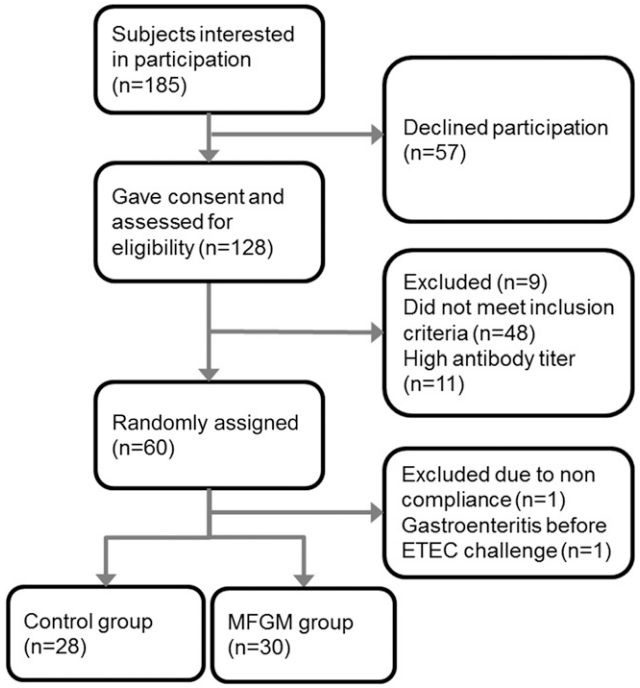

FIGURE 1 Flow diagram of study participants. ETEC, enterotoxigenic *Escherichia coli*; MFGM, milk-fat-globule membrane.

(g fecal wet weight/d). On the basis of 2-sided statistical testing for unpaired data, $\alpha = 0.05$ (chance of type I error) and $\beta = 0.20$ (chance of type II error), and to compensate for dropouts, 30 participants per group were included. Participants were stratified according to age, gender, fecal lactobacilli, and detectable antibody titers against CFAII (determined at screening) and randomly assigned to the MFGM or control group. Stratification and randomization was performed by a nonblinded person not involved in the study. The randomization code of each participant was kept in sealed envelopes, and the code was broken after finishing all laboratory and statistical analyses.

Dietary guidelines. A randomized, placebo-controlled, double-blind, 4-wk parallel-intervention study was performed. Participants were instructed to maintain their usual pattern of physical activity and their habitual diet throughout the entire study, but to abstain from dairy products and from products containing pre- and probiotics. Participants received a list of dairy products and products rich in pre- or probiotics in their paper subject diary. Dairy foods were not included in the diet because they contribute substantially to total daily calcium intakes (23). In a previous human trial, dietary calcium increased resistance to an oral diarrheagenic *E. coli* challenge (23). Dairy products were replaced by providing participants with low-calcium soy drinks. During the entire study, participants consumed daily a 250-mL soy-milk drink (Alpro-Soya Bio Nature). Drinks were consumed in the morning (125 mL) and evening (125 mL) during breakfast and dinner. The macronutrient composition (g/100 mL) of these soy-milk drinks was 3.8 g/kg protein, 2.3 g/kg fat, and 10.4 g/kg carbohydrates.

Two days before and 2 d after the diarrheagenic *E. coli* challenge, participants quantitatively reported all food and drinks consumed in an online nutrition diary. The weight of the food and drinks consumed was noted. If unknown, consumed amounts were expressed by household measures (e.g., a cup, a slice). Mean daily energy and macronutrient intakes in each period were calculated with the use of a computerized food-composition table [NEVO online version 2011/3.0; RIVM (National Institute for Public Health and the Environment)].

Dietary supplements. Participants were requested to mix 10 g of the milk concentrate rich in natural bioactive phospho- and sphingolipids (Lacprodan PL-20; MFGM group) (**Table 1**) or Miprodan 30 powder (control group) (Arla Foods Ingredients Group P/S) twice daily in their soy-milk drink during

TABLE 1 Nutritional composition of the supplements in the control and MFGM groups[1]

	Control, %	MFGM, %
Carbohydrates (lactose)	0.2	6.0
Protein	92	51
Fat	0.8	29
TGs	0.8	13
Phospholipids	0.0	16
Sphingomyelin	Not detected	4.3
Phosphatidylcholine	Not detected	4.3
Phosphatidylethanolamine	Not detected	3.5
Phosphatidylserine	Not detected	1.9
Phosphatidylinositol	Not detected	1.3
Other	Not detected	0.7
Minerals (ash)	3.7	6.0

[1] MFGM, milk-fat-globule membrane.

the entire study. Miprodan 30 is sodium caseinate manufactured from fresh pasteurized skimmed milk. The amino acid profile of Lacprodan PL-20 is similar to the amino acid profile in Miprodan 30.

Compliance with dietary guidelines and supplement intake. Participants were requested to daily record supplement intake and indicate whether they complied with the dietary guidelines in their online diary. To verify whether participants kept to the low-calcium dietary restrictions, fecal calcium was analyzed in homogenized wet fecal samples before the diarrheagenic *E. coli* challenge (on study days −1 or −2 depending on availability of fecal samples), as described previously (23).

Diarrheagenic E. coli challenge and fecal excretion. After an adaptation period of 2 wk to the intervention products, participants fasted for at least 4 h before the oral challenge with a live, but attenuated oral diarrheagenic *E. coli* strain (1.3×10^{10} CFUs) under supervision at NIZO Food Research, as previously described (23, 24). The *E. coli* strain used (E1392/75-2A) is a heat-labile/heat-stable (LT/ST) CFAII-positive variant of a previously enterotoxigenic O6:H16 LT'/ST' strain (25). CFAII-expressing strains are common in all regions of the world (26). Before (on day −1) and after (on days 1, 2, 3, 4, and 14) the diarrheagenic *E. coli* challenge, DNA was isolated from homogenized wet fecal samples, and diarrheagenic *E. coli* was quantified by qPCR as described previously (27).

Infectious diarrhea. Before (on days −1 and −2) and after (on days 1, 2, 3, 4, and 14) the diarrheagenic *E. coli* challenge, 24-h fecal samples were collected. Fecal samples were frozen at −20°C immediately after defecation and transported to the laboratory under frozen conditions, weighed, and homogenized by a Stomacher 3500 (Seward Limited, Worthing, West Sussex, United Kingdom); aliquots were stored at −20°C for later analyses. Diarrhea was determined by daily total fecal wet weight excretion (total fecal output), by the percentage of fecal dry weight as determined after freeze-drying (fecal consistency), and by having ≥3 loose stools/d together with a stool consistency ≥5 (Bristol Stool Scale score) (28).

Reported stool consistency, stool frequency, and gastrointestinal symptoms. During the entire study, participants daily reported information on stool consistency by using the Bristol Stool Scale (29) and on stool frequency in an online diary (LimeSurvey, An Open Source survey tool; LimeSurvey Project). Moreover, in the online diary, participants daily recorded symptoms according to the validated Gastrointestinal Symptom Rating Scale (GSRS) (30). The GSRS is a disease-specific instrument of 15 items combined into 5 symptom clusters: reflux, abdominal pain, indigestion, diarrhea, and constipation. The domain "diarrhea" consists of increased passage of stools, loose stools, and bowel urgency. The GSRS has a 7-point graded Likert-type scale where 1 = no discomfort, 2 = minor discomfort, 3 = mild discomfort, 4 = moderate discomfort, 5 = moderately severe discomfort, 6 = severe discomfort, 7 = very severe discomfort.

Immune response. Blood samples (10 mL) were taken by qualified staff of a local hospital at one time point before (day −4) and at 2 time points after (days 3 and 14) the diarrheagenic *E. coli* challenge. Sera were prepared by low-speed centrifugation (20 min at 3000 × *g* at 10°C) and stored at −80°C. Concentrations of specific IgG against CFAII in sera were determined by direct ELISA as described elsewhere (23). Concentrations of fecal calprotectin were determined by ELISA [CalproLab (ALP) Calprotectin ELISA Test; CalproAS], according to the manufacturer's instructions.

Data and statistical analysis. Per protocol analysis was performed for all outcomes and for all participants. Continuous variables are presented as means ± SEMs. The variables were analyzed by using general linear mixed models (data collected through questionnaires) or linear mixed models (data collected through biological sample analysis). If data did not follow a Gaussian distribution, they were transformed before modeling by using, e.g., logarithmic (used with the variables fecal wet weight and IgG) or square root transformation (used with the variable calprotectin) to obtain a good model fit to the data. The models had terms for time point, dietary treatment, and their interaction. In most cases, a covariate for the baseline value was also used. The mixed model had a random subject-wise intercept term. Hypotheses were tested using model contrasts, and *P* values were adjusted to avoid false-positive findings.

The analyses were conducted with R: A Language and Environment for Statistical Computing (version 3.0.1; R Development Core Team). The linear mixed models were computed using the R package Linear and Nonlinear Mixed Effects Models (version 3.1-109; J Pinheiro, D Bates, S DebRoy, D Sarkar, and R Development Core Team). The general mixed models were computed using the R package MASS (Modern Applied Statistics with S; WN Venables and BD Ripley, 4 ed., Springer, New York). Two-sided testing was used to detect the differences between means before compared with after *E. coli* challenge and between means of the MFGM group compared with the control group for all study outcomes. For both, *P* values <0.05 were considered significant.

Previous studies with the same challenge strain (23, 24, 27) indicated that the diarrheagenic *E. coli* challenge induces mild and short-lived symptoms. The severity of symptoms reaches a maximum at day 1 after challenge, with complete recovery within 2 d. Therefore, only the clinical symptom data obtained within 2 d after challenge were analyzed for their significance. Although we expected only to observe effects within the first 2 d after challenge, clinical outcomes were measured at various time points throughout the 14 d after challenge to confirm that all clinical outcomes returned to baseline values and to monitor any (unintended) symptoms induced by diarrheagenic *E. coli*.

Results

Baseline characteristics and compliance with diet and supplement intake

Two participants from the control group were excluded from further analysis (Figure 1) due either to insufficient compliance with study guidelines or reported gastroenteritis just before the

TABLE 2 Baseline characteristics of the healthy adults in the control and MFGM groups[1]

	Control (*n* = 28)	MFGM (*n* = 30)
Age, y	22 ± 1.1	22 ± 0.9
Weight, kg	70 ± 2.0	70 ± 2.0
Height, m	1.8 ± 0.0	1.8 ± 0.0
BMI, kg/m^2	22 ± 0.4	22 ± 0.4
Serum IgG CFAII, $\log_2$ dilutions	8.9 ± 0.4	9.0 ± 0.3
Fecal lactobacilli, $\log_{10}$ copies/g	9.0 ± 0.2	8.8 ± 0.2
Fecal calcium, mg/d	274 ± 44	331 ± 55

[1] Values are means ± SEMs. Participants were stratified according to age, gender, *Lactobacillus* spp. count, and CFAII titers at study start. CFAII, colonization factor antigen II; MFGM, milk-fat-globule membrane.

oral diarrheagenic *E. coli* challenge. Baseline characteristics are presented in **Table 2**. Participants were stratified by age, gender, lactobacilli counts, and serum CFAII titers. Diarrheagenic *E. coli* was not detected in fecal samples of any of the participants before challenge. The participants in the dietary groups did not differ in BMI.

Two days before and 2 d after the diarrheagenic *E. coli* challenge, participants quantitatively reported all food and drinks consumed in an online nutrition diary (**Table 3**). No significant difference in daily energy and macronutrient intake was found between the MFGM and control groups at day 2 after the diarrheagenic *E. coli* challenge.

Compliance with the dietary instructions was checked by analysis of calcium excretion in feces at day 2. No significant difference in total daily fecal calcium excretion was found between the MFGM and control groups at day 2 after the diarrheagenic *E. coli* challenge.

Primary outcomes

The diarrheagenic *E. coli* challenge significantly increased daily fecal wet weight at day 1 after challenge in both the control and MFGM groups ($P < 0.001$; **Figure 2**A).

The diarrheagenic *E. coli* challenge also resulted in a significant decrease in relative fecal dry weight at day 1 after the challenge compared with the relative fecal dry weight before the challenge, in both control and MFGM groups ($P < 0.001$; Figure 2B). Both daily fecal wet weight and relative fecal dry weight returned to baseline values after day 2 in both groups. No significant differences between the control and MFGM groups were detected (Figure 2A, B). However, a trend for a lower fecal output was seen in the MFGM group at day 2 compared with the control group ($P = 0.08$).

As anticipated, fecal diarrheagenic *E. coli* was detected in all of the participants at day 1 after challenge in both the control and MFGM groups. Concentrations of excreted diarrheagenic *E. coli* gradually decreased the first days after challenge. No significant differences in fecal diarrheagenic *E. coli* excretion between the control and MFGM groups were detected (Figure 2C).

Secondary outcomes

Immune response. Fecal calprotectin excretion was found to increase at day 1 after the diarrheagenic *E. coli* challenge ($P < 0.001$) (Figure 2D). Thereafter, the concentrations gradually decreased and returned to baseline values.

TABLE 3 Effect of an oral diarrheagenic *Escherichia coli* challenge on daily energy and nutrient intakes of healthy adults in the control and MFGM groups[1]

	Control ($n = 28$)		MFGM ($n = 30$)	
	Before	After	Before	After
Energy, kcal/d	2827 ± 162	2524 ± 164	2494 ± 118	2526 ± 115
Carbohydrates, % of energy	49 ± 2	49 ± 2	49 ± 1	50 ± 1
Fat, % of energy	31 ± 2	29 ± 1	32 ± 1	31 ± 1
Protein, % of energy	16 ± 1	16 ± 1	14 ± 1	15 ± 1
Alcohol, % of energy	3 ± 1	3 ± 1	3 ± 1	3 ± 1
Dietary fiber, % of energy	2 ± 0	2 ± 0	2 ± 0	2 ± 0

[1] Values are means ± SEMs. After a dietary adaptation period of 2 wk, participants were orally challenged with 1.3×10^{10} CFUs diarrheagenic *E. coli* on day 0. Two days before and 2 d after the diarrheagenic *E. coli* challenge, participants quantitatively reported all food and drinks consumed in a nutrition diary. Macronutrient intake was determined, assuming that the supplied soy products were consumed as instructed. MFGM, milk-fat-globule membrane.

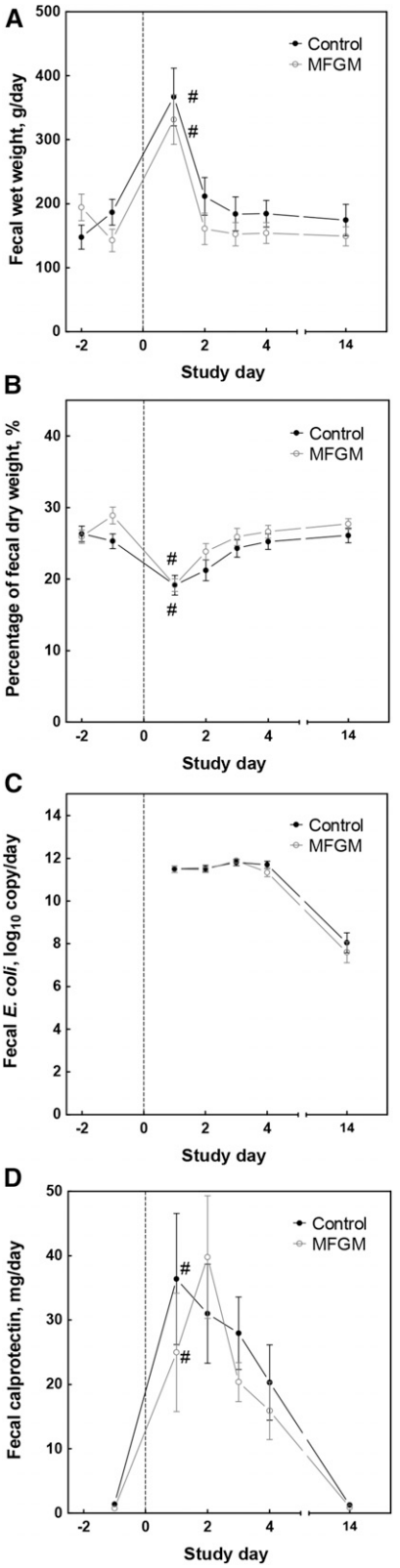

FIGURE 2 Effect of dietary MFGM and an oral diarrheagenic *Escherichia coli* challenge on total fecal wet weight (A), percentage of fecal dry weight (B), fecal diarrheagenic *E. coli* excretion (C), and fecal calprotectin (D) in healthy adults. After a dietary adaptation period of 2 wk, participants were orally challenged with 1.3×10^{10} CFUs diarrheagenic *E. coli* on day 0. Results are means ± SEMs, $n = 28$ (control) or 30 (MFGM). #Different from prechallenge, $P < 0.05$. MFGM, milk-fat-globule membrane.

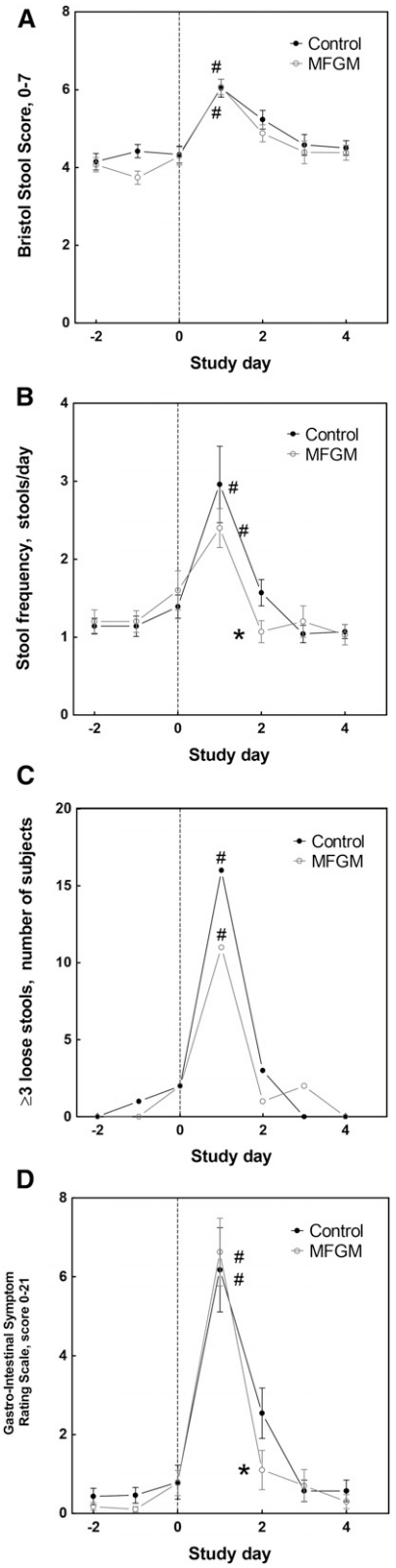

FIGURE 3 Effect of dietary MFGM and an oral diarrheagenic *Escherichia coli* challenge on daily stool consistency (daily maximum reported Bristol Stool Scale score (A), daily stool frequency (B), frequency of WHO-defined diarrhea [≥3 loose stools/d + stool consistency ≥5 (Bristol Stool Scale score)] (C), and daily gastrointestinal symptoms (Gastrointestinal Symptom Rating Scale domain diarrhea; score of 0–7 for increased passage of stools, loose stools, and urgent defecation) (D) in healthy adults. After a dietary adaptation period of 2 wk, participants were orally challenged with 1.3×10^{10} CFUs diarrheagenic *E. coli* on day 0. Results are means ± SEMs, $n = 28$ (control) or 30 (MFGM). #Different from prechallenge, $P < 0.05$. *Different from control at that time, $P < 0.05$. MFGM, milk-fat-globule membrane.

Serum CFAII-specific IgG also increased significantly 14 d after the diarrheagenic *E. coli* challenge when comparing baseline (354 ± 61 $\log_2$ dilutions in the control group and 375 ± 70 $\log_2$ dilutions in the MFGM group) and day 14 (6279 ± 2068 $\log_2$ dilutions in the control group and 2912 ± 489 $\log_2$ dilutions in the MFGM group) ($P < 0.001$). No effect of dietary treatment was observed.

Stool consistency, stool frequency, and WHO-defined diarrhea. The diarrheagenic *E. coli* challenge resulted in a significant increase in reported Bristol Stool Scale score at day 1 after challenge ($P < 0.001$) (**Figure 3**A). The diarrheagenic *E. coli* challenge resulted in a significant increase in stool frequency ($P < 0.001$). At day 2 after challenge, stool frequency was lower in the MFGM group than in the control group ($P = 0.04$) (Figure 3B). Both stool consistency and stool frequency returned to baseline values after day 2.

The diarrheagenic *E. coli* challenge also increased the number of participants reporting WHO-defined diarrhea at day 1 after challenge ($P < 0.001$). Sixteen of 30 participants (53%) in the control group and 11 of 28 participants (39%) in the MFGM group reported WHO-defined diarrhea at day 1. However, no significant effect of dietary treatment was reached for this variable (Figure 3C).

Gastrointestinal symptoms. The diarrheagenic *E. coli* challenge increased the GSRS sum score at day 1 ($P < 0.001$). Most predominant effects were observed in the GSRS domains diarrhea (loose stools, increased passage of stools, and urgent defecation) and abdominal pain (abdominal pain and abdominal distention). The diarrheagenic *E. coli* challenge resulted in an increase in diarrhea scores at day 1. At day 2, diarrhea scores were significantly lower in the MFGM group compared with the control group ($P = 0.05$) (Figure 3D). Diarrhea scores returned to baseline values after day 2.

Expected adverse events reported the first day after the diarrheagenic *E. coli* challenge included abdominal pain (72% of participants), abdominal distention (52% of participants), borborygmus (67% of participants), flatulence (77% of participants), increased passage of stools (64% of participants), loose stools (64% of participants), nausea (55% of participants), and urgent defecation (57% of participants) (31). The frequency of reported adverse events (other than reported through the GSRS) did not differ between the control and MFGM groups. No serious adverse events were reported during the study.

Discussion

In the present randomized, placebo-controlled, double-blind, 4-wk parallel-intervention study in healthy adults, a diarrheagenic *E. coli* challenge transiently induced mild symptoms of a food-borne infection. The severity of symptoms induced by the diarrheagenic *E. coli* challenge reached a maximum at day 1 after challenge, with complete recovery within 2 d. The diarrheagenic *E. coli* challenge resulted in increased fecal output, lower relative fecal dry weight, increased fecal diarrheagenic *E. coli* numbers (Figure 2), and an increase in reported stool frequency and gastrointestinal complaints at day 1 after the

challenge (Figure 3). Moreover, the diarrheagenic *E. coli* challenge resulted in an increase in specific antibody titers (Table 2) and fecal calprotectin (Figure 2) in the present study. Observed effects of the diarrheagenic *E. coli* challenge were similar to those observed in previous studies (23, 24, 27).

The milk protein concentrate rich in MFGM significantly decreased the diarrheagenic *E. coli*–induced changes in reported stool frequency and gastrointestinal complaints at day 2 after challenge. Although a trend was observed in the MFGM-induced decrease in fecal wet weight after the oral diarrheagenic *E. coli* challenge (AUC), dietary MFGM did not significantly affect the primary outcomes of fecal wet weight and fecal diarrheagenic *E. coli* excretion.

The mechanism by which MFGM could have modulated resistance to the diarrheagenic *E. coli* infection was not specifically addressed. However, because the tested milk protein concentrate is rich in the bioactive phospho- and sphingolipids derived from the MFGM, one might speculate that these specifically contributed to this protective effect. The MFGM is highly structured and contains unique polar lipids and membrane-specific proteins. Phospholipids and glycosphingolipids are quantitatively the most important polar lipids in MFGM. The phospholipids include phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine, whereas glycosphingolipids include sphingomyelin as the dominant species and gangliosides to a lesser extent (8).

To identify the mechanisms of action, the effects of phospholipids and MFGM on cytokines and chemokines was evaluated in Caco-2 cells in vitro (E van Hoffen, J Schloesser, unpublished results, 2013), but the observed effects were limited. This suggests that other mechanisms may be more important for the observed effects in the present study, such as reduction in pathogen adherence to the intestinal epithelium (decoy effect) or a direct bactericidal activity. In previous studies, components of the MFGM were shown to have antimicrobial activity and decoy activity and may improve gut barrier function (4–7). In vitro experiments and animal studies have shown that C10:0, C12:0, unsaturated C18 FAs, and sphingolipids can affect survival of *Campylobacter jejuni*, *Listeria monocytogenes*, *Clostridium perfringens*, *E. coli*, and *Salmonella enteritidis* (6, 11, 32). In addition to an antimicrobial and prebiotic activity, glycosylated and sialic acid–containing milk compounds are known to act as decoys for bacterial pathogens and toxins, which may prevent adherence of these pathogens to intestinal epithelial cells and subsequently protect against toxins and a broad range of bacterial pathogens in vivo (4, 33). In vitro experiments have shown that mucin (34), glycosylated sphingolipids, glycomacropeptides (13), and sialic acid (35) are able to prevent bacterial gut pathogens from binding to the epithelium.

In vivo evidence of the effects of MFGM on resistance to infections is scarce. In a previous study in children, whole-milk consumption was associated with fewer gastrointestinal infections than the consumption of low-fat milk (21). Children >1 y of age who were consuming low-fat milk as their only milk source in the 3 wk before illness had 5 times the risk of a doctor's visit for acute rotaviral or nonrotaviral gastrointestinal illness compared with children who consumed only whole milk during the same period. Moreover, the consumption of MFGM-enriched milk by young children was shown to have a protective effect against gastrointestinal infections, producing a significant decrease in the number of short febrile episodes (22).

In the present study, fecal diarrheagenic *E. coli* numbers were not affected by the milk protein concentrate rich in MFGM. However, stool cultures of infected participants might not necessarily reflect small intestinal colonization, but rather survival and multiplication of diarrheagenic *E. coli* in the large intestine. Indeed, in a previous study, dietary milk calcium decreased infectious diarrhea, whereas only marginal effects on fecal diarrheagenic *E. coli* excretion were observed (23). This discrepancy between fecal pathogen excretion and infectious diarrhea questions the current status of fecal pathogen excretion as a highly valid marker for nutritional immune enhancement (36).

The experimental human diarrheagenic *E. coli* challenge has proved to be invaluable as an important tool to evaluate the efficacy of vaccines or other interventions to protect against enteric diseases before moving on to field trials. However, when translating obtained results from experimental infections to natural infections, it should be noted that experimental infection studies are performed in adults. This fails to represent the population with the highest disease burden, children living in ETEC-endemic regions. In addition, the frequency and severity of disease observed in ETEC challenge studies are higher than those seen in naturally acquired illness associated with travel, likely due to the higher concentrations of inocula used (37). Finally, unlike wild-type strains, the attenuated diarrheagenic *E. coli* strain used in this study does not produce LT and/or ST enterotoxins (25).

In conclusion, compared with previous studies (23, 24, 27), the attenuated diarrheagenic *E. coli* strain transiently induced mild and short-lived symptoms of a food-borne infection with complete recovery of reported clinical symptoms within 2 d. The milk concentrate rich in phospho- and sphingolipids from the MFGM slightly inhibited the diarrheagenic *E. coli*–induced increase in reported stool frequency and gastrointestinal complaints, and a trend was observed for decreased fecal wet weight at day 2 after *E. coli* challenge. The present diarrheagenic *E. coli* challenge trial conducted in healthy adults indicates that a milk concentrate rich in natural bioactive phospho- and sphingolipids from the MFGM may positively affect in vivo resistance to attenuated diarrheagenic *E. coli*.

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