RESEARCH METHODOLOGY/STUDY DESIGN

CURRENT DEVELOPMENTS IN NUTRITION

Research Methodology and Study Design

Alleviation of Metabolic Endotoxemia by Milk Fat Globule Membrane: Rationale, Design, and Methods of a Double-Blind, Randomized, Controlled, Crossover Dietary Intervention in Adults with Metabolic Syndrome

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ABSTRACT

Background: Milk fat globule membrane (MFGM) is a phospholipid-rich component of dairy fat that might explain the benefits of full-fat dairy products on cardiometabolic risk. Preclinical studies support that MFGM decreases gut permeability, which could attenuate gut-derived endotoxin translocation and consequent inflammatory responses that impair cardiometabolic health.

Objectives: To describe the rationale, study design, and planned outcomes that will evaluate the efficacy of MFGM-enriched milk compared with a comparator beverage on health-promoting gut barrier functions in persons with metabolic syndrome (MetS).

Methods: We plan a double-blind, randomized, crossover trial in which people with MetS will receive a rigorously controlled eucaloric diet for 2 wk that contains 3 daily servings of an MFGM-enriched bovine milk beverage or a comparator beverage that is formulated with nonfat dairy powder, coconut and palm oils, and soy phospholipids. Compliance will be monitored by assessing urinary para-aminobenzoic acid that is added to all test beverages. After the intervention, participants will ingest a high-fat/high-carbohydrate meal challenge to assess metabolic excursions at 30-min intervals for 3 h. Nondigestible sugar probes also will be ingested prior to collecting 24-h urine to assess region-specific gut permeability. Intervention efficacy will be determined based on circulating endotoxin (primary outcome) and glycemia (secondary outcome). Tertiary outcomes include: gut and systemic inflammatory responses, microbiota composition and SCFAs, gut permeability, and circulating insulin and incretins.

Expected results: MFGM is expected to decrease circulating endotoxin and glycemia without altering body mass. These improvements are anticipated to be accompanied by decreased gut permeability, decreased intestinal and circulating biomarkers of inflammation, increased circulating incretins, and beneficial antimicrobial and prebiotic effects in the gut microbiome.

Conclusions: Demonstration of improvements in gut barrier functions that limit endotoxemia and glycemia could help to establish direct evidence that full-fat dairy lowers cardiometabolic risk, especially in people with MetS. The clinical trial associated with this article has been registered at clinicaltrials.gov (NCT03860584). *Curr Dev Nutr* 2020;4:nzaa130.

Keywords: MFGM, LPS, dairy, glucose tolerance, inflammation, obesity, cardiometabolic risk, sphingomyelin, phosphatidylserine, leaky gut

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Abbreviations used: COM, comparator; GIP, gastric inhibitory peptide; GLP-1, glucagon-like peptide 1; MetS, metabolic syndrome; MFGM, milk fat globule membrane; MPO, myeloperoxidase; PABA, para-aminobenzoic acid; rFC, recombinant factor C; TLR4, Toll-like receptor 4; 3NPH, 3-nitrophenylhydrazine.

Introduction

Metabolic syndrome (MetS), which affects ∼35% of American adults (1), is diagnosed based on the presence of ≥3 of 5 cardiometabolic risk factors: increased blood pressure, waist circumference, blood glucose, triglyceride, and low HDL cholesterol (**Table 1**) (2). Especially concerning is that MetS increases all-cause mortality risk (3) by driving the progression of cardiometabolic disorders (e.g., type 2 diabetes mellitus, nonalcoholic fatty liver disease, cardiovascular disease) (4). Because most people with MetS lack adequate medical intervention beyond encouragement of lifestyle modifications, effective dietary strategies are needed to alleviate the early etiologic responses that otherwise provoke MetS-associated risk of life-threatening diseases.

TABLE 1 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Age: 18–65 y	Unstable body mass (±2 kg during prior 3 mo)
BMI ≥25.0 kg/m^2	Dietary allergies or restrictions
Metabolic syndrome (≥3 of the following established criteria) (2):	Use of dietary supplements within previous 30 d
• Systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg	Use of antibiotics or probiotics within previous 30 d
• Waist circumference ≥88 cm (female) or ≥102 cm (male)	Use of anti-inflammatory agents within previous 30 d
• Blood glucose ≥100 mg/dL	History of cardiovascular disease or cancer
• Blood triglycerides ≥150 mg/dL	Pregnancy
• HDL cholesterol <50 mg/dL (female) or <40 mg/dL (male)	Lactation
	Changes in birth control (within past 6 mo)
	Gastrointestinal disorder or chronic diarrhea
	Smoking within the past 3 mo
	>2 alcoholic drinks/d
	>5 h/wk aerobic exercise

Metabolic endotoxemia, which is defined as a 2–3 times increase in circulating endotoxin (5), is an early etiological factor in MetS that initiates insulin resistance and obesity (6). Endotoxemia results from the excess absorption of gut-derived endotoxins (e.g., LPS derived from the membrane of Gram-negative bacteria) (6, 7). Although transcellular chylomicron-mediated absorption of endotoxin helps to limit its proinflammatory effects, paracellular absorption of endotoxin resulting from "leaky gut" is highly implicated in cardiometabolic risk (8). Indeed, endotoxemia occurs in association with impaired immune function, gut dysbiosis, and increased gut permeability (9). Nonetheless, after being absorbed, endotoxin becomes bound by the LPS-binding protein and is transferred to sCD14 (10), which facilitates Toll-like receptor-4 (TLR4) signaling to activate NFκB-dependent inflammatory responses (e.g., TNFα, IL-1, IL-6) that mediate metabolic derangements (e.g., insulin resistance, lipogenesis) (11). Preventing gut-derived endotoxin translocation is therefore expected to alleviate endotoxemia-associated inflammation that otherwise increases cardiometabolic risk.

The cardiometabolic benefits of full-fat dairy products are controversial due to equivocal outcomes of observational studies (12–14). In the absence of clear evidence, but in an effort to limit excess energy and saturated fat consumption, the Dietary Guidelines for Americans recommends nonfat or low-fat dairy foods over full-fat dairy foods (15). However, this does not preclude the possibility that full-fat dairy foods afford benefits consistent with the limited evidence from controlled trials examining their consumption on cardiometabolic health (12, 13). Indeed, full-fat dairy contains milk fat globule membrane (MFGM), which potentially reduces cardiometabolic risk in association with improved gut health (16). MFGM is a unique trilayer membrane that surrounds secreted milk fat droplets and is rich in phospholipids, proteins, and glycoproteins (17) (**Figure 1**). Mice supplemented with milk fat isolate, which is enriched in MFGM, are protected from LPS-induced inflammation (18). Supplementation also decreased gut barrier permeability and prevented LPS-induced mortality in association with lower circulating concentrations of proinflammatory cytokines (e.g., TNFα, IL-6, IL-10) (18). Others report that MFGM in rat pups improved gut development (e.g., increased expression of tight junction proteins) in association with increased gut microbiota species richness and evenness, favorable shifts in specific bacterial populations (e.g., lactobacilli), and protection against *Clostridium difficile* toxin-induced intestinal inflammation (17), although similar microbiota-related outcomes did not occur in overweight postmenopausal women (19). Anti-inflammatory activities of MFGM are partly attributed to sphingomyelin, which along with other glycolipids is absent in plant-derived phospholipids. This is consistent with sphingomyelin lowering dietary fat–induced increases in circulating endotoxin, adiposity, and liver steatosis in mice (20). Milk sphingomyelin also improves microbiota composition (21), likely by an antimicrobial activity that reduces pathogenic bacteria (22) and a prebiotic activity that increases SCFA-producing *Bifidobacterium* (20).

MFGM has received considerable study in preclinical models and is under active investigation in healthy cohorts (23, 24). Of the relatively few controlled human trials that have been conducted, none have examined the putative gut-level benefits of MFGM in people with MetS in relation to cardiometabolic risk with a focus on endotoxin-TLR4-NFκB inflammation. Thus, the objective of this article is to describe the experimental rationale, study design, and outcomes of a planned investigation that will evaluate the efficacy of an MFGM-enriched milk on health-promoting gut barrier functions in persons with MetS. We will conduct a double-blind, randomized, controlled crossover intervention to examine MFGM-enriched bovine dairy milk compared with a comparator (COM) beverage formulated with nonfat dairy milk powder, soy phospholipids (lecithin), and a blend of palm and coconut oils (75:25) to decrease endotoxemia-associated inflammation in association with improved gut barrier function and glucose tolerance. We hypothesize that MFGM-enriched dairy milk will decrease endotoxemia and improve glucose tolerance in adults with MetS by increasing gut barrier permeability and alleviating gut dysbiosis and inflammation (**Figure 2**). To test this, we will conduct a rigorously controlled crossover intervention in which participants with MetS are provided MFGM-enriched milk or a COM beverage along with all foods in a eucaloric manner for 2 wk prior to assessing microbiota composition, fecal SCFAs, intestinal permeability and inflammation, endotoxemia, glucose tolerance and incretins, and systemic TLR4/NFκB inflammatory responses.

Methods

Study design

All study procedures have been approved by the Institutional Review Board at The Ohio State University (2018H0564), and the trial has

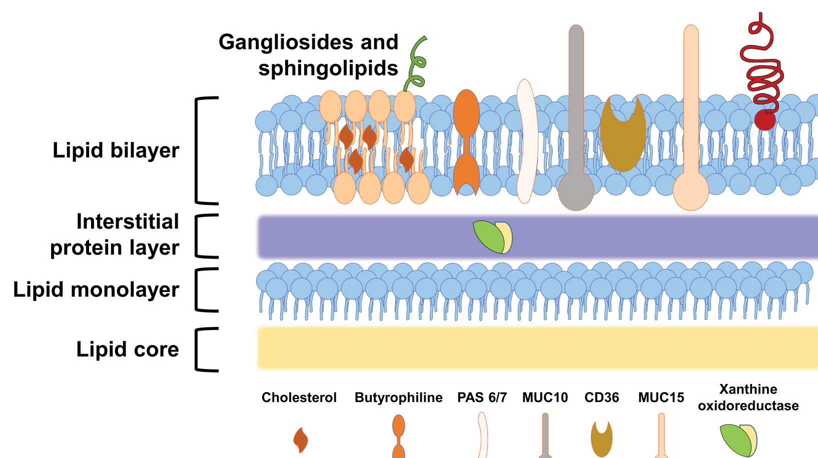

FIGURE 1 Structure of milk fat globule membrane. Milk fat globule membrane is a unique trilayer membrane that protects the lipid core of secreted milk fat droplets. It includes bioactive gangliosides, sphingolipids, glycoproteins, and proteins that are expected to function to mediate improvements in gut barrier function in humans. In preclinical studies, these bioactive components exhibit antimicrobial and prebiotic activities on the gut microbiome and anti-inflammatory effects on the intestinal barrier. Not drawn to scale. MUC, mucin; PAS, periodic acid SCHIFF. Adapted from reference 25.

been registered at clinicaltrials.gov (NCT03860584). Only preapproved procedures will be followed in the conduct of this study. Upon study entry, each participant will be assigned a code number that is used to annotate all study records and collected biospecimens. A key linking the study code to participants' identifying information will be maintained by the principal investigator or a designated member of the research team and stored in a locked filing cabinet or password-protected computer. After 5 y, the key linking study codes to identifying information will be destroyed in accordance with our preapproved procedures.

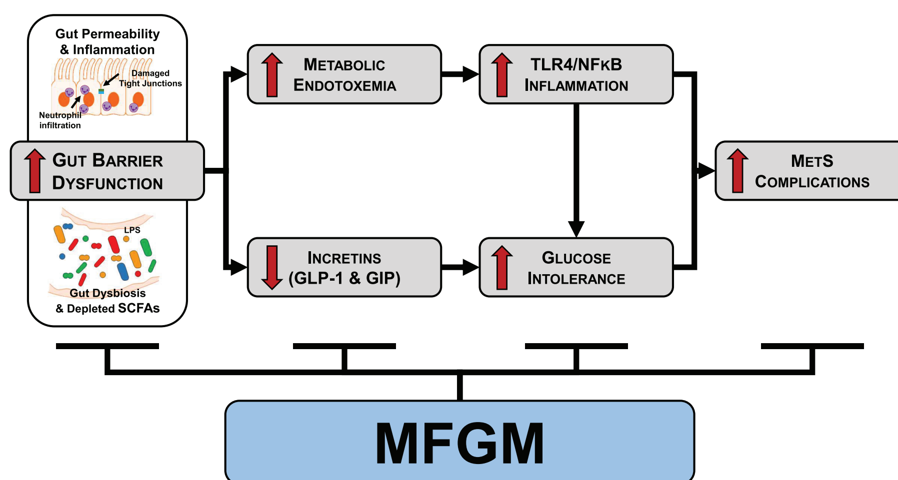

FIGURE 2 Hypothesized benefits of milk fat globule membrane (MFGM) on endotoxemia-associated inflammation and glucose intolerance. Our planned clinical trial in subjects with metabolic syndrome is expected to demonstrate that an MFGM-enriched beverage will alleviate gut barrier dysfunction (i.e., intestinal inflammation; gut dysbiosis, and depleted SCFAs; increased gut permeability) that otherwise provokes the translocation of gut-derived endotoxins (e.g., LPS) to initiate endotoxemia-associated TLR4/NFκB inflammation. Reduced SCFA production by the gut microbiota can also impair incretin production. Inflammation and limited incretin production contribute to glucose intolerance leading to advanced MetS complications. MFGM in the planned study is expected to alleviate endotoxemia-associated TLR4/NFκB inflammation in subjects with MetS by improving gut barrier function in association with reduced intestinal inflammation and prebiotic and antimicrobial activities that increase commensal bacteria and decrease pyrogenic populations. Restored biosynthesis of SCFAs (e.g., butyrate) by the gut microbiota is also expected to enhance incretin production to contribute to improved glucose tolerance. GIP, gastric inhibitory peptide; GLP-1, glucagon-like peptide 1; MetS, metabolic syndrome; TLR4, Toll-like receptor 4.

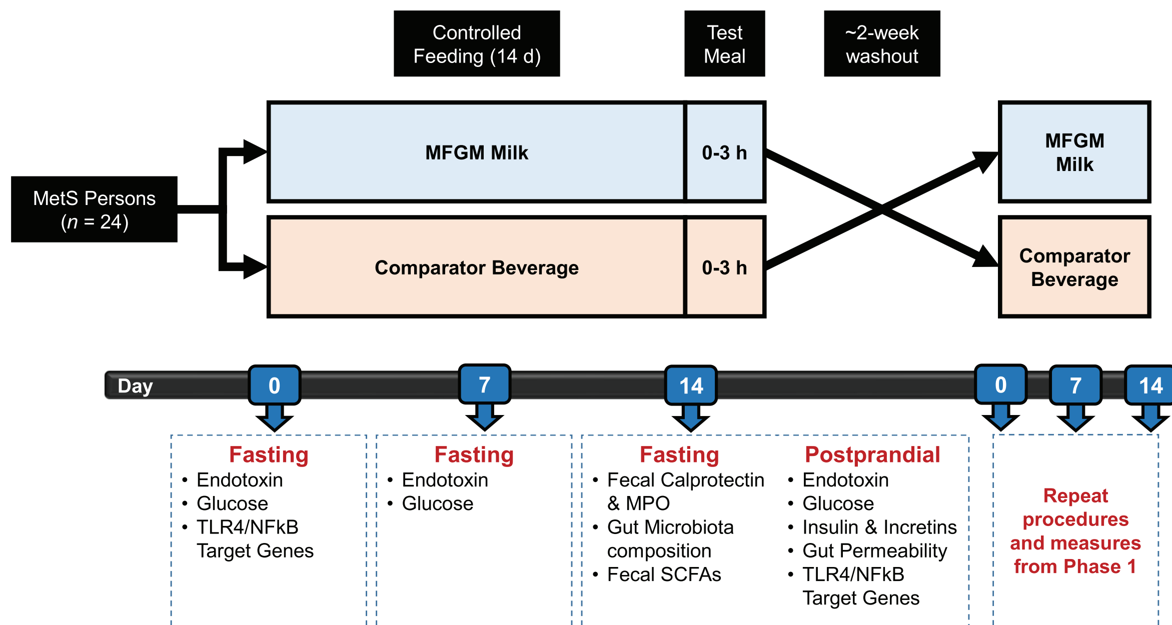

FIGURE 3 Study design of the planned double-blind, randomized, controlled, crossover dietary intervention. Adults with MetS will be randomly assigned to receive either MFGM-enriched milk or a comparator beverage formulated with nonfat dairy milk powder, soy phospholipids, and a blend of palm and coconut oils (75:25) for 14 d while following a prescribed, eucaloric diet in which all foods are provided. Fasting blood samples will be collected on days 0, 7, and 14 for the measure of metabolic chemistries. On the morning of day 14, participants will provide a fecal sample that was collected during the preceding 24 h for the analysis of gut microbiota composition, SCFAs, and neutrophil-derived proinflammatory proteins. In the fasted state, participants will receive a high-fat/high-carbohydrate meal challenge that also contains nondigestible sugar probes. Blood will be collected prior to (0 min) and at 30-min intervals for 3 h following the test meal for metabolic assessments. Urine will be collected for 24 h to assess region-specific gut permeability. Upon completing the first study intervention, participants will complete an ∼2-wk washout before receiving the alternative treatment and completing the second intervention in an identical manner. MetS, metabolic syndrome; MFGM, milk fat globule membrane; MPO, myeloperoxidase; TLR4, Toll-like receptor 4.

The randomized controlled trial will be conducted in male and female adults with MetS ($n = 24$; ages 18–65 y) recruited from the Columbus, Ohio, area. All subjects will provide a written informed consent form prior to completing any research procedures. In a double-blind, crossover design involving 2 treatment arms (**Figure 3**), participants fulfilling study entry criteria (Table 1) will be randomly allocated to receive a bovine milk test beverage enriched with MFGM-derived phospholipids or a closely matched COM milk beverage for 2 wk. Participants will be instructed to consume the test beverage (3 servings/d; 250 mL each) with each meal and/or snack throughout the day as part of a prescribed eucaloric diet. Beverage compositions and their respective phospholipid profiles are presented in **Tables 2** and **3**. After the 2-wk intervention (day 14), participants will ingest a high-fat/high-carbohydrate meal challenge containing nondigestible sugar probes. Metabolic excursions will be assessed for 3 h and urine will be collected for 24 h to assess region-specific gut permeability.

During each study arm, diet will be rigorously controlled by providing all foods to ensure weight maintenance and to improve homogeneity of study outcomes. Data collection on day 0, day 7, and day 14 of each study arm includes anthropometrics, blood pressure, and a fasting blood sample for metabolic assessments. On day 14, following a meal challenge, blood samples also will be collected at 30, 60, 90, 120, 150, and 180 min to assess postprandial metabolic responses. Blood collected on day 0 and day 14 (0 min and 180 min) also will be used to isolate RNA for gene expression studies. In addition, a fecal sample will be collected on day 13 for determinations of microbiota composition, fecal SCFAs, and intestinal inflammatory markers. Stool characteristics will also be recorded using a 7-point Bristol stool scale (26). Lastly, on day 14 following the ingestion of the test meal challenge containing sugar probes, participants will collect a complete 24-h urine sample to assess gut permeability based on the urinary excretion of sugar probes that are ingested with the test meal challenge (described in detail below). Upon completing these procedures, participants will undergo an ∼2-wk washout before repeating the study identically, but with allocation to the alternative milk test beverage (Figure 3). A 2-wk intervention and subsequent washout is expected to be sufficient to induce changes in the planned study outcomes because the human intestine is a rapidly renewing tissue (27), and prior studies using a 2-wk intervention have shown significant changes in microbiota composition (28).

Following completion of the study, subjects will be provided with honoraria for their participation. They also will be provided with a copy of their test results collected at screening phase (e.g., blood glucose, anthropometric measures) and encouraged to discuss these measures with their physician. Additionally, participants will have the option to pro-

TABLE 2 Energy and macronutrient content per 250-mL serving of each test beverage[1]

	MFGM milk	Comparator beverage
Total carbohydrates,[2] g	13.2	14.8
Total protein,[3] g	8.4	8.4
Total fat,[4] g	8.5	8.8
Saturated, mg	4434.7	5260.7
Monounsaturated, mg	2103.8	1836.2
Polyunsaturated, mg	593.0	339.2
trans fat, mg	4.6	0.0
C4:0, mg	205.5	0.0
C6:0, mg	143.7	0.0
C8:0, mg	99.2	112.7
C10:0, mg	216.3	112.0
C12:0, mg	261.0	939.8
C14:0, mg	779.6	490.8
C16:0, mg	2048.6	2537.4
C18:0, mg	800.4	402.6
C20:0, mg	3.2	2.5
C14:1, mg	26.1	0.0
C16:1, mg	115.2	0.7
C18:1, mg	1596.0	2186.7
C18:2, mg	223.2	650.0
C18:3, mg	35.8	3.6

[1]Participants will be instructed to consume 3 daily servings of 250 mL each.
[2]Carbohydrate content was estimated by difference in solids, and total solid content is determined by water evaporation using dual-frequency drying on a Smart 6 module (CEM Corp.) (29).
[3]Total protein content is determined by Dumas combustion on an N-Analyzer (Elementar Americas Corp.) (30, 31).
[4]Total fat content is determined by selective low-resolution NMR on an Oracle Rapid Fat Analyzer (CEM Corp.) (32).

vide their mailing and/or email address if they wish to receive future publications directly arising from the study.

Test meal challenge

On day 14, participants will ingest a high-fat/high-carbohydrate test meal challenge containing 1200 kcal. The meal will consist of 3 pieces of toast (45 g), margarine (100 g), and a glucose tolerance beverage (75 g; Fisher Diagnostics) that also contains gut permeability probes [lactulose (5 g), mannitol (1 g), sucralose (1 g), and erythritol (1 g)] (33). The high-fat/high-carbohydrate test meal model was designed based on an in-house pilot test showing that this approach increases postprandial circulating endotoxin and glucose while also permitting the assessment of gut barrier permeability from urinary sugar probes excreted over 24 h.

TABLE 3 Phospholipid content per 250-mL serving of each test beverage[1]

	MFGM milk	Comparator beverage
Total phospholipids,[2] mg	772.5	770.0
Phosphatidylcholine, mg	171.7	185.2
Phosphatidylethanolamine, mg	260.2	206.9
Phosphatidylinositol, mg	63.7	175.4
Phosphatidylserine, mg	108.7	0.0
Sphingomyelin, mg	125.9	0.0
Others,[3] mg	42.3	202.3

[1]Participants will be instructed to consume 3 daily servings of 250 mL each.
[2]Phospholipids are extracted (34) and analyzed as described (35) with minor modification to use an Ultimate 3000 UHPLC system (Thermo Scientific) equipped with a charged aerosol detector.
[3]Others refers to unidentified phospholipids, monoglycerides, and diglycerides that elute under these HPLC conditions in a manner that does not differ from commercial milk (36).

Test beverages

MFGM and COM beverages will be prepared in the pilot test plant at The Ohio State University (Columbus, OH) in accordance with good manufacturing practices. Both beverages will be formulated with nonfat dairy milk powder (Dairy America) to match the protein and carbohydrate content of commercially available milk (Table 2). For the MFGM-enriched beverage, a modified cream will be prepared with 40% butter fat (NutraPro International) and 10% MFGM ingredient (Lipid 100; Fonterra Co-operative Group) that is added to yield a final lipid content of 3.5% (w/v) consistent with commercial full-fat dairy milk. The selection of MFGM ingredient at 10% provides ∼10 times the total phospholipid content that is present in a serving of full-fat dairy milk (37). This dose of MFGM, although high in our formulated beverage, reflects the acute nature of the intervention and that dietary MFGM phospholipids are present in commonly consumed dairy foods other than dairy milk. For the COM beverage that is also formulated to contain 3.5% (w/v) total lipid, a modified cream will be prepared using 40% palm oil:coconut oil (75:25; Columbus Vegetable Oils) and 10% soy lecithin granules (General Nutrition Corporation). Thus, our test beverage formulations are closely matched in total lipid content, including saturated and unsaturated lipid, but differ in the composition of phospholipids and fatty acids (Table 3). To ensure formulation consistency and beverage safety, both beverages are homogenized (2-stage at 1500 psi and 500 psi) and pasteurized under high-temperature short-time conditions at 85°C for 15 s. Following pasteurization, beverages are bottled (250 mL/serving), stored at 4°C, and used within 15 d. Both beverages are also formulated with small (0.04%, v/v) amounts of vanilla extract for flavoring, to ensure study blinding.

Compliance to test beverages

To verify participants' compliance to test beverages, USP-grade para-aminobenzoic acid (PABA; Glenwood LLC) will be added at 100 mg/250-mL serving during product reconstitution. Its urinary excretion will be assessed as an objective measure of test beverage consumption consistent with reports that nearly 100% of PABA is excreted within 24 h (38). Thus, in the present study, spot urine samples will be collected on days 0, 3, 7, 11, and 14 for measures of PABA using an established spectrophotometric procedure (39). In brief, urine samples are mixed with sodium hydroxide and incubated in a water bath (100°C, 1 h) to hydrolyze *N*-acetylated and *C*-amidated metabolites of PABA. Then, a diazonium salt is formed in the presence of hydrochloric acid and sodium nitrate followed by the addition of ammonium sulfamate and *N*-naphthylethylenediamine dihydrochloride to generate a chromophore that is quantified at 540 nm against standards prepared in parallel. Compliance to test beverage consumption will be defined as urinary PABA concentrations that are either 2.5 times greater than baseline concentrations on day 0 or >30 mg/L, consistent with previously established criteria (40, 41). These thresholds recognize that other sources of aromatic amines, which are reported at low levels of 13–24 mg per 24-h urine collection (39, 42), can contribute to urinary PABA concentrations. In addition, a cut-off of >30 mg/L considers that

spot urine samples rather than complete 24-h urine samples will be collected in the present study. Because 80% of PABA is excreted within 5 h of consumption (39, 41), this threshold permits adequate detection from spot urine samples that are collected under conditions when test beverages could be last consumed >10 h prior to urine collection (e.g., in the morning after first void and prior to first daily test beverage ingestion).

Dietary control

Participants will be provided a controlled eucaloric diet during each 2-wk study arm. Daily energy intake of each participant will be calculated using the Harris–Benedict equation with appropriate adjustment for physical activity (43). Identical foods will be provided to all participants, but portion sizes will be adjusted to best meet individualized energy requirements. To promote compliance to controlled diets, we have established a 4-d rotating menu of different meals and snacks each day; all foods have been piloted for acceptability. Participants will be instructed to consume only foods and beverages provided, and any deviations will be recorded in food logs. In addition, participants will return empty containers or uneaten food portions to assess actual consumption (by weight difference) and to determine energy and nutrient intakes using Nutrition Data System for Research dietary analysis software (University of Minnesota). The daily macronutrient distribution of the prescribed diet is 51–57% of total energy from carbohydrate, 26–31% from fat, and 17–20% from protein. Total energy in the prescribed diet ranges from 2200 to 3500 kcal/d, with the test beverages providing 22–39% of total daily lipid depending on total energy intake. The range of fat-derived energy results from a basal diet that is adjusted to be eucaloric with a fixed beverage content. Controlled diets are also devoid of dairy foods, as well as fermented products and probiotics that could otherwise influence gut microbiota composition. We also considered that a high-fiber diet could limit hypothesis testing by potentially masking the gut-level antimicrobial and prebiotic activities of MFGM. Controlled diets therefore contain fiber at concentrations (7.8 g/1000 kcal) consistent with the usual low-fiber intakes of Americans (∼15 g/d) (44) that are below current recommended intakes (15).

Experimental procedures

Anthropometrics and blood pressure.

Participants' height will be measured on a wall-mounted stadiometer (Seca Model 216), weight on a digital calibrated scale (Seca Model 869), and waist circumference at the level of the umbilicus with a fiberglass tape measure. Blood pressure (Omron BP760) will be measured at the time of screening, and on days 0, 7, and 14 study visits during each trial. Blood pressure will be measured twice, separated by at least 1 min, following a minimum 15-min rest in the seated position in accordance with recommended procedures (45).

Endotoxemia.

Serum endotoxin will be measured using a high-sensitivity fluorometric kit (PyroGene recombinant Factor C Assay; Lonza), as we described (46), from fasting blood samples collected on days 0, 7, and 14 and throughout the 3-h meal challenge on day 14. In brief, serum diluted in endotoxin-free water is incubated prior to mixing with the provided working reagent containing recombinant factor C (rFC). Sample fluorescence is then measured at 380 nm/420 nm (excitation/emission) prior to and following an incubation period in which sample endotoxin activates rFC to induce cleavage of a fluorogenic substrate. Serum endotoxin is then determined from the change in sample fluorescence against an endotoxin standard curve prepared in parallel.

Gut permeability.

On day 14, as part of the test meal challenge, participants will ingest sugar probes [lactulose (5 g; Akorn Pharmaceuticals), mannitol (1 g; Spectrum), sucralose (1 g; Spectrum), and erythritol (1 g; Spectrum)] as described (33). Urinary excretion of sugars will be assessed by LC-MS as described (47) with minor modification to use a Shimadzu Prominence LCMS-2020 instrument. In brief, urine samples are diluted in acetonitrile:water (85:15) prior to mixing with a mixture of internal standards—[^{13}C]-glucose (Sigma); [^{13}C]-sucrose, and [^{13}C]-mannitol (Cambridge Isotopes Laboratories)—and centrifuging (10,000 × *g*, 15 min, 4°C). The supernatant is then injected on the LC-MS system for isocratic separation on an Acquity UPLC BEH Amide column (100 × 2.1 mm; 1.7 μm; Waters Corp.) using a mobile phase consisting of acetonitrile:water (65:35) that contains 0.1% (v/v) ammonium hydroxide. Single-ion monitoring will be used to detect each compound at their mass/charge ratios. Each analyte will be quantified on the basis of peak area relative to internal standards—[^{13}C]-glucose for erythritol and sucralose; [^{13}C]-sucrose for lactulose; and [^{13}C]-mannitol for mannitol—and against authentic standards (Sigma). Intestinal permeability is then defined by the urinary excretion (percentage of dose) and excretion ratios of lactulose:mannitol and sucralose:erythritol at 0–5 h and 6–24 h to reflect proximal and distal gut permeability, respectively (48–50). In brief, lactulose and mannitol are absorbed in the small intestine, but not the colon due to microbial fermentation, and urinary lactulose normalized to mannitol is therefore used to assess small intestinal permeability (51). Likewise, sucralose and erythritol are absorbed in the colon where they are unaffected by the gut microbiota, and the assessment of urinary sucralose normalized to erythritol reflects colonic permeability (50, 52).

Microbiota composition and function.

Microbiota composition will be assessed from fecal samples collected on day 13 of each study period as we described (53). In brief, total DNA is extracted and subjected to MiSeq sequencing on an Illumina platform using the 2 × 300 paired-end protocol (54). Sequence data will be analyzed using QIIME2 (55). α-Diversity (operational taxonomic unit richness, Shannon–Wiener diversity index, evenness) and β-diversity will be calculated using the Bray–Curtis dissimilarity (56), followed by multivariate analysis (i.e., principle component analysis, permutational multivariate analysis of variance, analysis of similarity, partial least squares discriminant analysis) to determine between-treatment effects. Microbial functions will also be predicted using PICRUSt2 (57) and comparison with the KEGG Orthology classification scheme and MetaCyc pathway database prior to statistical analysis of the functions with STAMP (version 2) (58). Current releases of all software will be used when conducting all microbiota-related analyses. Welch *t* test with Benjamini–Hochberg false discovery rate correction will be used to assess pair-wise differences using a q-quality filter ($P < 0.05$). Correlation analysis will also be used to identify the genera and functional features that correspond to treatment-specific changes in biomolecular endpoints.

Fecal SCFAs and lactate.

SCFAs will be analyzed from fecal samples collected on day 13 using LC-MS as described (59). In brief, 10 C2–C6 straight-chain SCFAs (e.g., butyrate, acetate, propionate) and branched-chain SCFAs (e.g., isobutyric acid, isovaleric acid) will be measured from fecal samples homogenized in propanol. Following centrifugation (4000 × *g*, 4°C, 10 min), the supernatant is mixed with 3-nitrophenylhydrazine (3NPH) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide, and incubated (30 min, 40°C) to generate 3NPH-SCFA derivatives. The sample is then analyzed on a Vanquish HPLC system equipped with a Q-Exactive hybrid mass spectrometer operated with electrospray ionization in negative mode (ThermoFisher Scientific). SCFAs will be quantified against area ratios of derivatized authentic standards prepared in parallel relative to 3NPH-[^{13}C]-butyrate (internal standard).

Glucose tolerance and incretins.

On days 0 and 14 and throughout the 3-h meal challenge on day 14, blood will be collected for measures of plasma glucose, insulin, and incretins. Glucose will be assessed by clinical assay (Pointe Scientific). Plasma insulin (ALPCO, #80-INSHU-E10.1) as well as the gut incretins gastric inhibitory peptide (GIP, #EZHGIP-54K; Millipore) and glucagon-like-peptide-1 (GLP-1; #EGLP-35K; Millipore) will be assessed using separate ELISA kits in accordance with the manufacturer's instructions.

Gut and host inflammation.

Intestinal inflammatory responses will be assessed from fecal samples obtained on day 13 of each study arm. Fecal protein concentrations of calprotectin (Hycult Biotech) and myeloperoxidase (MPO; Eagle Biosciences) will be measured by ELISA to assess between-treatment differences in gut-level inflammatory responses. Systemic inflammatory responses also will be evaluated on day 0 and day 14 using total RNA from whole blood samples by qRT-PCR to assess within- and between-treatment differences in expression levels of TLR4/NFκB signaling genes (*TLR4*, *CD14*, *MD2*, *myeloid differentiation primary response 88*, *p65*, *IL-1*, *IL-6*, *IL-8*, *TNFα*, *monocyte chemoattractant protein-1*) as we have described (60). In brief, blood samples will be mixed with RNAlater (Thermo Fisher) prior to cryogenic preservation. RNA will be isolated using a RiboPure-Blood Kit (Life Technologies) and RNA purity will be assessed at 260 nm/280 nm and 260 nm/230 nm on a BioSpec-nano spectrophotometer (Shimadzu). cDNA will then be synthesized for qRT-PCR analysis using an iScript cDNA synthesis kit (Bio-Rad) for the measurement of target genes using SYBR Green Supermix (Bio-Rad).

Study Powering and Data Analysis Plan

Power analysis

The primary outcome of this study is between-treatment differences in circulating endotoxin on day 14, and the predefined secondary outcome is between-treatment differences in glycemic responses on day 14. No prior studies have examined MFGM on endotoxin in people with MetS. We therefore utilized data from our cross-sectional study showing that people with MetS have greater fasting serum endotoxin than healthy persons (32.4 ± 4.4 compared with 16.4 ± 7.8 EU/mL; means ± SD) (61). Using these data, we estimated a conservative 50% improvement in the difference of mean endotoxin between people with MetS and healthy persons [(32.4 − 16.4) × 50% = 8.0 EU/mL] to predict a potential treatment effect of MFGM in the present study. Using this estimate and the more conservative SD = 7.8, our power analysis that considered within-subject treatment effects indicated that 13 participants would be needed to achieve 90% power ($\alpha = 0.05$). We therefore plan to enroll 24 persons with MetS to account for potential study attrition and to consider a potential gender × treatment interaction. With this plan to enroll 24 participants, and using the difference in AUC responses of postprandial glucose in response to high- and low-glycemic milk beverages in people with MetS (62), a predicted MFGM-mediated improvement of 50% in the difference of glycemic responses also provides ∼90% power ($\alpha = 0.05$) for this study end point.

Statistical analysis

Statistical analyses will be performed using the R statistical package for Windows (The R Foundation). AUC for all postprandial measures (0–180 min) will be calculated based on the trapezoidal rule. Linear mixed models accounting for repeated measures will be used to assess between-treatment effects. Assumptions of regression (i.e., linearity, normal distribution of errors, homoscedasticity) will be evaluated prior to analysis. If any assumptions are not adequately met, appropriate transformations and/or alternative modeling approaches might be used. Potential covariates (e.g., age, BMI, gender) also will be considered in the regression model to better define the benefits of MFGM on experimental outcomes.

Data management and privacy

Some assays will be performed immediately upon biospecimen collection to consider analyte stability whereas others will be performed after all samples are collected to minimize assay variability. The principal investigator (RSB) will be ultimately responsible for data quality and control and will oversee all aspects of the clinical trial including study execution, regulatory compliance to protect human subjects and preserve data confidentiality, and most biomolecular studies. Persons having expertise in dairy beverage processing (RJ-F and JO-A) will formulate and prepare all test beverages under good manufacturing practices. To facilitate data recording, archival, and analysis, a data management plan accessible to all study personnel will be stored on a cloud-based service (BuckeyeBox) at The Ohio State University. BuckeyeBox utilizes the advanced encryption standard 265-bit encryption and 2-layer authentication (i.e., password protection plus a system-compliant secondary verification method). These files are accessible only to those who have been granted permission by the principal investigator. The data management plan contains standard operating procedures for biospecimen collection and reporting procedures for experimental outcomes (e.g., file naming, data format). Each investigator is responsible for data quality control, maintaining original data sources, and uploading data to the cloud-based service in a format readable by all team members. Following dissemination of primary and secondary outcomes in the peer-reviewed literature, deidentified data will be provided upon request.

Discussion

Outcomes of this planned double-blind, crossover intervention are expected to demonstrate that MFGM, as part of an enriched dairy milk, relative to a plant lipid–containing COM beverage, alleviates endotoxemia-associated inflammation and glucose intolerance in people with MetS by improving health-promoting gut barrier functions. In support, MFGM is expected to decrease intestinal permeability, demonstrated by reduced urinary excretion of sugar probes, in association with prebiotic and antimicrobial activities that improve gut microbial community structure. Further, fecal MPO and calprotectin are expected to be attenuated whereas fecal SCFAs (e.g., butyrate) are expected to be increased, thereby suggesting gut-level anti-inflammatory activities of MFGM. Not only could these gut-level benefits help to reduce endotoxin translocation and consequent endotoxemia-associated inflammation, but also they are expected to increase circulating gut-derived incretins (GLP-1, GIP) that improve glucose tolerance. Thus, this intervention is expected to provide novel evidence supporting MFGM to help reduce MetS risk consistent with a mechanism that improves intestinal barrier functions to reduce endotoxin-TLR4-NFκB inflammation. These outcomes are expected therefore to support research translation of earlier preclinical investigations indicating that MFGM or its bioactive constituents protect against metabolic derangements resulting from obesity and insulin resistance (20, 53, 63).

Studies in preclinical models have demonstrated that MFGM and its bioactive components (e.g., sphingomyelin, polar lipids) have antimicrobial (22, 64), prebiotic (20, 64), and anti-inflammatory effects (18, 65). In the present study, the dose of MFGM is higher than that in commercial milk, but is sufficiently high to induce expected improvements in diversity and richness of gut microbial populations; future dose–response studies would be needed to determine the lowest effective dose. Specifically, MFGM is expected to decrease specific populations of pyrogenic *Proteobacteria* (22), and increase certain commensal bacteria populations. The latter is especially important for increasing the biosynthesis of SCFAs, which are often depleted with gut dysbiosis and known to help alleviate "leaky gut" (66). However, whereas we hypothesize that MFGM will increase fecal SCFAs in the present study, others have suggested that higher fecal SCFA concentrations are associated with cardiometabolic risk factors (67–69). Fecal SCFAs have also been reported to increase without affecting cardiometabolic outcomes, whereas butyrate-producing bacteria (e.g., *Lachnospiraceae*) (70) are associated with improved gut barrier function likely by upregulating intestinal tight junction protein expression (71, 72). Our study will therefore assess fecal SCFAs and region-specific biomarkers of gut permeability to consider the potential benefit of MFGM to alleviate metabolic endotoxemia by limiting endotoxin translocation from the gut consistent with a mechanism of butyrate-mediated improvements in gut barrier health (73). Our parallel assessment of fecal calprotectin and MPO is expected also to help establish an anti-inflammatory benefit of MFGM via a decrease in neutrophil-mediated oxidative distress responsible for injuring the intestinal epithelial barrier (74). This is consistent with the known activities of polar lipids present in MFGM that reduce epithelial barrier stress by limiting neutrophil infiltration (75, 76). Importantly, SCFAs such as butyrate also promote the secretion of the incretins GLP-1 and GIP (77). Hence, MFGM is expected to improve glucose tolerance in people with MetS by upregulating these incretins to improve insulin sensitivity (78). Together, these gut-level changes are expected to provide evidence that MFGM exerts health-promoting antimicrobial, prebiotic, and anti-inflammatory activities, thereby supporting long-term and dose–response interventions aimed at alleviating MetS risk.

Strengths

A primary strength of our study is an approach to implement rigorous dietary control combined with a double-blind, crossover randomized controlled trial to examine the benefits of MFGM in people with MetS. By administering closely matched beverages enriched with either MFGM phospholipids or soy phospholipids, and providing identical dairy-free diets to each participant during both study periods, we expect to demonstrate benefits of the MFGM test beverage on gut barrier functions that can enhance cardiometabolic health. Further, compliance to test beverages will be verified objectively based on urinary PABA excretion. Dietary control in a eucaloric manner and assessment of actual consumption by weighed records also will circumvent misreporting of self-reported dietary intakes (79). This dietary approach allows for the examination of MFGM effects independent of any alterations in body mass that could otherwise influence experimental outcomes. This approach will also permit an understanding of whether full-fat dairy foods can be incorporated effectively into the diet despite current recommendations that encourage limiting the intakes of these foods (15). Lastly, our study of MFGM in people with MetS will directly address observational findings that have suggested an inverse association between full-fat dairy products and MetS incidence (80–82). Thus, this study aims to establish evidence of how MFGM is functionally responsible for the putative benefits of full-fat dairy foods. These outcomes would be important for research translation in the context that the prevalence of MetS has increased over recent decades (83) whereas milk consumption often declines after childhood (84).

Limitations

Although the crossover study is well designed to test the efficacy of our MFGM-enriched beverage on primary and secondary outcomes, a limitation of our "food-based" approach is that it precludes an understanding of the specific component(s) of MFGM responsible for the expected benefits. Indeed, MFGM contains several phospholipids (e.g., sphingomyelin, phosphatidylserine) and proteins (e.g., mucin-1, lactadherin, butyrophilin) that might help to improve cardiometabolic health. Secondly, consistent with our expectations that MFGM will improve microbiota composition (20, 22, 64) and host inflammatory responses (18), it will not be possible to establish whether the expected anti-inflammatory activities of MFGM are attributed to prebiotic and/or antimicrobial activities on gut bacteria. For example, the SCFA butyrate helps to maintain gut integrity (71, 72) and provides anti-inflammatory function (85). However, sphingomyelin can also protect against inflammation (86). Thus, complementary studies in preclinical models will be needed to establish the mechanism of action for each bioactive constituent of MFGM. Thirdly, the MFGM beverage is expected to reduce endotoxemia-associated inflammation by limiting gut-derived endotoxin translocation. Although we will examine gut permeability as a likely mediator of endotoxemia, additional investigation will be needed to assess whether any decreases in circulating endotoxin are attributed to increased systemic clearance and reduced biosynthesis by gut bacte-

ria. Thus, we will integrate biochemical and metagenomics datasets for detailed statistical assessment of relations between/among study variables that can serve as the basis for future hypotheses testing. Additionally, our measures of endotoxin do not discriminate between free and chylomicron-bound endotoxin. Although MFGM constituents could potentially limit endotoxin incorporation into chylomicrons, our proposed measures of gut permeability will permit inferences that expected decreases in circulating endotoxin are attributed to reduced paracellular absorption of LPS. Other studies have also suggested that endotoxin might not be responsible for inducing postprandial inflammation (87). Thus, findings from the planned study might reveal decreases in circulating endotoxin independent of changes in inflammation. However, we will also consider performing a statistical mediation analysis to determine the causal framework by which treatment influences endotoxemia relative to changes in gene expression.

Conclusion

In conclusion, we expect this investigation to provide the first translational evidence in people with MetS of the benefits of MFGM acting at the gut to improve cardiometabolic health. This planned controlled trial will therefore address several knowledge gaps relating to MFGM, and more broadly full-fat dairy milk, that can support evidence-based dietary recommendations that supersede those that are largely based on outcomes of observational studies. Such findings are important for advancing an understanding of the health benefits of dairy foods but also for health conditions such as MetS in which metabolic endotoxemia is implicated (6, 7, 9, 11).

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