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Exogenous myrosinase from mustard seed increases bioavailability of sulforaphane from a glucoraphanin-rich broccoli seed extract in a randomized clinical study

Angela Mastaloudis[1,17], Lola Holcomb[2,3,17], Jed W. Fahey[4,5], Camila Olson[6], David C. Nieman[6], Colin Kay[7,8], Robert O'Donnell[9], Alessandra Pecorelli[10,11], Marissa Kinney[12], Yanyan Li[5,13], Suzanne L. Ishaq[3,5,14]✉ & Giuseppe Valacchi[10,15,16]✉

Inactive glucoraphanin (GR) in broccoli is converted to the antioxidant, anti-inflammatory, and anti-bacterial sulforaphane (SF) by cruciferous vegetable enzyme myrosinase (Myr), or similar enzymes from specific gut bacteria; both sources have variable efficiency. The effects of exogenous Myr on the conversion efficiency of GR to SF was compared to gut microbial Myr-like activity. In a randomized, double-blind, crossover study, sixteen subjects (9 F: 7 M) received a single oral dose of GR in broccoli seed extract with Myr-containing mustard seed powder, or broccoli seed extract alone, both with ascorbic acid. GR + Myr, on average, *doubled the* bioavailability of SF (39.8 ± 3.1%) compared to GR alone (18.6 ± 3.1%), and increased the conversion rate in the first 8 h (25.4% ± 2.7%) compared to GR alone (8.0% ± 2.7) based on measurement of urinary metabolites. There were no differences in fecal bacterial communities after the single dose; however, four bacterial GR-converting genes significantly correlated with GR conversion ($p < 0.0155$). To our knowledge, this is the first human study to simultaneously investigate (1) a well-defined Myr source, (2) broccoli seeds as source of GR, (3) prediction of gut microbial responsiveness to GR.

Keywords Bacterial metabolism, Glucosinolate metabolism, Diet and gut microbiome, Dietary supplement, anti-inflammatory, Anti-oxidant

[1]Dynamis Nutrition Science, Salt Lake City, UT, USA. [2]Graduate School of Biomedical Sciences and Engineering, University of Maine, Orono Maine 04469, USA. [3]One Health and the Environment, University of Maine, Orono Maine 04469, USA. [4]Departments of Medicine, Physiology Pharmacology & Therapeutics, and Psychiatry & Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21218, USA. [5]Institute of Medicine, University of Maine, Orono Maine 04469, USA. [6]Human Performance Laboratory, Department of Biology, Appalachian State University, North Carolina Research Campus, Kannapolis, NC 28081, USA. [7]Arkansas Children's Nutrition Center, Little Rock, AR 72202, USA. [8]Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA. [9]RMS Consulting, Forest Grove, OR 97116, USA. [10]Department of Environmental and Prevention Sciences, University of Ferrara, Ferrara, Italy. [11]Department of Food, Bioprocessing and Nutrition Sciences, Plants for Human Health Institute, North Carolina State University, Kannapolis, NC 28081, USA. [12]Molecular and Biomedical Sciences, University of Maine, Orono Maine 04469, USA. [13]School of Pharmacy and Pharmaceutical Sciences, SUNY Binghamton University, Johnson City, NY 13790, USA. [14]School of Food and Agriculture, University of Maine, Orono Maine 04469, USA. [15]Department of Animal Science, Plants for Human Health Institute, North Carolina State University, Kannapolis, NC 28081, USA. [16]Department of Food and Nutrition, Kyung Hee University, Seoul, South Korea. [17]Angela Mastaloudis and Lola Holcomb contributed equally. ✉email: sue.ishaq@maine.edu; gvalacc@ncsu.edu

The protective effects of a diet rich in fruits and vegetables have long been recognized[1]. Broccoli, in particular, is considered a 'superfood', with abundant epidemiological evidence for its health benefits and protection against the development of chronic diseases which involve inflammation[2,3]. Broccoli's benefits have been primarily attributed to its high content of glucoraphanin (GR), the biogenic precursor of sulforaphane (SF)[2]. GR supplementation results in upregulated expression of genes related to antioxidant defense and detoxification[4,5], direct protection against oxidative damage[6–8], improvement of cardiovascular health biomarkers[8–11], promotion of liver health, healthy glucose metabolism[7,12,13] and modulated inflammation by downregulating several genes[14,15], among other benefits.

The consensus of the scientific community has long been that GR exerts its effect only *after* conversion to SF, based on the evidence that GR itself is poorly absorbed: less than 5% of dietary GLR is absorbed systemically in rats[16] and less than 1–3% is absorbed systemically in humans[17,18]. Moreover, a recent study in Caco2 cell monolayers, a validated model of human small intestinal absorption, we observed no detectable absorption of GR at 10 μM[19]. However, when the cell monolayers were treated with broccoli seed extract digesta, prepared through an in vitro small intestinal digestion process, ~ 4.9% of GR absorption was observed at a GR concentration of ~ 300 μM, consistent with animal and human study results[19]. While many assert that GR is biologically inert[20], this does not appear to be the case. The recent Caco2 cell monolayer study by Zhu et al.[21], demonstrated that GR exerted anti-inflammatory benefits and protected intestinal barrier function, despite not being absorbed by the cells. For the majority of parameters, the same benefits were observed for SF and for GR, but in some cases, GR elicited benefits at concentrations 2–5 times higher than SF. These data are consistent with human studies that have reported that higher doses of GR compared to SF are required to elicit the same positive health effects[22]. Whereas GR is poorly absorbed in humans, it can be hydrolyzed by the plant enzyme myrosinase (Myr) and/or by several enzymatic pathways in specific bacteria, to form the isothiocyanate (ITC) sulforaphane, a well-absorbed (70–90%)[23] and highly bioactive compound[24] (Fig. 1). SF is a potent natural inducer of Nuclear factor erythroid 2-related factor 2 (Nrf2), and through this mechanism, has been demonstrated to increase intrinsic antioxidant status, upregulate detoxification pathways, and modulate inflammatory balance[24,25]. Further, SF inhibits inflammatory factor NF-κB and potentially cancerous proteins such as HER2[26], and downregulates proinflammatory pathways implicated in inflammatory bowel diseases[27,28] and tumorigenic pathways specific to a number of cancers[29]. The reduction in inflammation can derive from a direct effect of SF or from the crosstalk between the transcription factors Nrf2 and NF-κB as they are able to inhibit each other[30]. Similarly, ingestion of both GR and SF have been studied individually and combined in large- and small-scale where they consistently increase the urinary excretion of glutathione conjugates of environmental toxins including benzene, crotonaldehyde, aflatoxin, phenanthrene, and acrolein[22,31–34].

GR to SF conversion can be achieved by plant enzymes, or by gut microbiota, however; disparity in plant and bacterial enzyme activity, as well as cooking preparation, makes this process highly variable. Myrosinase (Myr) (β-thioglucoside glucohydrolase; EC 3.2.147) is an enzyme found in 16 families of higher plants, most notably the *Brassicaceae* plant family. This family includes cruciferous species (e.g., broccoli, cabbages, cauliflower, radishes, and Brussels sprouts) commonly used in pungent condiment spices including mustard (*Sinapis alba*) and wasabi (*Wasabia japonica*), among others[3,35,36]. Myr is naturally present in plant cells where it is structurally or cellularly segregated from GR[37] until the barrier or cell membranes are ruptured via cell damage during attack by plant damage and pathogens, by food processing such as chopping, freeze-thawing, and blending, and including chewing by insects, herbivorous animals, and humans. SF damages microbial pathogens and insect pests[38,39], but acts beneficially in mammals and is highly bioavailable in humans. Once absorbed, SF is rapidly metabolized by initial conjugation with the tripeptide endogenous nucleophile glutathione (GSH or -glutamyl cysteinyl glycine)[40–42]. The glutathione conjugate then undergoes successive steps of hydrolysis to form SF-cysteinylglycine (SF-Cys-Gly), SF-cysteine (SF-Cys) and ultimately SF-*N*-acetylcysteine (SF-NAC) (Fig. 1) as recently reviewed[20]. Following ingestion of SF or GR, the primary compounds found in the blood and later excreted in the urine are free SF and the SF metabolites SF-Cys and SF-NAC[32,43]. It has been consistently demonstrated that following intake of a bolus of either GR or SF, in a food matrix or as a supplement, the vast majority of free SF and SF metabolites are cleared within 24 h[32,44,45].

Conversion to SF can occur in the absence of plant-sourced Myr, via microbially mediated activity in the colon[46,47]. Preparations of broccoli sprouts which contain naturally high concentrations of GR have been used to enrich bacterial communities to produce SF, as well as other antioxidant and anti-inflammatory compounds from other vegetable sources[3,48,49]. This is achieved by steaming sprouts to inactivate enzymes, allowing GR to persist into the colon for bacterial transformation[47,48]. Consuming the volume of sprouts needed to achieve this may be unrealistic for some, especially patients with oral or gastroenteric disease, metabolic disease, or inappetence (e.g. associated with cancer treatments), thus an active encapsulated GR would be highly beneficial. However, there are current challenges with studying and implementing GR delivery for health benefits. While Myr-like activity has been identified in the human gut microbiota[50,51], the abundance and efficiency of microbial Myr-like enzymes exhibits large interindividual variability[52,53]. The GR conversion microbial pathway in the human gut remains largely unknown, and there are likely strain-specific patterns responsible for the variability in GR conversion in people[54]. Presently, only the *Bacteroides thetaiotaomicron* (*B. theta*) taxon has a fully described gene pathway for GR conversion[55]; and a different pathway has been proposed for several other taxa[56].

Additionally, dietary sources of GR are highly variable and therefore, there is a need for consistent, stable sources of GR and of SF in order to deliver efficacious levels to consumers. While GR is present in mature broccoli, many broccoli cultivars (a.k.a. "varieties") found in grocery stores may not contain any meaningful levels of GR. Surveys of up to 30 different varieties obtained from grocery stores were reported to contain anywhere from 0.2 to 50 mg GR/100 g serving of fresh broccoli (average 16.6 mg GR per 100 g serving)[2] and frozen broccoli has been reported to contain substantially less GR than fresh broccoli[57]. Therefore consumption of the recommended 2 servings per day could result in a huge range of GR intake, anywhere from only 0.4 mg

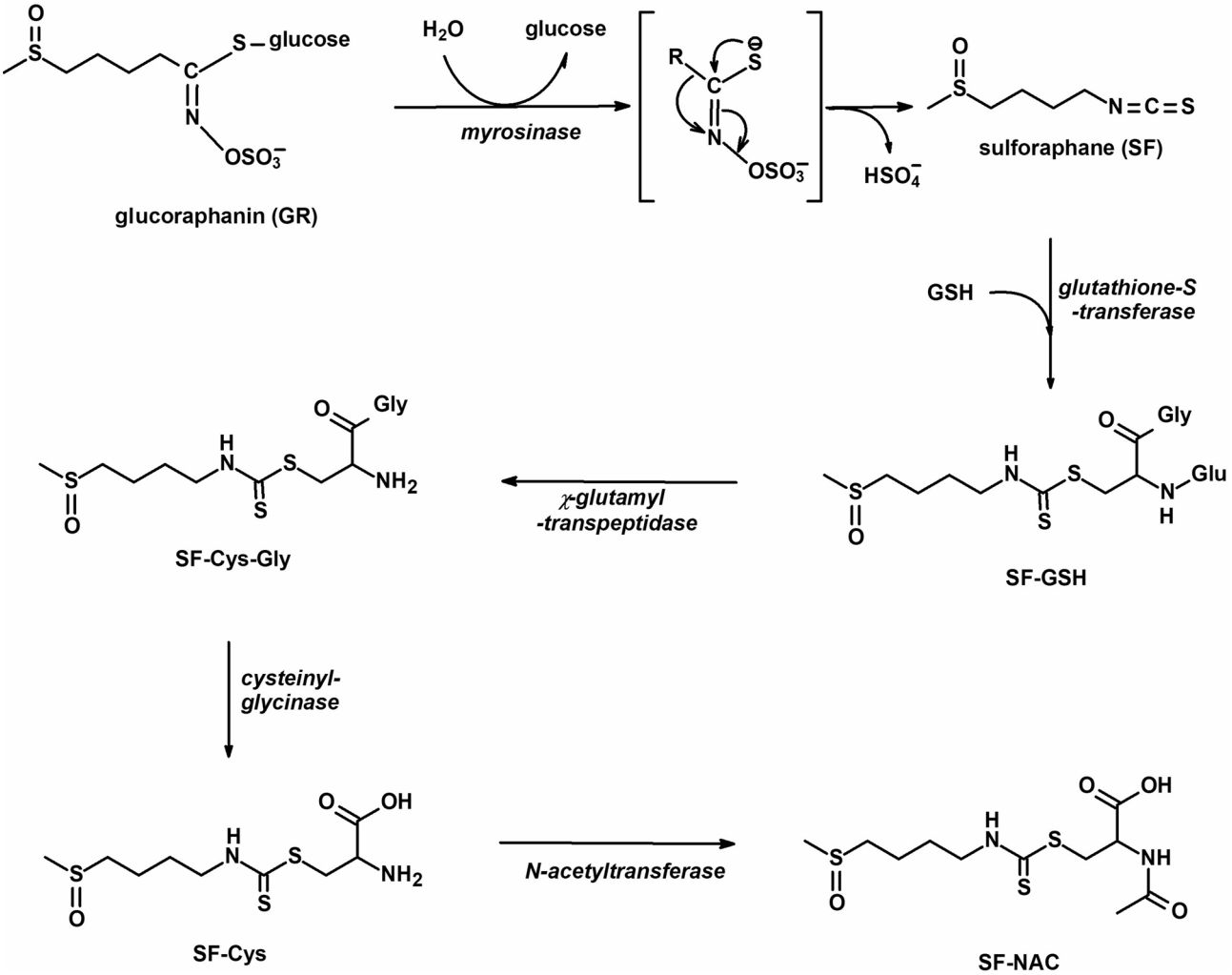

Fig. 1. Glucoraphanin conversion to sulforaphane and its metabolites: The β-glucosyl moiety is cleaved from glucoraphanin by the β-thioglucosidase myrosinase, to form an unstable thiohydroximate-O-sulfonate. In a following non-enzymatic rearrangement the sulfate moiety is released producing sulforaphane (SF) and other breakdown products including SF-nitrile. SF then can be converted at extremely low levels to SF-glucuronide or SF-sulfate (not pictured), but primarily it enters the mercapturic acid pathway and sequentially forms one of 4 metabolites -- sulforaphane glutathione (SF-GSH), sulforaphane-cysteinylglycine (SF-Cys-Gly), sulforaphane-L-cysteine (SF-Cys), sulforaphane *N*-acetyl-L-cysteine (SF-NAC).

of GR, up to 100 mg GR/day (average 33.2 mg). In contrast to fresh broccoli florets, broccoli sprouts contain at least 20–50 times more GR than found in mature fresh broccoli sold in supermarkets[18,52,58], however, there is still enormous variability in the content of GR in sprouts depending on the genetics of the seed used for production, farming practices, and environmental and production methods. Clinical trials have investigated GR doses varying between ~ 6 and 350 mg/person/day for a variety of health outcomes, however, only doses > 30 mg have reported efficacy, such as upregulation of Nrf2 regulated genes and many studies have reported dose response effects of greater efficacy with higher doses of $GR^{2}_{\text{convert to REF [2]}}$. The broccoli seed extract (BSE) evaluated in the present study is derived from the seed of a proprietary broccoli cultivar that is especially high in GR (*Brassica oleracea* L. var. *italica*, brand name TrueBroc®, Brassica Protection Products LLC, Baltimore, MD). The extract is standardized to contain a minimum of 13% GR, allowing for the delivery of a consistent, stable form of GR at a calibrated dose, which is ideal for use in clinical studies, as well as in foods and dietary supplements.

In the past decade, there has been great interest in the development of BSEs rich in GR, and the primary challenges have been stability and bioavailability, with mixed success. The major disadvantage of extracts that deliver only GR is that conversion of GR to SF and its metabolites in the absence of exogenous Myr is relatively low and highly variable, ranging from 1 to 40% in healthy volunteers[18,22,24], but up to 30–40% when GR is co-delivered with exogenous Myr from daikon radish or broccoli seeds and sprouts[59,60]. In contrast, 70–90% of oral SF is consistently excreted as SF and SF metabolites in people[23]. To overcome these limitations, we combined BSE with exogenous Myr from mustard seeds using validated protocols to ensure stable and high yields of both enzyme and GR. Further, we included ascorbic acid in our formulation, as it is essential for human health, is present in abundance in the Brassicaceae family, and has been definitively identified as a required cofactor for

Myr activity based on enzymology and X-ray crystallography in a reaction that is independent of its reducing capacity[35,61].

The aims of the present study were to determine in healthy human volunteers the oral bioavailability of SF derived from BSE standardized to its precursor, GR, with and without the addition of exogenous active Myr delivered in the form of mustard seed powder. In addition, we correlated the SF conversion rate to trends in fecal microbiota, collected prior to the GR intervention, which might predict which participants would respond to GR alone based on their existing microbial community. To our knowledge, this is the first study to investigate the co-administration of GR from BSE and mustard seed powder with known Myr activity alongside predicting gut microbial responsiveness to GR.

Methods

Subjects and study design

This randomized, double-blind (investigators and volunteers), crossover study protocol (Figs. 2, Supplemental Material 1, Figure S1) was registered at www.clinicaltrials.gov under number NCT04946526 and approved by the Institutional Review Board of Appalachian State University (protocol number # 21-02216) where all interventions were conducted and samples were collected. Clinical Trial Registration Dates include (DD/MM/YYYY): First Submitted, 17/06/2021; First Submitted that Met QC Criteria, 29/06/2021; and First Posted, 01/07/2021. All methods were carried out in accordance with relevant guidelines and regulations. Voluntary, written, informed consent was obtained from all subjects prior to study enrollment.

Healthy volunteers (free of any medical conditions that might affect study measures) of both sexes, ranging in age from 25 to 65 years, with a Body Mass Index (BMI) of 20–29.9 kg/m^2, who were not smokers, were eligible; a total of sixteen volunteers were recruited (9 females and 7 males). Anthropometric data can be found in Table 1. All volunteers indicated a willingness to adhere to dietary restrictions: refraining from consuming cruciferous vegetables and condiments that might contain glucosinolates or isothiocyanates (e.g., prepared mustard aka deli mustard, mustard greens, horseradish, wasabi, mayonnaise; Supplemental Material 1) for 3 days before and for the 9-day duration of the study; to fast overnight before taking the supplement and at the testing facility to consume the light breakfast consisting of a muffin and 12 oz black coffee (Supplemental Material 1, Tables S1–S3) along with the supplement; to collect all urine for 24 h on two separate occasions during the study; to record their diet (food items and quantity) for the 24 h prior for visit 1 in order to replicate the meals prior to visit 2 (Supplemental Material 1); and to collect a fecal sample at baseline. Exclusion Criteria: diarrhea or oral antibiotic intake within the previous 4 weeks; use of proton pump inhibitors (PPIs), antacids or other medications that influence stomach acidity; use of laxative medications or other products that promote colon cleansing in the previous 4 weeks; history of malabsorption or GI tract disorders or GI surgeries (i.e. lapband, gastric bypass, etc.); supplementation with broccoli sprout, seed or powder extracts, or other over the counter supplements including

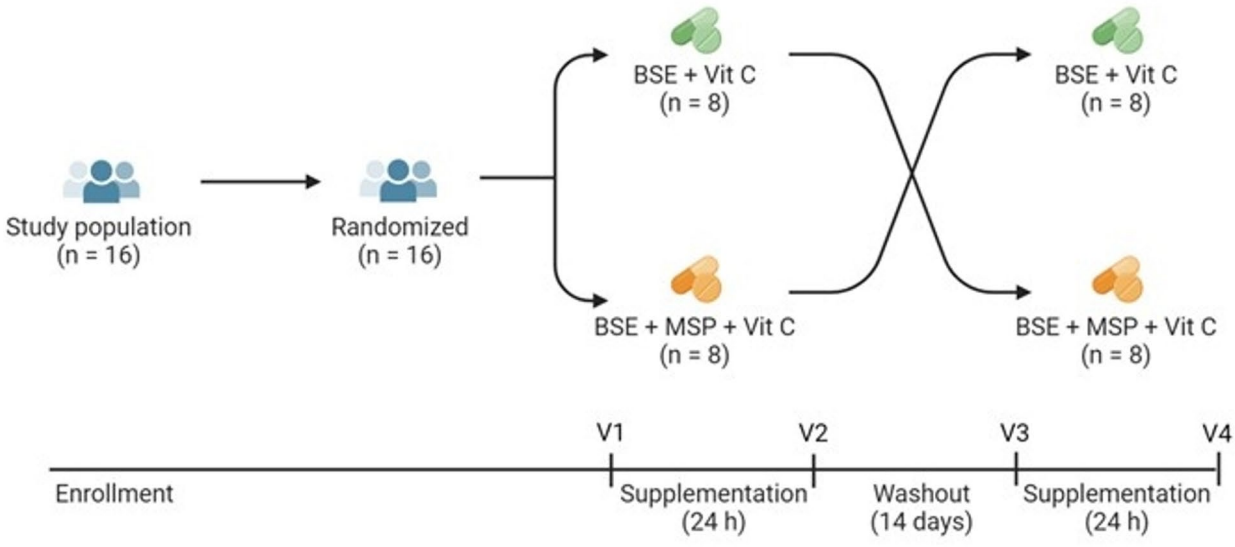

Fig. 2. The study design used a crossover treatment of Broccoli Sprout Extract (BSE) containing glucoraphanin (GR) alone, or with exogenous Myrosinase Protein (MSP) added. Both treatments included ascorbic acid.

Characteristics	Values (Mean ± SD)
Population	16
Female (n, %)	9 (56.2%)
Male (n, %)	7 (43.8%)
Age (years)	40.3 ± 8.7
Weight (kg)	73.5 ± 12.1
Height (cm)	171.3 ± 9.0
BMI (kg/m^2)	25 ± 3.0
Body fat (%)	30.8 ± 8.3
Resting heart rate (beats per minute)	69.4 ± 9.0
Systolic blood pressure (mmHg)	126.0 ± 15.3
Diastolic blood pressure (mmHg)	78.5 ± 10.1

Table 1. Characteristics of the study participants.

the other bioactive ingredients in the test supplement, antioxidants and multi-vitamin mineral supplements, including vitamin C, for the previous 2 weeks; a known allergy or intolerance to any of the ingredients contained in the test supplements (e.g. broccoli, broccoli seeds, mustard seeds, vitamin C); currently pregnant, planning to become pregnant, or breastfeeding; any other condition which in the Investigator's opinion may adversely affect the subject's ability to complete the study or its measures or which may pose significant risk to the subject.

The number of subjects chosen was guided by our previous demonstration of bioavailability of SF from GR alone and GR + Myr broccoli seed and sprout preparations[18,35,59]. Sixteen (16) subjects were determined to provide 90% power to detect a mean difference of 10% of the dose (or more) converted to SFN (average rate for GR conversion in people)[18,35,59] with an alpha test level of 0.05. Since the excretion of SF and SF metabolites in the urine of human subjects who do not consume cruciferous vegetables or condiments is negligible (less than 0.2 nmol/mL)[24,43], increases resulting from glucoraphanin with or without the presence of Myr rise well above the baseline concentrations and therefore, a placebo group was not necessary.

Subjects were randomly assigned to receive a single oral encapsulated dose of one of two treatments in random order, described in *Interventions*, followed by a washout period of 14 days; a fecal sample was collected on Day 14 of the washout period. The following day, Visit 3, subjects returned to the facility where they consumed an encapsulated dose of the second treatment (Figs. 2). All 16 subjects completed the study; no adverse events were reported.

Interventions

Treatment A consisted of 385 mg Broccoli Seed Extract (BSE) which contained 115 µmol (50 mg) GR, along with 145 mg mustard seed powder containing enough active Myr (enzyme) to fully hydrolyze the GR in the capsule to SF post-ingestion (~ 50 units Myr activity) and 100 mg vitamin C (as ascorbic acid) to improve Myr activity[21,35,62]. Treatment B consisted of 385 mg BSE which contained 115 µmol (50 mg) GR and 100 mg vitamin C (as ascorbic acid), and was reliant on gut bacteria to perform the conversion, which typically results in higher SF in colon and systemic locations, but overall lower total conversion[63,64]. The amount of GR supplemented was similar to that which can be obtained from a serving of fresh broccoli. Both treatments were delivered in two size-0 vegetarian hypromellose capsules and contained the following inactive excipients to aid in the blending of actives and manufacturing of the capsules: microcrystalline cellulose and calcium laurate. Capsules were filled by APS BioGroup (Phoenix, AZ) under CGMP conditions.

BSE, a refractance window dried (RWD) hot water extract of broccoli seed *Brassica oleracea L.* var. *italica* (brand name TrueBroc® from Brassica Protection Products LLC, Baltimore, MD, USA) was standardized to no less than 13% GR by weight (> 130 mg GR/1000 mg BSE powder). Mustard Seed Powder, a milled white mustard seed (*Sinapis alba*; syn. *Brassica hirta*) guaranteed to contain no less than 100 Units of Myr activity/gram powder was provided by Brassica Protection Products LLC, Baltimore, MD. Vitamin C, in the form of United States Pharmacopeia (USP) grade ascorbic acid, was provided by APS BioGroup (Phoenix, AZ), the manufacturer of the capsules. The size 0 vegetarian hypromellose capsules that were used here are designed to disintegrate in the upper GI tract and release contents in the small intestine.

Urinary SF and SF metabolites

First, a recovery study was conducted using internal standards (ISs), phlorizin (Sigma-Aldrich), sulforaphane-d8 (Toronto Research Chemicals, Canada) and D, L-sulforaphane-d8-N-Acetyl-L-cysteine (Toronto Research Chemicals, Canada), to validate acceptable recovery relative to previously reported solid phase extraction (SPE) methodology[65]. Briefly, urine metabolites were purified using Discovery C18 SPE cartridges (SPE 96 well plate, 100 mg/well, 2 ml). Cartridges were pre-conditioned with 2% acetic acid (AA) in methanol (2 ml) and water (2 ml), and equilibrated with 1 ml 2% AA in water. Each urine sample (200 µl) was diluted with 2% AA in water (1:2, v/v), and mixed with 20 µl IS, phlorizin (200 µM) before loading. The sample was loaded on the cartridges and washed with 2 ml 2% and 2 ml 0.2% AA in water, respectively. Internal reference standards sulforaphane-d8 (50 µM) and sulforaphane-sulforaphane-d8-N-Acetyl-L-cysteine (10 µM) were added to each well of the collection plate before eluting the samples with 0.2% AA in methanol. The resultant recovery was within the acceptable

limits (100–106%). The assay was established as acceptable and applied to the clinical samples and followed the protocol of Langston-Cox et al.[65] with modification.

Using the above protocol, excretion of free SF and SF metabolites and GR were analyzed in each urine sample: 6 per subject, from two crossover periods with a baseline, 0–8 h, and 8–24 h sample in each period. Metabolites were analyzed using a targeted metabolite analysis method optimized to detect GR, SF and the six SF metabolites (sulforaphane N-acetyl-L-cysteine (SF-NAC), sulforaphane glutathione (SF-GSH), sulforaphane-L-cysteine (SF-Cys), sulforaphane-cysteinylglycine (SF-Cys-Gly), sulforaphane-glucuronide and sulforaphane-sulfate) by LCMS. Five of these compounds had commercially available authentic reference standards available (Sigma-Aldrich, US; Cayman Chemical Company, Michigan, US): D,L-Sulforaphane (4478-93-7), DL-Sulforaphane N-acetyl-L-cysteine (334829-66-2), D, L-Sulforaphane Glutathione (289711-21-3), D, L-Sulforaphane-L-cysteine (364083-21-6) and glucoraphanin (21414-41-5) (Fig. 1). The remaining two metabolites, SF-glucuronide and SF-sulfate, are produced by the body's Phase 2 detoxification enzymes UDP glucuronidases (EC 2.4.1.17) and UDP-sulfotransferases (EC 2.8.2), respectively[63,64]. These conjugates had no commercially available reference standards and were first characterized based on their MS fragmentation patterns in pooled study biospecimens following broccoli feeding. The relative quantification of the putatively identified metabolites (i.e., having no available reference standards) were established using surrogate reference standard curves from structurally similar compounds.

The quantitative analysis of each sample was performed by Exion ultra-high performance liquid chromatography (UPLC) coupled with a triple quadrupole mass spectrometer (SCIEX, QTRAP 6500+), equipped with electrospray IonDrive Turbo-V Source. The manual optimization of *source parameters* for the LC was carried out by flow injection analysis to maximize response. The optimized source parameters were: curtain gas, 45; ion-spray voltage, 4500; temperature, 650; and ion source gas 1 and 2, 80. Advanced scheduled MRM™ algorithm was used at positive and negative ionization mode toggling for analysis using Analyst v.1.6.3 software (SCIEX). MRM transitions and collision energies are reported in Supplemental Material 1. The separation was carried out using Waters Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm particle size). The oven temperature was maintained at 37 °C. Mobile phase A and B was 0.2% (v/v) AA in water (Optima grade, Fisher Scientific, USA) and 0.2% (v/v) AA in LC-MS grade acetonitrile (Honeywell, USA), respectively. Injection volume was 5 µl. The binary gradient used was as follows: 0–1.0 min (0–16% B), 1.0–4.5 min (16–22% B), 4.5–4.6 min (22–100% B), 4.6–6.6 min (100% B), and 6.7–8.0 min (0% B). Flow rate was maintained at 0.55 ml/min. Calibration curves containing 11 concentrations (1 nM to 10 µM) were established for five reference standards by spiking reference standards in synthetic human urine (Surine™ Negative Urine Control; S-020, Sigma-Aldrich Corporation, St. Louis, MO, USA). The optimized *analyte specific* quadrupole voltages (mean ± SD) for negative mode were: declustering potential (DP), 75 ± 19; entrance potential (EP), 9 ± 1; collision energy (CE), 10 ± 1; collision cell exit potential (CXP), 11 ± 1. Similarly, for positive mode, the optimized analyte specific quadrupole voltages (mean ± SD) were as below: DP, -70 ± 20, EP, -10 ± 0; CE-40 ± 20; and CXP,-11 ± 1.

Conversion efficiency of GR to SF was calculated as follows: µmol of SF + SF metabolites collected in urine over 24 h as a percentage of the total µmol of GR ingested in the capsules provided in either Treatment A or B. Urine volumes were recorded in order to calculate urinary SF and SF metabolite quantities. Urinary creatinine was measured: (1) in order to identify non-compliance, for example, urine collections that may have been improperly performed (e.g. incompletely collected) and (2) to adjust SF and SF metabolite results for differences in hydration. To account for the variation in urinary concentrations between subjects, the creatinine-normalized excretion of the urine metabolites was determined. Urine creatinine concentrations were measured by a colorimetric assay using a commercial kit (catalog number EIACUN, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The method involves the reaction of creatinine reacts with picrate to form a chromophore under alkaline conditions. The absorbance of the creatinine-picrate chromophore is then detected spectrophotometrically. In brief, urine samples were diluted 1 to 20 with distilled water. Then, diluted urine samples and standards were incubated with 100 µL creatinine reagent for 30 min at room temperature. The absorbance was measured at 490 nm. Samples were analyzed in triplicate.

A Crossover design model with Treatment and Time Period as the experimental factors was used to analyze the SF plus metabolites data. Studentized residual plots were generated for the models in order to look for the presence of outlier observations that might (if not detected and removed from the analysis) produce misleading conclusions. The model test was followed by a Tukey-HSD comparisons procedure to further interpret the interaction. Data are presented as averages ± standard error (SE). A p-value cut-off of < 0.05 was used to determine statistical significance.

Post hoc examination of the data suggested that there might be some impact of gender on the trial data. So, in a secondary analysis the possible impact of gender and treatment in combination on total SF conversion percentage was analyzed using a Split Plot layout with gender as the whole plot factor and Treatment as the subplot factor. Since the primary analysis including both genders indicated that the impact of treatment was focused on the initial 0–8-hour time period, this analysis was carried out only on that portion of the data. There were no outliers in the analysis. The model test was followed by a Tukey-HSD comparisons procedure to further interpret the interaction.

DNA extraction from fecal samples

DNA was extracted from sixteen human fecal samples and two control samples (water and RNA-later) using the Zymo Quick-DNA™ Fecal/Soil Microbe MicroPrep Kit, according to kit instructions. The protocol was performed in a BSL2 hood, and all biosafety work was approved by the University of Maine Institutional Biosafety Committee. DNA extract concentration was determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific).

qPCR of select GR-metabolizing genes

To determine which fecal microbial communities have a higher quantity of GR-metabolizing genes, primer sets targeting known genes of GR-converting bacteria (Supplemental Material 2) were selected for quantitative polymerase chain reaction (qPCR), which was performed using the Applied Biosystems QuantStudio 6 Flex Real-Time PCR system and Luna® Universal qPCR Master Mix (New England BioLabs). Primers were diluted to 10 µM, for a final concentration of 0.25 µM in each well. For all primer sets, a standard curve for each gene was created using a set of 6 serially diluted Geneblocks (IDT). Primer sequences, Geneblock sequences, and protocols are detailed in Supplemental Material 2. All samples, standards and negative controls were run in triplicate. Quantstudio analysis software was used to calculate sample gene copy numbers from the standard curve. Data were visualized in the R software environment ver. 4.3.2[66], and plots were made using the ggplot2 package[67], and statistically analyzed in R using linear regression analysis, with and without log transformation.

The primer targets included the following: *Bacteroides thetaiotaomicron* VPI-5482 (*B. theta*) which contains a set of linked genes called operon BT2160-2156 using previously published primers and protocols[55] and geneblocks (Supplemental Material 2 Table S6[48]); *Ligilactobacillus agilis* strain R16 (formerly *Lactobacillus agilis*) which uses a set of three methionine sulfoxide reductase (Msr) genes, primers and geneblock for Msr3 were designed using PrimerBLAST; *Enterococcus casseliflavus* strain CP1 which uses three β-glucosidase genes using a combination of published[56] and PrimerBLAST-designed primers; *Citrobacter* spp. strain Wye1 which uses a GH3 β-O-glucosidase gene[56]; and *Escherichia coli* which uses a β-glucosidase gene[56].

16 S rRNA bacterial community sequencing analysis

Samples underwent DNA amplicon sequencing of the 16 S rRNA gene V3-V4 region, using primers 341 F and 806R and protocols consistent with The Earth Microbiome Project[68], and sequenced on an Illumina MiSeq platform using the 2 × 300-nt V3 kit (Molecular Research Labs, Clearwater, TX, U.S.). Raw sequence data (fastq files and metadata) is available from NCBI through BioProject Accession number PRJNA1189833. Amplicon sequence data were processed using previously curated workflows[49] in the Ishaq Lab (Supplemental Material 3) which used the DADA2 pipeline ver. 1.22[69] in the R software environment ver. 4.3.2[66]. The data set started with 8,334,957 raw reads from 16 samples and 2 negative controls. Trimming parameters were designated based on visual assessment of the aggregated quality scores at each base from all samples (plotQualityProfile in DADA2): the first and last 10 bases were trimmed, and sequences were discarded if they had ambiguous bases, more than two errors, or matching the PhiX version 3 positive controls (Illumina; FC-110–3001). After filtering, 7,853,583 paired non-unique reads and 16 samples remained.

The DADA algorithm was used to estimate the error rates for the sequencing run, dereplicate the reads, pick sequence variants that represent "microbial individuals," and remove chimeric artifacts from the sequence table. Taxonomy was assigned using the Silva taxonomic training data version 138.1[70] and reads matching chloroplasts and mitochondria taxa were removed using the dplyr package[71]. No-template control samples were used to remove contaminating sequences from the samples by extraction batch[72]. The sequence table, taxonomy, and metadata were combined using the phyloseq package[73] to facilitate statistical analysis and visualization, representing 16 samples and 2,941 taxa. Due to the variability in sequences per sample which passed quality assurance parameters (range 12,060–157,040 sequences/sample), the data were rarefied[74] to 12,060 sequences/sample.

Normality was checked using a Shapiro-Wilks test on alpha diversity metrics generated from rarefied data; observed richness ($W = 0.96522$, P-value = 0.7565); evenness ($W = 0.93122$, P-value = 0.2548; and Shannon diversity were normally distributed ($W = 0.95075$, P-value = 0.5016). Linear models compared alpha diversity metrics (lme4 package[75], in which GR conversion group rankings and/or percentages were used as factors. Jaccard unweighted similarity was used to calculate sample similarity based on community membership (species presence/absence), and non-parametric multidimensional scaling and tested with permANOVA using the vegan package[76]. Random forest feature prediction with permutation was used to identify differentially abundant SVs based on factorial conditions[77]. Plots were made using the ggplot2[67], ggpubr[78], and phyloseq packages.

Results

Bioavailability/conversion efficiency

The combination of BSE and mustard seed powder containing active Myr (GR + Myr), on average, doubled bioavailability of GR to SF and SF metabolites as a percent of the 115 µmol (50 mg) GR dose: from 18.6 ± 3.1% (range 2.2–38.1%) without myrosinase to 39.8 ± 3.1% (range 5.3–76.9%) with exogenous Myr from mustard seed (11.30, df = 1, 43, $p = 0.0016$) (Fig. 3A). Moreover, inclusion of exogenous Myr significantly altered the pharmacokinetics of conversion as evidenced by the finding that conversion efficiency as measured by composite of SF + SF metabolites in the first 8 h urine collection (Time 8 h), which was significantly higher in the GR + Myr treatment compared to GR alone: 25.4 ± 2.7% vs. 8.0 ± 2.7% (F = 20.60, df = 1, 14, $p = 0.0005$) (Fig. 3B). In contrast, no differences were observed in the 8 to 24 h urine collection (Time 24 h) between the two treatments: 11.7 ± 1.3% for GR + Myr vs. 8.9 ± 1.3% for GR alone (F = 9.45, df = 1, 43, $p = 0.0037$). One outlier was identified and removed from the subsequent analysis. The analysis was repeated with the outlier included, but it did not alter the results (data not shown). No irregularities were noted in dietary recalls.

Concentrations of GR, the compound delivered in the capsules, were below the limit of detection for all urine samples for all subjects at all timepoints evaluated (data not shown). Data on urinary excretion of SF and its individual metabolites is contained in Supplemental Material 1, Table S4. Urinary creatinine was measured as a means of adjusting for differences in hydration status within and between subjects. Adjustment for urinary creatinine concentrations had no effects on the outcomes evaluated (data not shown), therefore, the unadjusted data is presented throughout.

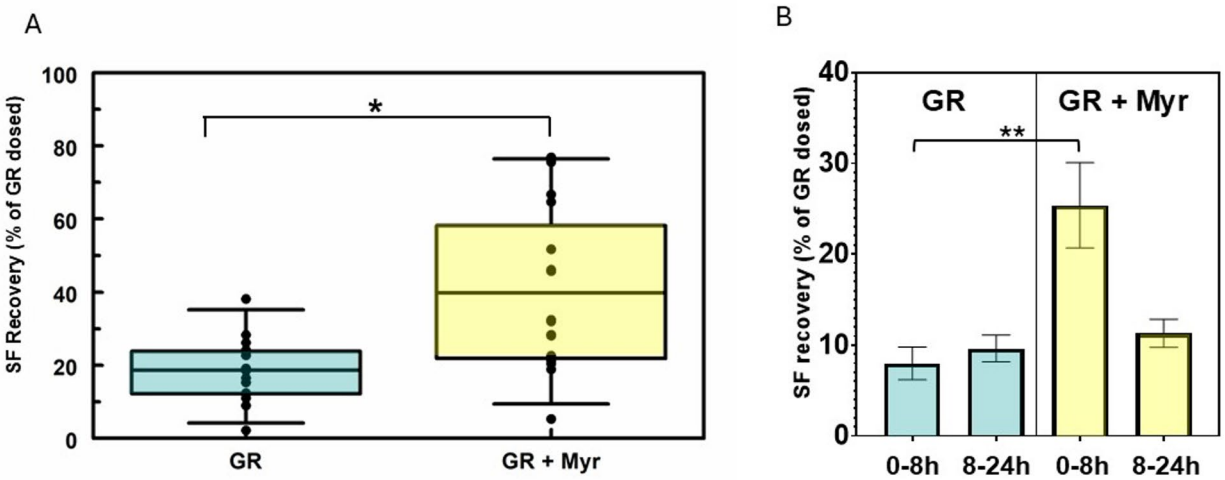

Fig. 3. Bioavailability of glucoraphanin (GR) as measured by recovery of sulforaphane plus its major metabolites (SF) excreted in (**A**) a complete 24-hour period of urine collection, or (**B**) in the first 8 h and the following 16 h of urine collected. Both treatments included ascorbic acid. For panel A, all data were grouped as recovery of sulforaphane plus its major metabolites (SF) excreted in a complete 24-hour urine collection following administration of an oral bolus of GR from BSE and the treatments were significantly different (*$p = 0.0016$). In panel B, data are shown as recovery of SF and metabolites excreted in the first 8 h versus 8 to 24 h after a single dose. The recovery in the first 8-hour urine collection following administration of an oral bolus of GR from BSE with Vit C was 3.2-fold higher with- than without added myrosinase (**$p = 0.0005$), and there were no significant differences between treatments in the following 16 h of urine collected.

Post-hoc analysis on the effect of gender on GR conversion

Post hoc examination of the data suggested that there might be some impact of gender on the trial data. Thus, a secondary analysis of the possible impact of gender and treatment in combination on total SF conversion percentage was performed. Since the primary analysis including both genders indicated that the impact of treatment was focused on the initial 0-8-hour time period, this analysis was carried out only on that portion of the data.

The effect of gender on conversion was explored using a Split-Plot model with Gender as the whole plot factor and Treatment as the subplot factor. Studentized residual plots identified no outliers. The analysis identified multiple significant effects: for Treatment (as expected), $F = 21.40$, $df = 1,14$, $p = 0.0004$, and for Gender, $F = 5.62$, $df = 1, 14$, $p = 0.0326$; with a borderline significant Gender by Treatment interaction, $F = 3.57$, $df = 1, 14$, $p = 0.0796$). Thus, males given GR alone had significantly greater conversion during the 0–8-hour urine collection than did females, whereas female subjects showed no treatment effect during either time period and did not differ significantly from male subjects receiving GR + Myr in either time period.

Order of supplementation affects GR conversion

GR conversion percentage was quantified for each participant following supplementation. Eight participants received GR + Myr first, followed by a 14 day washout period, then received GR alone second. The other eight participants received GR alone first, followed by the washout, then GR + Myr. The addition of exogenous Myr significantly increased conversion efficiency compared to GR-only, as shown by the bioavailability data. The two groups of participants had similar GR conversion when comparing the GR-only treatment, regardless of whether it was their first or second treatment period (Fig. 4, Supplemental Material 1 Figure S2 for individual responses). Interestingly, the order of treatments mattered for the response to the second treatment. Participants who received GR alone first, then GR + Myr second, had significantly higher conversion than the GR + Myr-first group when comparing the conversion for the GR + Myr ($p = 0.0089$) and the GR-alone treatment ($p = 0.033$) (Fig. 4; two-tailed unpaired t-test of unequal variance).

Bacterial GR-converting gene copy number

Bacterial GR-converting gene levels correlate with GR conversion. Conversion of GR to SF and its metabolites was calculated for participants in the first period of the crossover design, in which only samples from people who received GR alone as the first treatment (Fig. 5) were utilized to measure the effect on gut bacteria using a linear model regression model (lm). Conversion was compared to the copy number of selected bacterial genes known to be involved with GR conversion[55,56,79]. These included 5 genes from the *Bacteroides thetaiotaomicron*

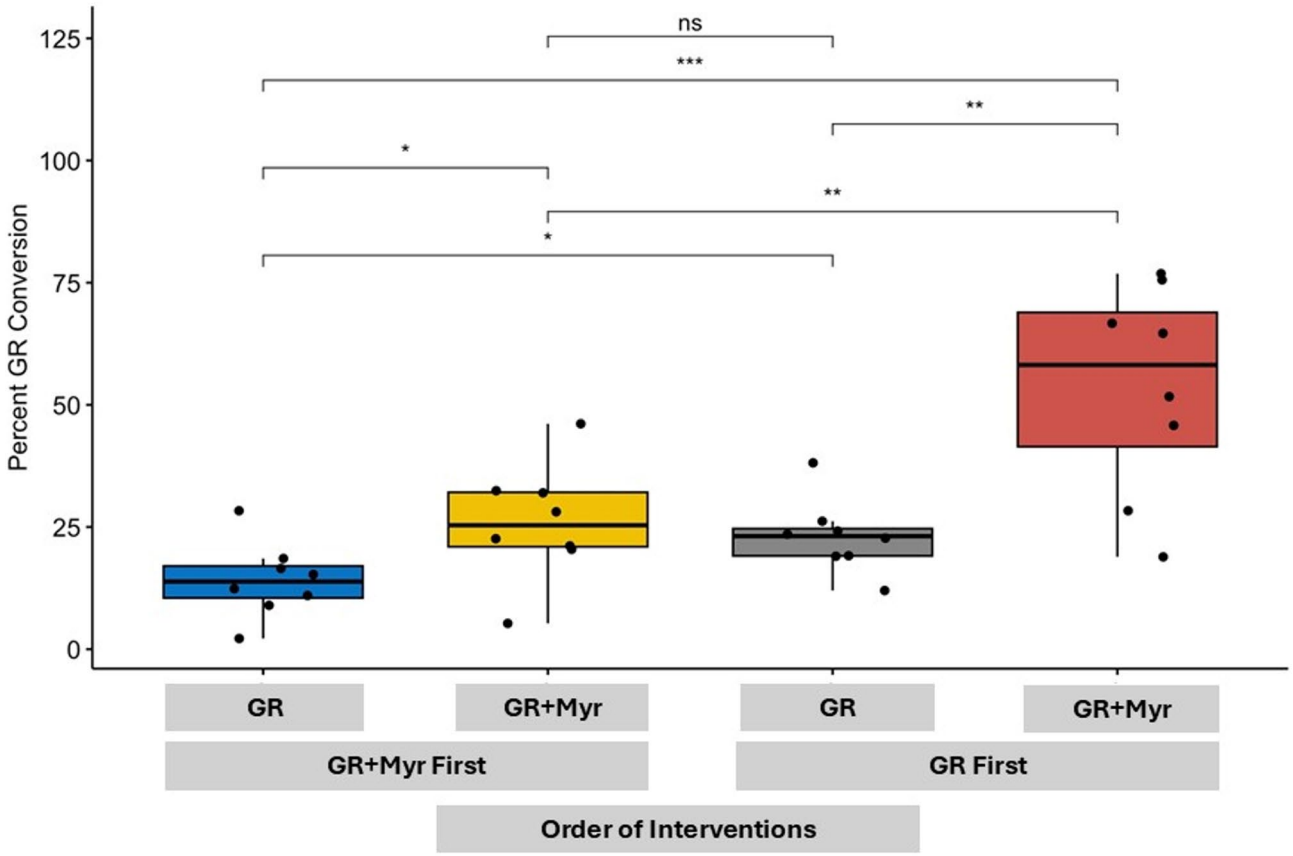

Fig. 4. Glucoraphanin conversion percentages from all participants following supplementation of GR + Myr or GR alone, by participants' first treatment in accordance with study crossover design. The group of participants who received GR alone first, then GR + Myr second, had significantly higher GR conversion percentages following either GR + Myr or GR alone ($p = 0.0089$ and $p = 0.033$, respectively; two-tailed unpaired t-test of unequal variance). Pairwise comparisons by condition (i.e., group (GR + Myr First or GR First) and treatment (GR + Myr or GR)) also demonstrate significant differences in GR conversion percentage between experimental conditions.

(*B. theta*) GR-converting operon, as well as genes from bacteria which have not had the full GR metabolism pathway identified: two from *Escherichia coli*, two from *Enterococcus casseliflavus*, and one from *Ligilactobacillus agilis*. Four genes from the *B. theta* operon were significantly correlated with % GR conversion: BT2156 (lm, F = 3.439, adjusted $r^2 = 0.6073$, $p = 0.0138$); BT2157 (lm, F = 3.562, adjusted $r^2 = 0.6254$, $p = 0.0119$); BT2158 (lm, F = 3.984, adjusted $r^2 = 0.68$, $p = 0.00725$); and BT2159 (lm, F = 2.708, adjusted $r^2 = 0.475$, $p = 0.0352$). A previous *B. theta in vitro* culture study demonstrated that the expression of gene 2158 and at least one of either 2156 or 2157 was required for GR conversion, and expression of at least four correlated with GR conversion[55]. No other genes were significantly correlated with % GR conversion in people who received GR alone first, nor was the total count of these other genes significant (lm, $p > 0.05$). Bacterial gene counts were not correlated with % GR conversion of GR + Myr, in individuals who received that treatment first (Supplemental Material 4 Figure S3, $p > 0.05$), which was expected as this treatment did not rely entirely on gut bacteria.

Similarly, we used linear regression to test the correlation between GR conversion and bacterial GR-conversion genes in all samples (both crossover periods) for the GR alone or GR + Myr groups to test the capacity to predict response to GR regardless of the timing of the sample. Only two genes from the *B. theta* GR-converting operon were significantly correlated with conversion percent: *B. theta* 2158 (linear model, F = 3.548, adjusted $r^2 = 0.4358$, $p = 0.00322$) and *B. theta* 2159 (linear model, F = 2.756, adjusted $r^2 = 0.3054$, $p = 0.0155$), both of which code for oxidoreductase enzymes. There was no correlation between any of the genes and GR conversion when GR + Myr was given, which was expected as this treatment did not rely on gut bacteria.

Bacterial community similarity

Bacterial community changes were subtle, but not significantly different depending on supplementation order. The number of bacterial taxa present (i.e., bacterial richness) in feces 14 days after either GR + Myr supplementation or GR alone supplementation was not different between these two groups (Supplemental Material 4, Figure S4, Wilcoxon test $p > 0.05$). Further, bacterial community samples did not cluster by participants' first supplementation based on unweighted Jaccard Dissimilarity (Figure S5, GR + Myr first vs. GR first, permanova test $p = 0.6989$).

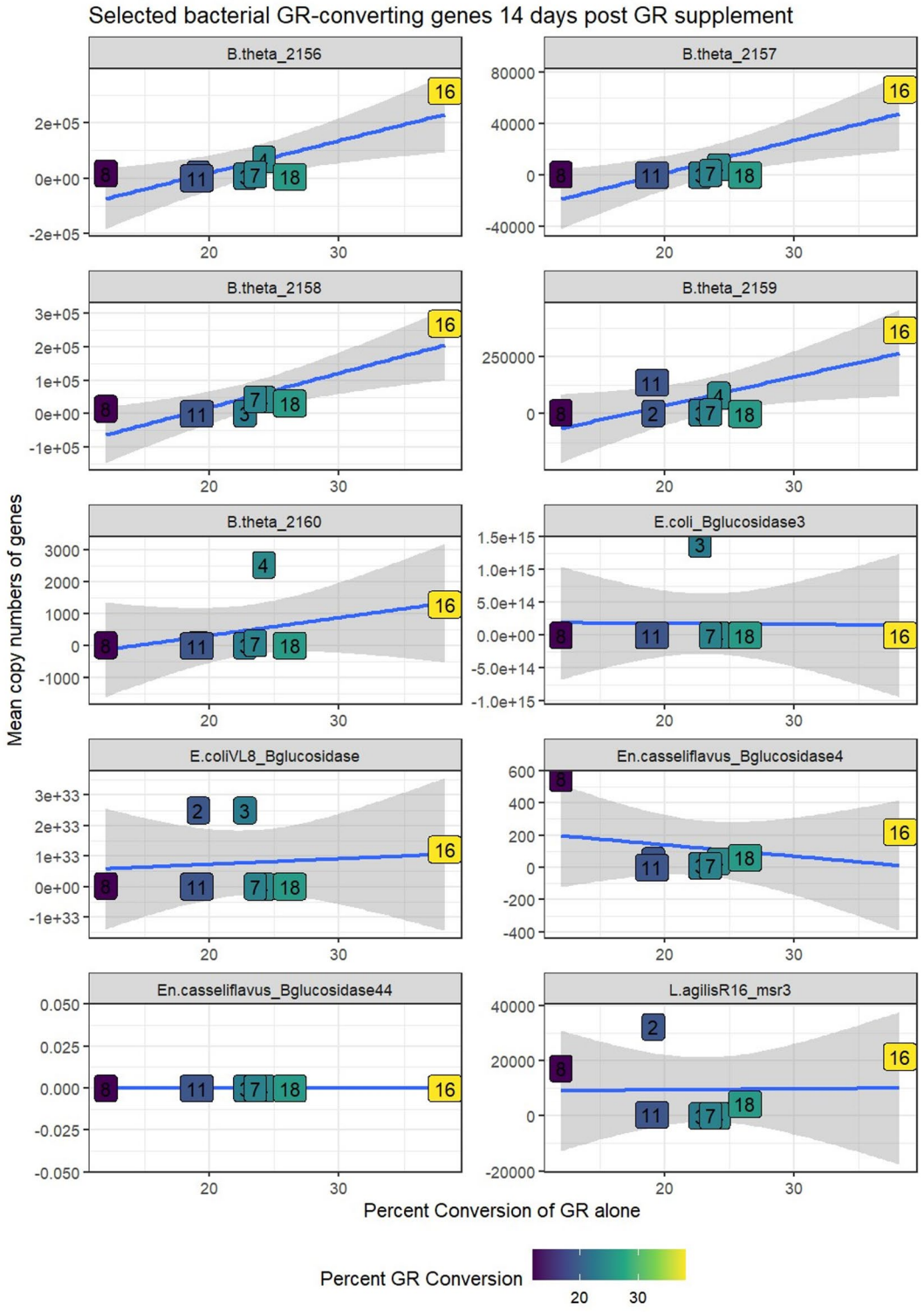

Fig. 5. Percent conversion of GR in people who received that treatment first, compared to copy numbers from selected genes involved in GR metabolism in fecal bacteria. Bacterial genes were amplified from fecal samples collected 14 days after the first period. Samples are labeled by participant number and colored by percent GR converted by gut bacteria. A trendline was added using linear regression, which was used to test for significance ($p < 0.05$). BT2156-BT2159 were significant.

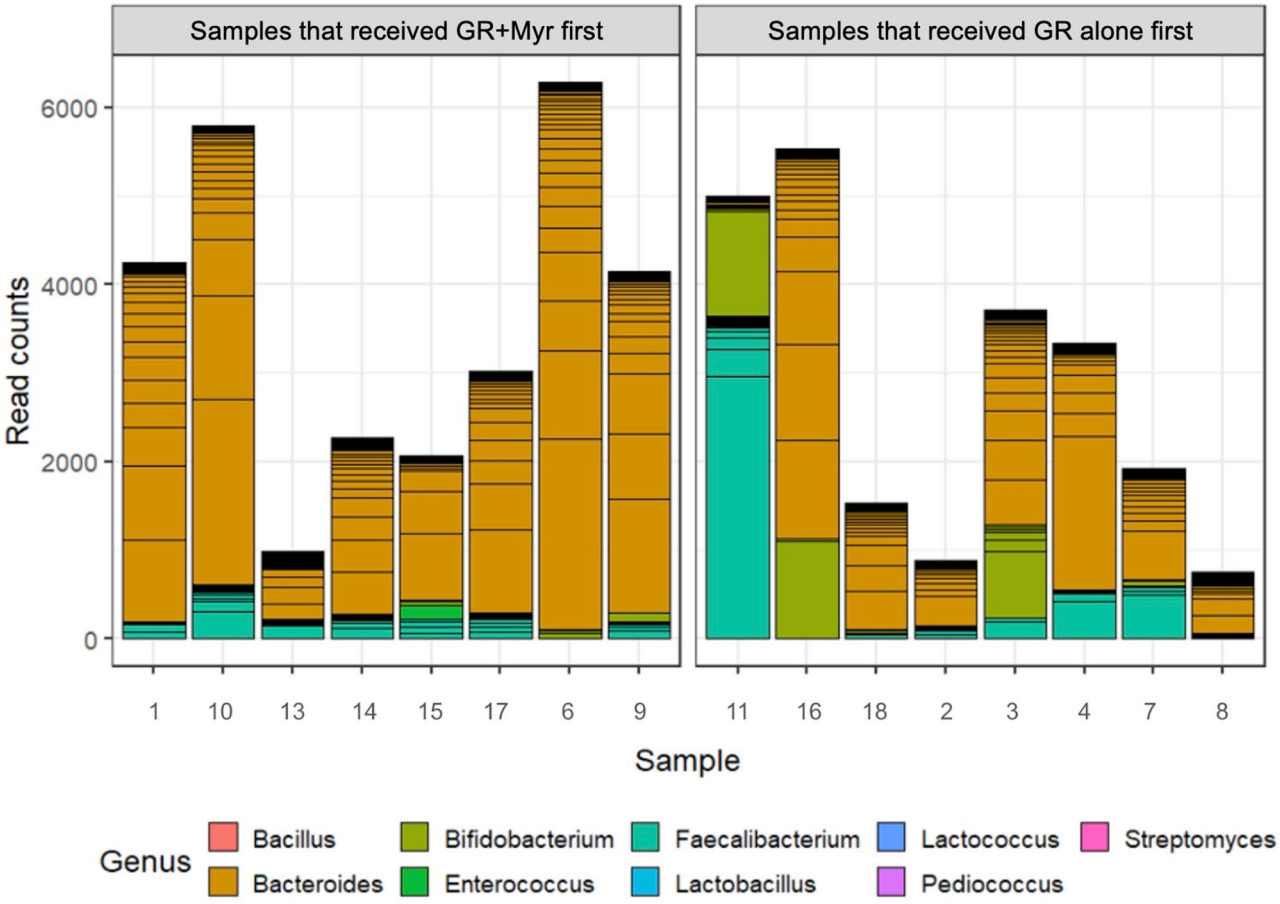

Fig. 6. Abundance (read counts) of genera with putative GR-metabolizing capacity for participants 14 days after GR + Myr or GR alone. Bacterial communities were amplified from fecal samples collected 14 days after GR + Myr, or after GR alone to examine the participants' existing potential for GR conversion. Samples are labeled by participant number and colored by bacterial genus for each sequence variant block in the columns.

There were subtle differences in the most prevalent and relatively abundant genera (i.e., core genera) of the bacterial communities from participants who received GR + Myr prior to fecal sampling versus those who received GR alone. Of note, *Bifidobacterium* was a more prevalent and abundant genus in feces from participants who received GR alone (Figure S6 and Figure S7). However, a random forest taxa classifier was unable to predict specific bacteria associated with treatment (data not shown, random forest model accuracy = 6.25%). Nor did DESeq2 negative binomial[80] reveal any significantly differentially abundant taxa between the two treatment groups (data not shown).

There are several commensal gut bacterial taxa that perform myrosinase-like enzymatic activity and metabolize GR to SF[55,56,79]. Nineteen taxa were identified for analysis in detail. Within our samples, we identified 244 bacterial sequence variants (SVs) belonging to the following nine genera: *Bacteroides*, *Bifidobacterium*, *Faecalibacterium*, *Enterococcus*, *Bacillus*, *Lactococcus*, *Lactobacillus*, *Pediococcus*, and *Streptomyces*. Interestingly, there were slight differences in bacteria; community composition with respect to treatment group and GR conversion percentage (Fig. 6). Three participants (#3, #11, #16) had a notable abundance of *Bifidobacterium*, which has been shown to metabolize glucosinolates in culture[81,82]. These 3 participants received GR alone prior to their GR + Myr supplementation, and this treatment group showed significantly greater GR conversion percentages following each treatment ($p = 0.033$ and $p = 0.0089$, respectively; unpaired t-test) (Fig. 6). Furthermore, participants 11 and 16 both achieved > 50% GR conversion following GR + Myr supplementation, ranking them as top converters.

Bacterial communities did not differ by conversion efficiency

Bacterial communities did not differ between low/high GR converters following supplementation. The bacterial richness in feces 14 days after GR supplementation was not different between people who were post-hoc classified as high/mid/low GR converters (Supplemental Material 4, Figure S8, Wilcoxon test $p > 0.05$). Nor did bacterial community samples cluster by post-hoc conversion rate based on unweighted Jaccard Dissimilarity (Figure S9, permanova test $p > 0.05$). A random forest classifier could not predict specific bacteria associated with post-hoc GR conversion percentage (data not shown, random forest model accuracy 12%).

The *Bacteroides* genus was most prevalent and abundant across all except one sample, regardless of participants' GR conversion ranking or treatment order. There were no notable differences in bacterial

community composition with respect to taxa that may perform GR conversion between high, mid, and low percentage GR-converting participants.

Bacterial richness, beta diversity clustering, and random forest taxa prediction were not significantly associated with post-hoc designation of GR-conversion percentage when participants were supplemented with GR and endogenous myrosinase to provide immediate SF and bypass the need for bacterial GR conversion. Some participants were low converters with GR alone but high converters when given endogenous myrosinase, however, we did not find any bacterial richness, beta diversity, or taxa associated with these participants which might indicate bacteria were associated with a poor response to the endogenous myrosinase treatment. Contrastingly, there was one participant who was a low GR converter when provided with endogenous myrosinase and slightly better when given GR alone, and one participant who converted < 5% on either treatment, but no microbial community assessment metrics indicated any bacterial taxa were associated with poor conversion. As a further post-hoc analysis, and because the values for some individuals' SF metabolites were surprisingly low, we performed a post-hoc analysis of SF-nitrile using stored urine samples (see Supplemental Material 1). Whereas some subjects produced almost no SF-nitrile, one subject produced almost exclusively SF-nitrile. GR to SF conversion approximately doubled when myrosinase was delivered along with GR, whether SF-nitriles were included in the sum or metabolites (1.9-fold) or not (2.1-fold) (Table S4). No bacterial genes were significantly correlated with SF-nitrile production, in either the GR alone (Figure S10) or the GR + Myr groups (Figure S11).

Discussion

Over 100 clinical studies have now investigated delivery of GR and SF, individually and combined[2,54]. However, interpretation of the scientific literature is often confused by the fact that many studies use the terms GR and SF interchangeably. For example, many studies supplement with GR, but incorrectly list SF in the title and body of the manuscript, leading to challenges in the interpretation of the data[80,59]. Nonetheless, both compounds studied independently have been shown to confer health benefits in humans. In this study, our primary outcome variable was the conversion efficiency of GR to SF, calculated as the composite of SF plus its metabolites measured in urine collected over 24 h, and expressed as a percentage of the total GR ingested. The amount of GR supplemented in the present study was 50 mg, in line with levels that could be readily obtained from a serving of fresh broccoli, depending on the cultivar and other conditions.The addition of exogenous Myr from mustard seed powder resulted in significantly increased conversion efficiency and therefore, bioavailability of SF within 24 h of ingestion. These clinical results are consistent with the published literature, and moreover they are congruent with the studies reporting the greatest increases in SF bioavailability. Concentrations of GR, the compound delivered in the capsules, were below the limit of detection for all urine samples for all subjects at all timepoints evaluated, consistent with previous studies reporting that GR itself is poorly absorbed, < 1–3%, unless it is first converted to SF[17,18].

Previous studies have reported conversion efficiencies in the range of ~ 30–40% (on a molar basis) when GR is co-delivered with exogenous plant Myr. For example, Fahey et al.[59] reported a mean conversion efficiency of 35.9% from broccoli seed powder with active Myr. Our results (39.8%) are at the highest end of this range. Differences in bioavailability could be due to a number of factors including the type of capsule and opening time in the GI tract, the source of Myr (broccoli seed vs. mustard seed), the amount of Myr activity, the subject population, the number of subjects ($N = 4$ vs. $N = 16$), and the fact that we provided exogenous ascorbic acid (AA), a cofactor for the myrosinase enzyme known to increase myrosinase activity[21,35,62], in both treatment arms. We recently published results from a dynamic gastric digestion model, Human Gastric Simulator (HGS), investigating the pharmacokinetics of the same base formula used in the present study (25 mg GR from BSE + 72.5 mg mustard seed powder with active Myr per capsule) without or with a range of ascorbic acid doses (0, 11, 44, 88, or 154 mg AA/capsule)[21]. The size 0 vegetarian hypromellose capsules used in the present study are designed to disintegrate in the upper GI tract and release contents in the small intestine. Similar capsules were investigated in the HGS model (size 0 vegetarian hypromellose capsules), but from a different manufacturer. In the HGS model, the capsules began to dissolve after 20 min of dynamic gastric digestion and at ~ 30 min, the capsule and the majority of its contents (98% of GR + Myr) passed out of the HGS into the simulated small intestine. In the absence of AA, conversion efficiency of GR to SF was calculated as 40% following 1 h and 70% after 2 h incubation in the simulated small intestine. In contrast, addition of 154 mg AA per capsule (compared to 50 mg AA/capsule in the present study) increased conversion efficiency from 40% up to 70% following 1 h incubation in the simulated small intestine, whereas lower levels of AA exhibited only modest effects. The effects of AA on conversion efficiency were even more apparent after 2 h incubation in the simulated small intestine, where 88 and 154 mg AA/capsule increased conversion efficiency from 70% to nearly 100%, confirming that inclusion of AA, a known cofactor of the myrosinase enzyme, has the potential to significantly enhance conversion efficiency. Notably, the lower doses of AA, including the dose closest to the amount studied here, 44 mg AA/capsule, did not significantly increase conversion efficiency at 2 h compared to the 0 mg AA capsules, suggesting an important dose response that should be considered in future studies. Moreover, these data suggest that the present study may not have included sufficient AA to optimize Myr enzyme activity in vivo, another consideration for future studies.

In the present study, when subjects received GR without Myr, the reported bioavailability of 18.6% was higher than the ~ 10% previously reported in the literature[44]. For example, when the same BSE was delivered in capsule format in a previous study, the mean conversion efficiency was only 11.4%[59]. In contrast, broccoli sprout extracts containing glucoraphanin supplemented as beverages every 8 h for 7 days, yielded a cumulative excretion of dose similar to the present study, 18.7%[83]. This could be explained by a number of factors, including differences in the capsules used and/or differences in the study populations and their native microflora. It is unlikely that any of the differences can be explained by differences in dose as previous studies, with or without the presence of exogenous Myr, have consistently demonstrated similar bioavailability, regardless of dose[59,83]. Of note, the abovementioned

studies did not supply exogenous AA with myrosinase whereas we did. These studies did, however, utilize the precise [commercial] BSE investigated in the present study. Shapiro et al.[83] compared the bioavailability of two doses of GR following supplementation with broccoli sprout extracts containing GR in a beverage consumed every 8 h for 7 days. Cumulative excretion of the two doses, 100 µmol (44 mg) vs. 25 µmol (11 mg), were not statistically different 17.8 ± 8.6% vs. 19.6 ± 11.7%, despite the 4-fold difference in dose.

The majority of subjects given GR as BSE with exogenous Myr and Vit C, converted GR to SF notably faster (e.g., within the first 8 h), than those given GR and Vit C alone. One of the most likely explanations for the pronounced differences is that when SF was produced as a result of added exogenous Myr, it was produced and absorbed in the small intestine and metabolized primarily to its GSH derivatives and excreted in the urine. This is a more rapid process than when GR passes into the large intestine and then is acted upon by the greater bacterial population within that terminal segment of the gastrointestinal system. Thus, the data is entirely consistent with the most plausible biological explanation, though this explanation was of course not proven as a result of our intervention. In line with this idea, a recent study evaluating the kinetics and bioavailability of SF from broccoli confirmed a quicker appearance of SF excretion peaks within 2–3 h after consumption of products with a high myrosinase content due to its absorption in the small intestine[65]. In contrast, a product with a minimal amount of myrosinase showed delayed peaks (7–8 h after consumption) reflecting SF formed by the gut bacteria and absorbed exclusively in the large intestine[84,85].

In addition to plant-derived Myr activity, there are several bacteria with capacity for metabolizing GR to SF[50,21], which are recruited in fermentations of crucifers as well as in the gut of mice or people consuming cruciferous vegetables[48,49]. Only one bacterial isolate, *Bacteroides thetaiotaomicron* (*B. theta*), has a fully described gene pathway for GR conversion[55]; and different pathways have been proposed for several other taxa[56,84] via β-glucosidase and other genes involved in fiber metabolism, as well as genes involved in sulfur metabolism. These taxa include strains of *Bifidobacterium*, *Citrobacter*, *Enterobacter*, *Enterococcus*, *Escherichia coli*, and *Ligilactobacillus agilis*[56,79].

The GR conversion microbial pathway in the human gut remains largely unknown, despite the in vitro use of colon-specific bacteria with fermented cruciferous vegetables[48,49]. While some genes have been identified as being upregulated or involved during GSL digestion, only one bacterial species, *B. theta*, has a full gene pathway described[55]. We documented four of the five *B. theta* GR-converting genes in fecal samples from our study subjects which were correlated with GR conversion. Two individuals (#4 and #13) had very low GR gene counts overall, and hosted only B. theta genes plus a small amount of one other bacteria's GR genes (beta-glucosidase genes from either E. coli VL8 or Enterococcus casseliflavus), but did not have the lowest conversion rate on GR supplementation alone, implying that GR-converting bacteria other than those tested for may be present. Contrarily, one individual with only a 2% conversion rate on GR supplementation alone hosted B. theta and smaller amounts of both E. coli VL8 and Enterococcus casseliflavus.

Many bacteria which persist in the small intestine and upper large intestine, as well as in mucosal-associated fractions, are not well represented in feces. The lack of anatomically specific microbial communities in the human gut limits our knowledge of GR conversion in people, and the microbial community of even specific pathogen-free mice is not complex enough to fully replicate the human gut, however some proof-of-concept studies exist. In a previous study, feeding healthy mice a chow diet containing 5% wt/wt steamed broccoli sprouts, which inactivated plant Myr and would therefore rely on gut microbiota to convert GR, resulted in increased SF content along the GI tract, peaking within the colon (6–23 ug/g of colon tissue vs. up to one hundred ng/g in upper intestine and cecum tissues). In contrast, GR was decreased throughout the GI tract[47]. These data imply that gut bacteria have location-specific conversion of GR. This was supported by additional work on gut biogeography in a DSS-mouse-model of ulcerative colitis[48], and an IL-10-ko-mouse-model of Crohn's Disease[49].

SF-nitrile has very limited biological activity and has typically not been reported in the literature in relation to GR to SF conversion which has focused only on the glutathione (GSH) mediated conjugation and excretion of SF, nor did we assess SF-nitrile content originally. However, since the conception of this study robust methods for the determination of SF-nitrile in urine have been published[85,86]. As yet, the reason for the high variability and inefficient conversion of GR to SF and its metabolites in the absence of exogenous Myr (ranging from 1 to 40% in healthy volunteers) is hypothesized to be, in part, a result of interindividual differences and strain-specific patterns in people[54,82,87]. For example, some species of *Bifidobacteria* make ITCs[88] while others produce GLS-derived-nitriles[81]. Similarly, SF-nitrile production was highly variable in our study and others[87], although SF-nitrile production is low in specific-pathogen-free rodents, e.g., rats[16], which lack the species of enterococci associated with nitrile production[87].

We discovered an effect of gender on GR conversion: males given GR alone had significantly greater conversion during the 0–8-hour urine collection than females, whereas female subjects showed no treatment effect during either time period and did not differ significantly from male subjects receiving GR + Myr in either time period. Since the gender effect in the model analysis was not a planned part of the original trial design and its presence precluded including the other effects that are part of the crossover design, these results must be regarded as suggestive and not conclusive. No gender-based differences were observed in other studies in people[89] and in mice[90]. However, our present results are consistent with our earlier results in which males (n = 47) converted 15.8% of dose and females (n = 84) converted 9.5% of dose[52]. This may be related to the different half-lives of SF in tissues, or differences in gut chemistry via dietary agonists (e.g., ascorbic acid[35]) or antagonists (e.g., amygdaline and arbutin[91]). For example, high-cysteine diets may liberate SF from its conjugated form and allow it to be re-used[92]. We observed no gender-based differences in the microbial community data which might explain the difference in conversion, although in other studies a slight increase in gut microbial diversity was linked to interactions between sex steroid hormones and specific bacteria[93], or linked to the effect of sex hormones on average height, weight, and by extrapolation, to the size and length of the intestines[94].

In our study, participants had similar GR conversion when comparing the GR-only treatment, regardless of whether it was their first or second treatment period. But, participants who received GR alone first, had significantly higher conversion of GR + Myr compared to the conversion efficiency in the GR + Myr-first group. This implies cooperation between bacterial conversion and Myr conversion in that second period, and an entrainment of the gut bacterial community. Even when the bacterial community structure (presence or abundance) is unchanged, activity levels can vary widely, which we were unable to capture in the present study. For example, bacteria with minimal abundance can dominate microbial community biochemistry, especially for niche metabolites where they have little competition[95]. Thus, our GR conversion activity could be increasing without increasing the abundance of GR-converting bacteria in people. A previous study in mice confirmed that a daily dose of GR for two weeks resulted in a progressive increase in GR conversion in the first week, as measured by the amount of GR and SF collected in daily feces[47], implying that previous treatment with GR can induce a better microbial response after multiple doses. After the first week in which conversion percent increased, mice in the previous study exhibited a consistent level of conversion for an additional week during daily GR dosing[47], which suggests that the gut microbiome is capable of maintaining this conversion. Future studies will need to focus on the metatranscriptome of the gut to better assess the microbial contribution to GR conversion.

To evaluate microbial trends in our study, we collected a single stool sample 14 days after the first treatment period and the day prior to the second treatment period of the study (the day prior to Visit 3), to generate hypotheses about the impact of GR supplementation. We would not expect a single dose of GR to influence the microbiota, although we know that our participants have already encountered GR and other glucosinolates through prior consumption of cruciferous vegetables or condiments (e.g. mustard). Thus, the fecal sample allows us to make limited suppositions about the effect of GR versus GR + Myr supplementation on gut microbiota. However, by that same principle of trainability, the fecal sample allows us to generate hypotheses about how a prior supplement might predispose the gut bacteria (e.g. *Bifidobacterium*[81,82] to react more strongly to a follow-up dose.

Our findings are consistent with previous studies that found that GR conversion in people was highly variable[52,82], and that fecal bacteria from people with high GR conversion were more capable of performing this function even though the microbial community as a whole was not different from people with high and low percent GR conversion[82]. The exciting baseline data described herein now provide encouragement for a more detailed examination of effects of the microbiota on bioavailability and conversely, of the effects of dose form (GR or GR + Myr, and supplemental Vit C) on the microbiota.

SF is only moderately stable over time when stored in refrigerated or frozen conditions, and unstable at room temperature, limiting shelf life and product quality. Furthermore, broccoli sprout powders rich in SF are extremely hygroscopic and the preparation of capsules is thus both challenging and expensive. In order to overcome these limitations, we combined BSE with exogenous Myr from mustard seeds. For the production of the BSE used in the present study, seeds undergo a hot water extraction technique in which the endogenous Myr is denatured (inactivated), without degrading GR, which is heat stable. At the same time, the GR is concentrated, resulting in a relatively high GR (> 13%) product that is shelf stable at room temperature and well-suited for inclusion in a variety of commercial food and supplement formats including drinks, capsules and tablets. Alternative sources of exogenous Myr (e.g. daikon, mustard seeds, etc.) can be combined with broccoli seeds, sprouts and whole broccoli to increase conversion of GR to SF[44,59,60]. Mustard seeds are a rich source of Myr and in fact Myr from mustard seed is more thermally stable than broccoli Myr[60].

Conclusions

To our knowledge, this is the first human study: (a) to investigate the co-administration of GR from BSE and mustard seed powder with known Myr activity, (b) to include a standardized dose of ascorbic acid (Vit C), a known cofactor to the Myr enzyme, (c) to evaluate the possibility of predicting gut microbial responsiveness to GR, and (d) to investigate the effects of a well-defined source of Myr on conversion of broccoli seed GR to SF and its ability to modulate gut bacteria. Administration of a single oral bolus of a highly standardized dose of GR with vitamin C resulted in conversion to SF and its glutathione metabolites at the very high end of the range reported previously by us and others. When a highly standardized dose of Myr derived from mustard seed powder was added to this mixture, conversion was more than doubled and was also at the very top of the previously reported ranges. Further testing for the far less biologically active SF-nitrile was conducted, which did not alter the conversion ratio (with-to-without Myr). Strikingly, the addition of exogenous Myr to GR with vitamin C resulted in a dramatic increase in the conversion rate compared to GR with vitamin C only, as measured in the first 0–8-hour urine collection. Differences did not persist in the 8–24-hour urine collection. Furthermore, four documented GR-converting genes identified in fecal samples from study subjects were correlated with GR conversion, and bacterial entrainment is hypothesized to contribute to increase conversion efficiency. Future studies should focus on the metatranscriptome of the gut to better assess the microbial contribution to GR conversion. In summary, inclusion of Myr derived from mustard seed powder doubled the conversion efficiency of GR to SF compared to GR from BSE and Vit C alone. Given the extensive evidence on the anti-oxidant and anti-inflammatory benefits of sulforaphane, the use of GR + Myr supplementation can proceed in clinical trials with healthy and populations with inflammation-based diseases to determine efficacious dosage and treatment recommendations.

Data availability

Raw 16S rRNA bacterial sequence data (fastq files and metadata) is available from NCBI through BioProject Accession number PRJNA1189833.

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Author contributions

AM, LH, JWF, DCN, AP, YL, SLI & GV conceived the study, analyzed and interpreted the data. AM, AP, YL, SLI & GV designed the experiments. AM, LH, JWF, AP, SLI, & GV wrote and edited the manuscript. LH, CO, CK, & MK performed the laboratory analyses.CO, DCN, & MK performed the clinical studies. RO, LH, and SLI performed statistical analyses. All authors read and approved the final manuscript.

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Declarations

Competing interests

Angela Mastaloudis was employed by Brassica Protection Products, LLC., Jed W. Fahey was a consultant to Brassica Protection Products, LLC. Lola Holcomb, Camila Olson, David C. Nieman, Colin Kay, Robert O'Donnell, Alessandra Pecorelli, Marissa Kinney, Yanyan Li, Suzanne L. Ishaq, and Giuseppe Valacchi declare no conflicts of interest.

Additional information

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Correspondence and requests for materials should be addressed to S.L.I. or G.V.

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