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Effect of supplemental prebiotics, probiotics and bioactive proteins on the microbiome composition and fecal calprotectin in C57BL6/j mice

Ye Li, Korry J. Hintze, Robert E. Ward*

Nutrition, Dietetics and Food Sciences, Utah State University, 8700 Old Main Hill, Logan, UT, 84322-8700, USA

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ABSTRACT

The composition and metabolic activity of the microbiome affect many aspects of health, and there is current interest in dietary constituents that may affect this system. The purpose of this study was to evaluate the effects of a mix of probiotics, a mix of prebiotics and a bioactive protein fraction on the microbiome, when fed to mice alone and in combination at physiologically relevant doses. Mice were fed the total western diet (TWD) supplemented with prebiotics, probiotics, and bioactive proteins individually and in combination for four weeks. Subsequently, effects on the composition of the gut microbiome, gut short-chain fatty acids (SCFAs) concentration, and gut inflammation were measured. *Ruminococcus gnavus* was increased in mice gut microbiome after feeding prebiotics. *Bifidobacterium longum* was increased after feeding probiotics. The treatments significantly affected beta-diversity with minor treatment effects on cecal or fecal SCFAs levels, and the treatments did not affect gut inflammation as measured by fecal calprotectin.

1. Introduction

Prebiotics and probiotics are two common dietary supplements that have been shown to affect gut health in both rodent and human studies. Prebiotics are substrates that are utilized by select gut microorganisms, and which confer a health benefit [1,2]. Probiotics are bacteria that improve gut health, and which come predominantly from the *Lactobacillus* and *Bifidobacterium* genera [3–5]. Most prebiotics are oligosaccharides, which pass undigested through the small intestine to the colon and are fermented by intestinal bacteria and stimulate the growth of specific microbial taxa [6–8]. Probiotics are taken as supplements. The bacteria are added or naturally present as starter culture for fermented foods like yogurt, kefir, or kimchi.

There have been many model rodent and human clinical studies that have investigated the health benefits of prebiotics, probiotics and/or synbiotics (prebiotics and probiotics administered together). Such health benefits include promotion of gut fermentation, modulation of the microbiome composition, reduction of inflammation, decreased susceptibility to food allergy and prevention of cancer [9,10]. Suggested benefits of probiotics include improvement of the gut barrier function, increased competitive adherence to the mucosa and epithelium, gut microbiota modification, and regulation of the gut associated lymphoid immune system [11]. In general, the effectiveness of probiotics and prebiotics have been more substantial in rodent studies [12]. Several factors may explain the discordance between rodent and human studies, including 1) the specific prebiotics and probiotics administered, 2) the method of delivery, 3) the duration of treatment, 4) the dosage used, and 5) fundamental differences between the two species. In addition, rodent studies often use inbred strains and thus genetic variability is lower.

In rodents, prebiotic supplementation of diets is common at levels between 5.5% and 15% on a mass basis [13–16], which is high, considering total dietary fiber in the purified rodent diet, the AIN-93G, is only 5% by mass. In human studies, prebiotics have been provided at levels between 5 and 20 g/day [17–24]. A possible explanation for differences in results between rodent and human studies may be the amount given, yet there has not been much discussion in the literature of how to translate prebiotic intakes

* Corresponding author.
E-mail address: robert.ward@usu.edu (R.E. Ward).

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between species. If dosage levels are compared on a gram of prebiotic consumed per kilogram of body weight in mice and humans, the levels are approximately 60 times higher in rodent studies. Yet, body weight normalization does not take into account the increased metabolic rate of rodents. Allometric scaling is a method of interspecies comparison of basal nutrient requirements [25], and may be more appropriate for translating intakes between species. One method of allometric scaling is nutrient density, wherein nutrients are expressed relative to calories. If the nutrient density of prebiotic supplementation is compared (mg prebiotic/kcal diet), then rodent studies typically supply 3–4 fold more that the human studies.

In rodent studies, probiotics have been given in a range of 10^8 and 10^9 CFU/day for mice [26–29], and around 10^9 CFU/day for humans [23,24,30–35]. If probiotic intakes between rodents and humans are compared on a mass basis (i.e. 10^9 CFU for 75 kg human vs. 10^8 CFU for 25 g mouse), rodents are typically given ~150X more. When CFUs are normalized to calorie intake (i.e. 10^9 CFU for 2500 kcal/d human vs. 10^8 CFU for 11 kcal/d mouse), rodents are given ~10X more. In rodent studies, probiotics may be mixed in the food pellets [13,14] or administered via oral gavage [26,27,29,36,37]. Additionally, probiotics have be added to the drinking water of the rodents [28,38].

Tri-Factor® is a proprietary blend of low molecular weight bioactive proteins isolated from bovine colostrum and egg yolks (www.4life.com). Tri-Factor contains two ultra filtrates of colostrum, one with a molecular weight cut off of 10 kDa, and a second at 3 kDa. Low molecular weight colostrum proteins and peptides are rich in proline, and low in glycine, alanine, arginine and histidine, and do not contain tryptophan, methionine or cysteine [39].

The overall objective of this study was to evaluate the effect of a dietary supplement designed to improve human gut health in a mouse model when provided at a dose allometrically scaled for mouse metabolism. As a control, mice were fed the Total Western Diet (TWD), a purified rodent diet that matches the average US intake of macro- and micronutrients [40]. The TWD is a semi purified diet that was designed by our group at Utah State University to provide rodents with a diet that reflects the average macronutrient and micronutrient intake of Americans [40–44]. One assumption made in rodent studies is that the results will be useful to improve human wellbeing [45]. However, as much as 80% of therapeutics have failed in human studies after being shown to be efficacious in rodents [46]. The discordance between results may be due to differences in physiology between mice and humans, or may be due to element of the experimental design, such as dose and length of treatment [45]. In formulating the diets for this study, we translated the dose of prebiotics, probiotics and bioactive proteins from humans to mice using nutrient density, which is essentially normalizing them to calories. The TWD was supplemented with either prebiotics, probiotics, or Tri-Factor, individually and in combination. The endpoints of interest were the effect on the composition of the gut microbiome, cecal and fecal short chain fatty acids (SCFAs), and gut inflammation.

The overall hypothesis was that the treatments would increase the diversity of the microbiome and be associated with more SCFAs, and less gut inflammation. Specifically, we predicted the prebiotics treatment would increase fecal microbiome diversity, cecal SFCAs content and decrease fecal calprotectin. We anticipated the probiotics treatment would increase the fecal levels of the probiotics administered and reduce fecal calprotectin. The Tri-Factor treatment would affect the microbiome composition, and the levels of gut inflammation. Last, the combined treatment was predicted to increase both the fecal microbiome diversity and SCFAs due to the presence of the probiotics and prebiotics, while it would reduce fecal calprotectin.

2. Method and materials

2.1. Diet formulation

The treatment dosages were calculated using a nutrient density approach to convert the dosage of the human supplement (Pre/o Biotics, 4 Life, Sandy, UT) to metabolically equivalent doses in mice (Table 1). The supplement, Pre/o Biotics contains 2.5 g of prebiotics with equal parts fructooligosaccharides (FOS), galactooligosaccharides (GOS) and xylooligosaccharides (XOS). In addition, Pre/o Biotics contains 0.5×10^9 CFU of *Bifidobacterium infantis* (M – 63), *Bifodobacterium longum* (BB536) and *Bifidobacterium lactis* (Bl-04), and 0.25×10^9 CFU *Lactobacillus rhamnosus* (Lr-32) and *Lactobacillus acidophilus* (NCFM). Last, Pre/o Biotics contains 100 mg of Tri-Factor.

To convert the dosages using nutrient density, an average caloric intake of 2500 kcal day was used for humans. For mice, 11 kcal was determined using a number or previous studies in our group [41,44]. The quantities of prebiotics, probiotics and Tri-Factor in Pre/o Biotics were normalized to an average human caloric intake (i.e. 2.5 g prebiotics/2500 kcal = 1 mg/kcal). This value was then used to determine the mass added to the TWD formulation, which has a 4400 kcal per kilogram. For the prebiotics, there should be 4.4 g of prebiotics per kg of diet (i.e. 1 mg/kcal * 4400 kcal), and similar calculations were made for the probiotics and Tri-Factor. To increase the likelihood of measuring treatment effects, the dose of prebiotics and Tri-Factor was increased 1.5-fold, and the probiotic treatment 3-fold (Table 1).

The control diet was the TWD, and for the treatment groups, a portion of maltodextrin was removed to account for the prebiotic, probiotic and Tri-Factor addition. The decision to replace maltodextrin was made as it has most often been used as a control in human prebiotic studies [47,48]. Diet assignments were as follows: 1) TWD: Total Western Diet as control: 2) PRE: prebiotics, 3) PRO: probiotics, 4) Tri-Factor, 5) COM: prebiotics, probiotics and Tri-Factor. The composition of the diets is shown in Table 2. All diets were stored with vacuum package at −20 °C until provided for feeding.

2.2. Study design

C57Bl/6 J male mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were randomly assigned to each treatment for 4 weeks. Mice were individually housed in HEPA-filtered micro isolator cages. A 12-h light/dark cycle was used, and the room temperature was kept between 18 and 23 °C with humidity between 20 and 50%. All animal care and husbandry procedures were performed under the Animal Welfare Act and the Public Health Service Policy on Humane Care and Use of Laboratory Animals, as well as USU Institutional Animal Care and Use Committee (protocol #2640). The experimental design is shown in Fig. 1.

Food intake and body weight were measured twice weekly. At the end of intervention, mice were killed by CO_2 asphyxiation. Blood was removed by cardiac puncture and plasma was separated from whole blood via centrifugation. Plasma was aliquoted into microcentrifuge tubes and snap frozen in liquid nitrogen. Both fecal and cecal samples were collected at the end of intervention and snap frozen in liquid nitrogen and stored at −80 °C until analysis.

2.3. Diet probiotic enumeration

Diet samples were sent to Covance Laboratory (Madison, WI) for Total Probiotic Enumeration using standard plate procedures [49].

Table 1
Translation of human to mouse intakes using nutrient density.

Nutrient intake		Prebiotics	Probiotics	Transfer factor
Human	Pre/o Biotics supplement*	2.5 g/d	2×10^9 CFU/d	100 mg/d
	Energy intake (kcal/d)	2500	2500	2500
	Nutrient density	1 mg/kcal	8×10^5 CFU/kcal	40 μg/kcal
Mice	Translated dose	11 mg/d	8.8×10^6 CFU/kcal	0.44 mg/d
	Energy intake (kcal/d)	11	11	11
	Nutrient density	1 mg/kcal	8×10^5 CFU/kcal	40 μg/kcal
	Actual dose§	16.5 mg/d	1.3×10^7 CFU/d	0.66 mg/kcal

*Pre/o Biotics contains 2.5 g of prebiotics with equal parts fructooligosaccharides (FOS), galactooligosaccharides (GOS) and xylooligosaccharides (XOS); contains probiotic as following 0.5×10^9 CFU of *Bifidobacterium infantis* (M − 63), *Bifodobacterium longum* (BB536) and *Bifidobacterium lactis* (Bl-04), and 0.25×10^9 CFU *Lactobacillus rhamnosus* (Lr-32) and *Lactobacillus acidophilus* (NCFM).
§The prebiotic and Transfer Factor were increased by 1.5-fold and the probiotics by 3-fold to increase likelihood of measurable effects.

Table 2
Composition of experimental diets.

Diet composition	TWD	PRE	PRO	TF	COM
Treatment (g/kg)					
Prebiotic	–	6.75	–	–	6.75
Probiotic[a]	–	–	0.15	–	0.15
Transfer factor	–	–	–	0.26	0.26
Carbohydrate (g/kg)					
Cellulose	30	30	30	30	30
Corn starch	230.0	230.0	230.0	230.0	230.0
Maltodextrin[b]	70.0	63.2	69.7	69.6	62.7
Sucrose	261.3	261.3	261.3	261.3	261.3
Protein (g/kg)					
Casein	190	190	190	190	190
L-cysteine	2.85	2.85	2.85	2.85	2.85
Fat (g/kg)					
Anhydrous milk fat	36.3	36.3	36.3	36.3	36.3
Beef tallow	24.8	24.8	24.8	24.8	24.8
Cholesterol	0.4	0.4	0.4	0.4	0.4
Corn Oil	16.5	16.5	16.5	16.5	16.5
Lard	28	28	28	28	28
Olive oil	28	28	28	28	28
Soybean oil	31.4	31.4	31.4	31.4	31.4
Vitamin, mineral, antioxidant (g/kg)					
Mineral mix	35	35	35	35	35
Vitamin mix	10	10	10	10	10
Sodium chloride	4	4	4	4	4
Choline bitartrate	1.4	1.4	1.4	1.4	1.4
TBHQ	0.028	0.028	0.028	0.028	0.028
% Kcal					
Protein	15.5	15.5	15.5	15.5	15.5
Carbohydrate	50.0	49.7	50.0	50.0	49.7
Fat	34.5	34.7	34.5	34.5	34.7
Calorie (Kcal/g)	4.4	4.3	4.4	4.4	4.3

[a] The prebiotics contained equal parts fructooligosaccharides, galactooligosaccharides, and xylooligosaccharides.
[b] Prebiotics, probiotics and Transfer Factor additions were balanced by removing maltodextrin.

2.4. SCFAs analysis

SCFAs were extracted from fecal and cecal samples at the end of intervention, and measured by gas chromatography with flame ionization detection (GC-FID) according to the method from Ward et al. [41].

2.5. Gut microbiome

Taxonomic measures of the fecal microbiome were performed using 16s rRNA sequencing. The fecal samples collected at the terminal necropsy were used for this analysis. Bacterial DNA from the fecal samples was extracted using the QIAGEN QIAamp DNA Stool Mini Kit (Hilden, Germany) according to the manufacturer's instructions. This extraction process involves homogenization and lysis of the stool using a stool lysis buffer and bead beater, and removal of inhibitors.

After DNA extraction, samples were analyzed by spectroscopy to determine the concentration of DNA for each sample and then diluted with TE buffer to a concentration of 1 ng/μL. Samples were amplified via PCR, using barcoded primers directed against the V3 region of the 16 S rRNA [50]. PCR amplification was performed using the following protocol: 5 min at 95 °C; 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s; final annealing at 72 °C for 10 min; hold at 4 °C.

Following PCR amplification, gel electrophoresis was performed to visualize amplicons. The PCR products were then purified using AMPure microbeads. Once all the samples were purified, DNA concentration was assessed using the Picogreen assay, which measures fluorescence via spectrophotometry to determine DNA concentration. Samples were then diluted to 1 ng/μL with TE buffer and pooled together into a single tube. Sequencing was performed

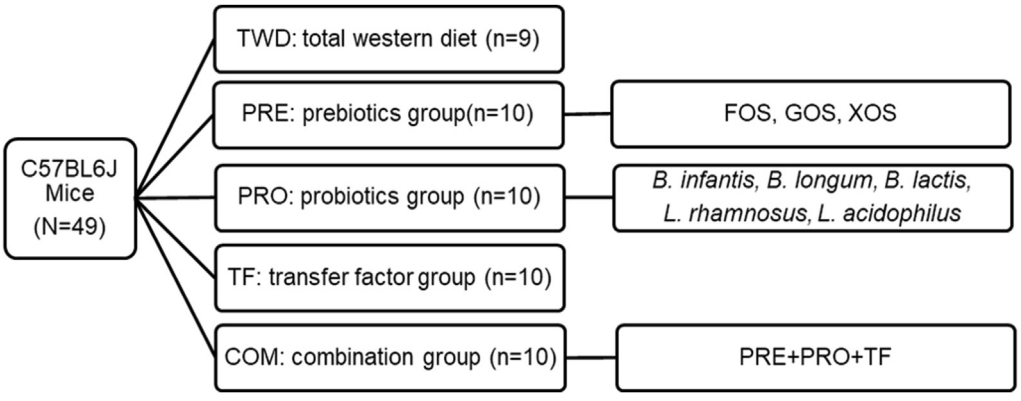

Fig. 1. Experimental design.

at the Utah State Center for Integrated Biotechnology core sequencing facility using the Ion PGM System and analyzed using Ion Reporter™ workflow.

Sequences were processed with the latest version of MacQIIME [51]. Sequences were filtered for quality and assigned operational taxonomic units (OTUs) [52] at a 97% sequence similarity as compared to a reference GreenGenes OTU database (gg_13_8_otus). Sequences were assigned using the open-reference OTU picking methodology with UCLUST [53]. Sequences at the highest levels of abundance were chosen as representative sequences, and these were checked for chimeras using uchime61 [54]. Alpha diversity, beta diversity, and taxonomic summaries were performed using the core_diversity_analyses.py script. For diversity analyses, sequence depth was rarified to the sample with the fewest sequences. Group and pair-wise comparison of the microbiome data was conducted with a free online software program, MicrobiomeAnalyst [55].

2.6. Gut inflammation

Fecal calprotectin was extracted by with the following extraction buffer: 0.1 M Tris, 0.15 M NaCl, 1.0 M urea, 10 mM $CaCl_2$, 0.1 M citric acid monohydrate and 5 g/L BSA (pH 8.0). After extraction and centrifugation, the supernatant was used for the ELISA analysis with a commercial kit following manufacturer's instructions (Hycult Inc, Wayne, PA).

2.7. Statistics analysis

Treatment effects and interactions were determined by one way-ANOVA with Dunnett's test. Beta Diversity was determined via Principle Coordinates Analysis (PCoA) using the Bray-Curtis Index at the OTU level with Permutational MANOVA. Pairwise analysis was used in microbiome results to compare each treatment with TWD group. For all statistical tests, a p value < 0.05 (two-tailed test) was considered as significant. Transformations were used to equalize variance prior to the statistical analyses in cases where variance assumptions were not met.

3. Results

3.1. Diet probiotic content

The probiotics were added to the PRO and COM diets as powders, and plate counts were conducted by a third party to enumerate the colony forming units (CFUs) in each diet. These numbers were then used to determine the average probiotic intake for each diet (Table 3). In the TWD and Tri-Factor diets, the probiotic plate counts were below the detection limit of the assay, which is not surprising, as probiotics were not added to the diets. The PRE diet did contain a measurable level of bacteria, which presumably were introduced in the prebiotic powders. The COM diet contained the highest level of probiotics, followed by the PRO diet.

Table 3
Probiotic enumeration for diets, and estimated probiotic intake/d.

Treatment	TWD	PRE	PRO	TF	COM
CFU/g diet	$<1 \times 10^4$	2×10^4	9×10^4	$<1 \times 10^4$	3.4×10^5
CFU/d[a]	$<2.4 \times 10^4$	5.5×10^4	2.6×10^5	$<2.7 \times 10^4$	9×10^6

[a] Probiotic intake was estimated using CFU/g content measured in diets and average mass of food consumed per group.

3.2. Food intake, weight gain, metabolic efficiency and probiotic intake

Mice consumed more calories on the PRO diet than the TWD (Table 4), but there were no other differences in intake among the diets. There was no treatment effect on weight gain, nor metabolic efficiency, which is the mass gain per calorie.

3.3. SCFAs

There were very few differences in the SCFA content of the cecal or fecal contents (Table 5). In the cecal contents, only caproic acid differed significantly between the treatments, with all treatments being higher than the control. In feces, there was a trend ($p < 0.1$) for differences in iso-butyric and valeric acids.

When the TWD and COM treatments are compared directly, there was more butyric and caproic acid in the cecal contents, and more acetic and butyric acid in the fecal content (Fig. 2).

3.4. Microbiome

3.4.1. Taxonomic summaries

After quality, chimera, and abundance filtering, sequences were assigned to OTUs using the pick_open_ref_otus command for an average of 46,853 sequences per sample assigned to 1546 OTUs. Compared to diet, PRE, PRO and COM treatments changed the microbiome composition. Fig. 3 showed the family level taxonomy.

Because the differences in taxonomic relative abundance did not follow a normal distribution, these analyses were performed using non-parametric, single factor comparisons. A complete summary of significant differences in relative abundance is given in Table 6.

Ruminococcus gnavus was increased after feeding prebiotics. The relative abundance increased approximately 2.4 fold for the COM diet and 3.4 fold for the PRE diet. *Bifidobacterium longum* was increased in both the PRO and COM treatments. As it was not detected in the TWD control diet, it is not possible to estimate the magnitude of the increase.

3.5. Microbiome diversity

Alpha diversity refers to within-habitat diversity. It is the component of total diversity that can be attributed to the average number of species found within homogeneous sampling units (i.e. habitats) [56]. Alpha diversity was determined using Chao 1 index. The analyses showed that no significant difference affected by diets in Fig. 4.

Beta diversity is referred to between-habitat diversity. It is the component of total diversity that can be attributed to differences in species composition among the homogeneous units in the landscape [56]. Fig. 5 is a spatial representation of beta diversity with Principle Coordinate Analysis. The treatment affected beta-diversity with exception of TF.

3.6. Gut inflammation

The effect of diets on fecal calprotectin is shown in Fig. 6. Prior to being randomized to the treatments, fecal samples were collected from mice consuming a standard laboratory chow diet. According to the data, mice consuming chow had lower levels of fecal calprotectin than mice on any of the treatment diets. After 4-week intervention on TWD and other treatments, mice showed higher level of fecal calprotectin. In comparison within treatments, there was no treatment effect among the test diets ($p = 0.1355$).

Table 4
Food intake, weight gain, and metabolic efficiency.

Treatment	TWD	PRE	PRO	TF	COM
Energy intake (Kcal/day)	10.8 ± 0.2[a]	12.1 ± 0.2[ab]	12.9 ± 0.5[b]	12.2 ± 0.4[ab]	11.7 ± 0.4[ab]
Weight gain (g)	6.6 ± 0.5	8.0 ± 0.5	7.7 ± 0.5	6.4 ± 0.4	7.4 ± 0.6
Metabolic efficiency (g/kcal)	0.61 ± 0.04	0.66 ± 0.04	0.60 ± 0.04	0.53 ± 0.03	0.63 ± 0.05

Values with different superscripts differed significantly ($p < 0.05$).

Table 5
SCFAs in cecal and fecal samples for treatments.

Cecal SCFAs (μmol/g)	TWD	PRE	PRO	TF	COM	p-value (ANOVA)
Acetic acid	27.8 ± 2.10	26.3 ± 2.50	26.4 ± 1.70	28.9 ± 2.10	28.3 ± 1.30	0.84
Propionic acid	3.87 ± 0.28	3.88 ± 0.29	4.58 ± 0.30	4.11 ± 0.28	4.61 ± 0.33	0.22
n-Butyric acid	3.02 ± 0.30	3.30 ± 0.43	3.38 ± 0.23	3.62 ± 0.35	4.00 ± 0.35	0.33
iso-Butyric acid	0.48 ± 0.01	0.44 ± 0.03	0.47 ± 0.01	0.44 ± 0.02	0.47 ± 0.01	0.47
iso-Valeric acid	0.53 ± 0.01	0.49 ± 0.02	0.51 ± 0.02	0.51 ± 0.03	0.52 ± 0.03	0.79
n-Valeric acid	0.51 ± 0.02	0.48 ± 0.04	0.49 ± 0.04	0.52 ± 0.05	0.52 ± 0.03	0.86
Caproic acid	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05
Fecal SCFAs (μmol/g)	**TWD**	**PRE**	**PRO**	**TF**	**COM**	**p-value (ANOVA)**
Acetic acid	20.4 ± 2.1	28.0 ± 2.5	25.5 ± 4.5	22.8 ± 1.8	27.2 ± 1.9	0.27
Propionic acid	2.2 ± 0.30	2.6 ± 0.20	2.1 ± 0.30	2.6 ± 0.30	2.8 ± 0.20	0.20
n-Butyric acid	0.70 ± 0.10	0.97 ± 0.17	0.87 ± 0.08	1.05 ± 0.12	1.03 ± 0.10	0.26
iso-Butyric acid	0.26 ± 0.03	0.37 ± 0.04	0.25 ± 0.03	0.35 ± 0.05	0.32 ± 0.02	0.06
iso-Valeric acid	0.43 ± 0.05	0.58 ± 0.05	0.45 ± 0.03	0.55 ± 0.07	0.52 ± 0.02	0.21
n-Valeric acid	0.19 ± 0.03	0.26 ± 0.04	0.14 ± 0.02	0.31 ± 0.08	0.19 ± 0.02	0.10
Caproic acid	0.06 ± 0.01	0.17 ± 0.06	0.09 ± 0.02	0.20 ± 0.10	0.11 ± 0.03	0.39

SCFAs are expressed as mean ± SE (μmol/g). P-value was calculated by one-way ANOVA.

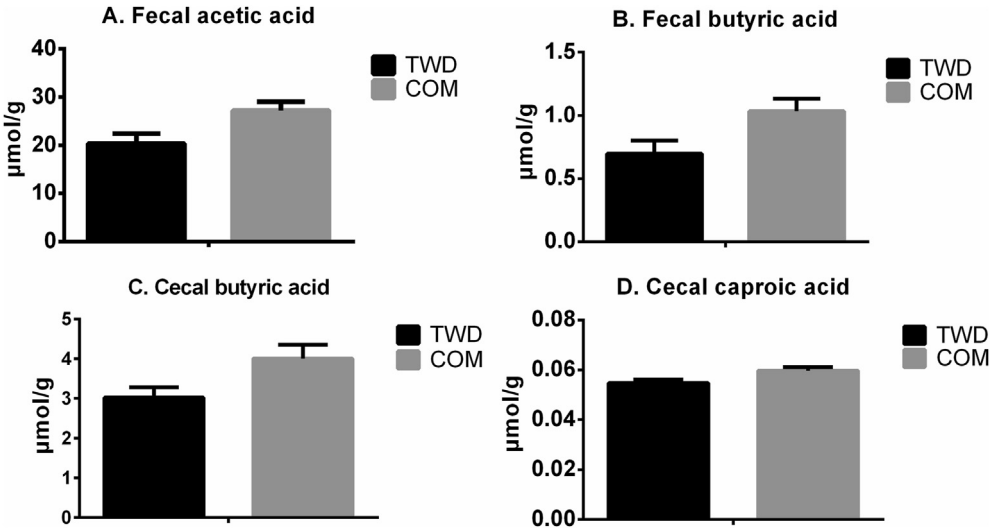

Fig. 2. SCFAs analysis, data represent as mean ± SE (μmol/g). A, acetic acid in fecal samples, $p = 0.025$. B, butyric acid in fecal samples, $p = 0.037$. C, butyric acid in cecal samples, $p = 0.044$. D, caproic acid in cecal samples, $p = 0.022$.

4. Discussion

In the current study, to investigate the potential effects of a supplement designed for humans, we estimated a suitable rodent dosage based used nutrient density, which is a method of nutrient and bioactive translation between species that normalizes intake to kcal. This method was used in the development of the Total Western Diet [40,43,44,57]. It has been suggested that other methods may be used to translate intakes of bioactive between rodents and humans, such as scaling by body surface area [58,59]. However, Blanchard and Smoliga point out that the use of body surface area scaling is based on antiquated science, and is primarily useful for determining safe starting doses in humans, and not pharmacologically active doses [60]. Furthermore, methods of bioactive dose translation between species should involve more comprehensive characterization including physiologic and pharmacologic parameters.

Enumeration of probiotic bacteria from the PRO and COM diets was lower than expected, as a 3-fold increase over the human dose was added to the diets (Table 3). It is likely the low recovery is due to the process of diet manufacture, and the labile nature of the added probiotics. Nonetheless, mice consuming these diets received levels between 10^5 and 10^6 CFUs per day, which have been associated with significant physiological effects in previous rodent

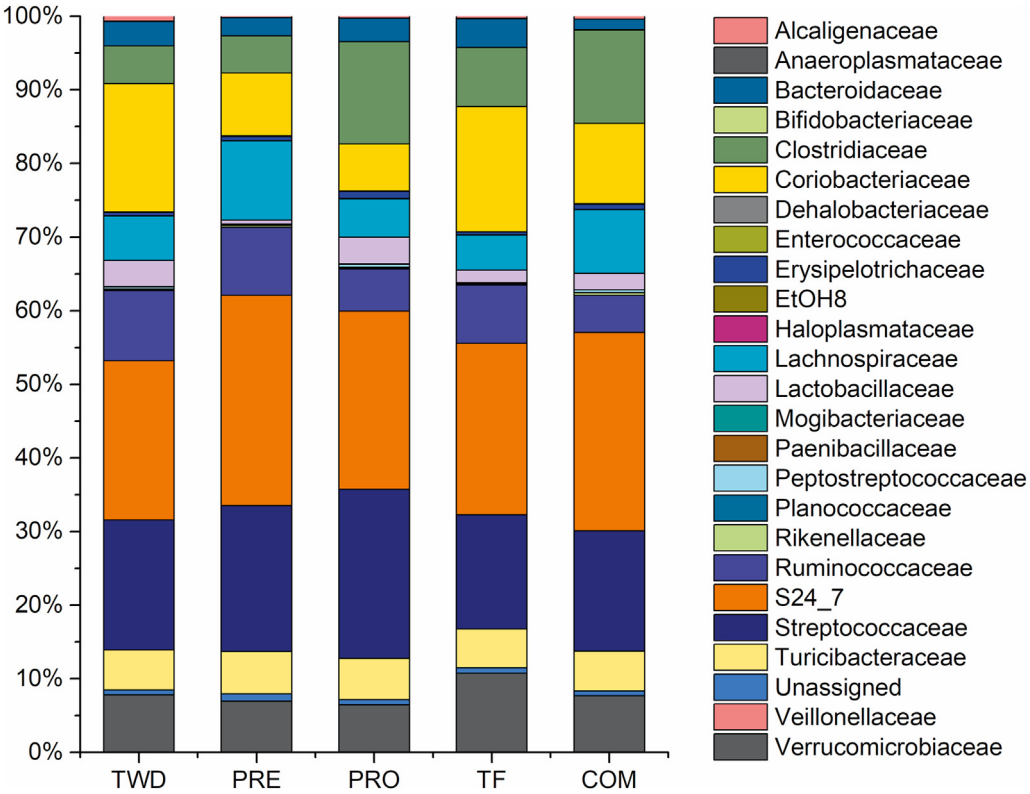

Fig. 3. Family level taxonomy, percentage of total OTUs.

Table 6
Significant effect of treatment on taxonomic abundance compared to TWD.

Taxonomic Abundance		p-value, each group compared to TWD				Direction*
		PRE	PRO	TF	COM	
Class	Actinobacteria	/	0.0059	/	/	PRO
Order	Bifidobacteriales	/	0.0047	/	0.0226	PRO, COM
Family	*Bifidobacteriaceae*	/	0.0078	/	0.0438	PRO, COM
Genus	*Ruminococcus*	0.0065	/	/	/	PRE
	Bifidobacterium	/	0.0081	/	/	PRO
Species	*gnavus*	0.0004	/	/	0.0017	PRE, COM
	longum	/	0.0019	/	0.0069	PRO, COM
	neonatale	/	0.0321	/	/	PRO

*Note: Direction denotes a greater relative abundance in the study group mentioned.

studies [61]. It was not expected that there would be measurable probiotics in the PRE diet, as the only addition to this diet were the prebiotics. It seems, therefore, that the added powders may contain microbes measured in the probiotic plate count method [49]. Further support for the prebiotic powders containing microbes is the difference between the PRO and COM probiotic levels. The COM diet contained 2.5×10^5 more CFUs per gram than the PRO diet, which may be partially accounted for by the prebiotic powders in the COM diet.

Mice consuming the PRO diet consumed more calories per day than mice consuming the TWD (Table 4). In fact, there was an increased intake of all the experimental diets, but the TWD vs. PRO was the only pairing that was statistically significant. It is unclear why addition of a small amount of bacteria, fiber, or bioactive proteins added to the TWD would increase intake. One possibility is that these extra ingredients contain some aroma volatiles that or taste-active molecules that increase the palatability of the diets. Conversely, these results may just be a random effect. Yet, the increased calorie consumption was not associated with a greater weight gain, nor metabolic efficiency. As the feeding period was only 28 days, it is not surprising there were no differences in weight gain between the groups.

There were few differences in either the cecal or fecal concentration of SCFA due to inclusion of either the prebiotics, probiotics or both. In the cecum, all treatments increased the level of caproic acid compared to the TWD diet ($p < 0.05$; Table 5). Both the PRE and COM diets contained prebiotics, and it was not unexpected to see changes in the cecal and fecal content of these groups. It is also possible that addition of the probiotics would cause an increase in the PRO group due to the addition of microbes to the diet, but it is unclear why the TF treatment would affect the cecal caproic acid content. A direct comparison of the control (TWD) diet and the COM diet indicated significant differences in fecal and cecal SCFA (Fig. 2). In the feces, both acetic and butyric acid levels were increased, and in the cecal contents butyric and caproic acids were more concentrated. Of the three treatments, the prebiotics would

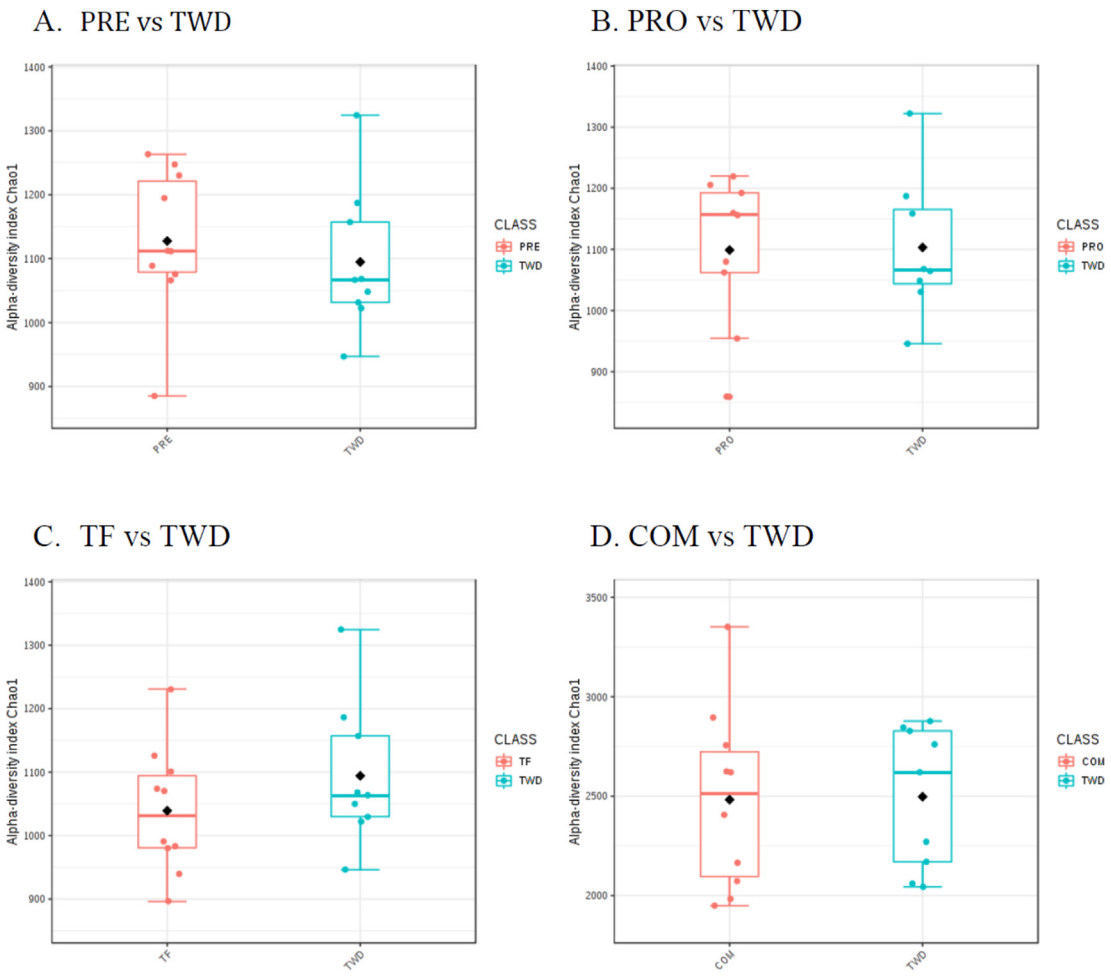

Fig. 4. Alpha-diversity of gut microbiome, expressed by OTUs. A, PRE vs TWD, p = 0.243: B, PRO vs TWD, p = 0.673: C, TF vs TWD, p = 0.277: D, COM vs TWD, p = 0.720.

be the most likely factor in causing increases in SCFA, as they are the substrates microbes use for fermentation. However, according to the data from all treatments (Table 5), SCFA levels in the PRE and COM treatments are not consistently the highest. This suggests some interaction between the three treatments contained in the PRO diet.

In mice, many studies have reported more substantial increases in cecal and fecal SCFA with prebiotic inclusion in the diet, which is likely due to the quantity. For example, mouse diets are often supplemented with 5–10% by mass of a prebiotic [14,62–64], whereas the inclusion in this study was at 0.675% across three different prebiotics. Hamilton et al. fed 10% inulin or bovine milk oligosaccharides to mice on a high fat diet (4500 kcal/kg) which increased cecal butyrate and propionate [62]. Weitkunat supplemented a high-fat diet in mice with 10% inulin, and acetate, propionate and butyrate were all increased in the cecum, as were total SCFA [14]. Nihei et al. include 5.5% cyclodextrin to a high-fat diet (~5250 kcal/kg) which was associated with an increase in all cecal SCFA except n-valeric acid. Last, Murakami added 10% epilactose to both low and high fat diets which increase all cecal SCFA except lactate [65]. In the studies above, prebiotics were associated with impressive health benefits. For example, supplementation prevented adiposity development [14,62], gut permeability [16], improved lipid metabolism [14,66], and increased energy expenditure [14,65].

Rodent studies suggest that substantial intakes of prebiotics may improve metabolic health, yet it is unclear if such levels can be achieved in human diets. To date, there has been little discussion in the literature on translating intakes of prebiotics between rodents and humans. A 25 g mouse consuming 2.5 g of food a day with 10% prebiotics will ingest 0.25 g, or 10 g/kg. For a 70 kg, that translates to 700 g of prebiotics per day. However, if nutrient density is used and the prebiotics are normalized to kcal, 2.5 g of a high fat diet (5000 kcal/g) with 10% prebiotics would deliver 20 mg/kcal. For a 2500 kcal diet, an equivalent intake would be 50 g/d, which is significantly higher than the Institute of Medicine's recommendation for total dietary fiber, which is 14 g/1000 kcal [67].

In human trials, prebiotics are typically supplemented between 5 and 20 g/d [17,19–24]. Fecal SCFA have been measured in some studies, and to date a clear effect has not been established. No change in fecal SCFA was determined after 1.4 or 2.8 g/d XOS for 8 weeks [20], or 5 and 7.5 g/d inulin for 21d [19]. Conversely, consumption of 5 g/d XOS for 4 weeks increased fecal butyrate and decreased acetate, while a mix of 3 g/d inulin and 1 g/d XOS resulted in an increase in propionate and total SCFA [21]. Childs et al. provided subjects with 8 g/d XOS and 10^9 CFT *Bifidobacterium animalis* subspecies *lactis* Bi-07, singly and in combination for 21d [24]. Individually, both treatments reduced fecal acetate and butyrate, but the combination did not. In addition, the combination increased fecal isovaleric acid. At higher intakes, prebiotics supplementation has been shown to increase fecal SCFA, but is also associated with an increase in gastrointestinal stress. Clarke et al.

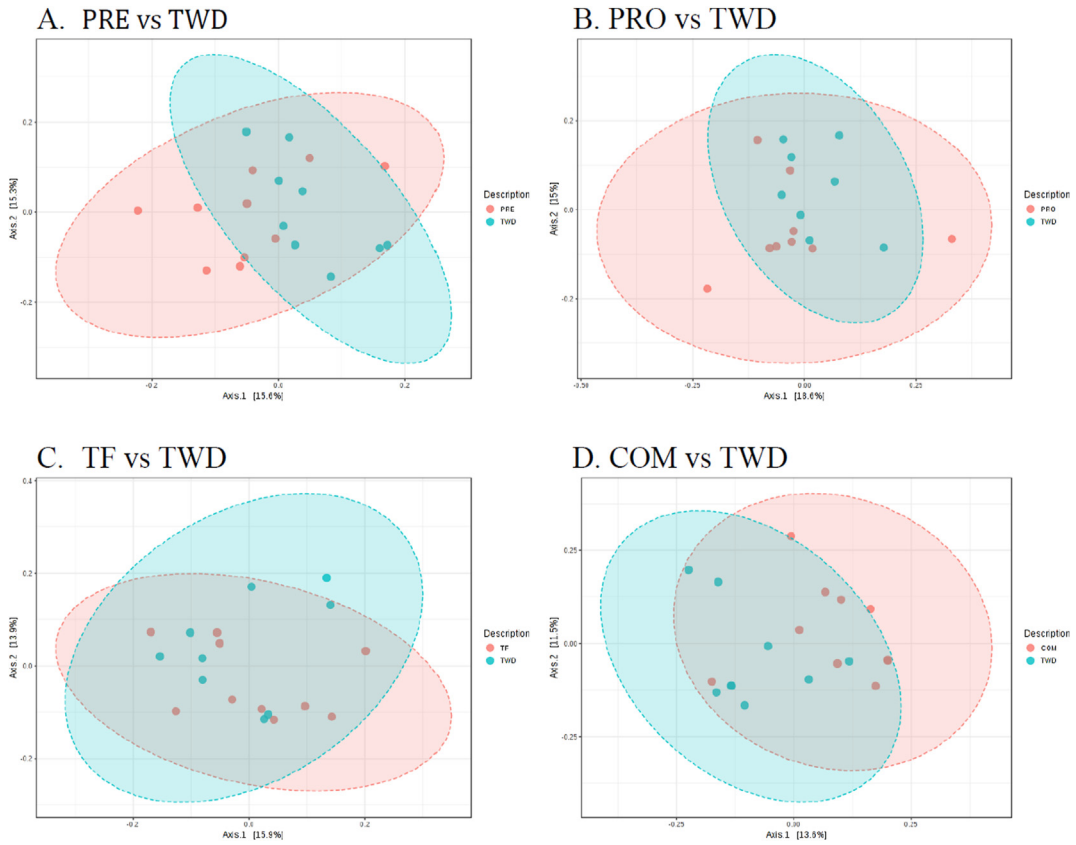

Fig. 5. Beta-diversity of gut microbiome, unweighted unifrac distance with non-parametric PERMANOVA test. A, PRE vs TWD, p = 0.021: B, PRO vs TWD, p = 0.015: C, TF vs TWD, p = 0.415: D, COM vs TWD, p = 0.005.

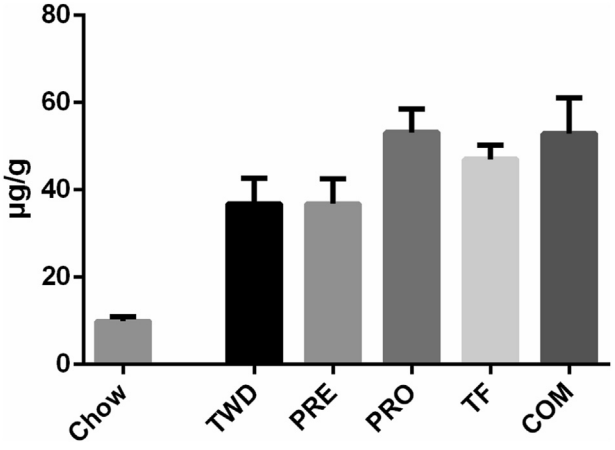

Fig. 6. Fecal calprotectin. Data represent mean ± SE (mg/g)

fed subjects either 3 × 5 g/d of a mixture of inulin and FOS or maltodextrin for 28d [18]. The prebiotic supplementation significantly increased total fecal SCFA, but was associated with significant increases in self-reported GI symptoms and headaches. More concerning, however, is the fact that the 15 g/d prebiotic supplementation increased circulating inflammatory cytokines, the proportion of immune cells that expressed TLR2 and TLR4, and the response to TLR2 agonists in an *ex vivo* assay. The authors suggested that increases in these markers, while moderate, were consistent with increased immune cell contact with microbial stimuli.

Differences in the gut microbiome can contribute to an increased susceptibility to diseases both within and outside the gut [68]. There have been a number of studies that have shown modification of gut microbiome when mice are supplemented with large doses of prebiotics, probiotics, individually or in combination [13–15,26,27,29,36,37,69–71]. Changes are typically an increase in abundance of fecal *Bifidobacterium*, *Lactobacillus* and *Alloprevotella* [13–16,37,69,70,72]. The Firmicutes/Bacteroidetes ratio has also been affected by treatments [36]. An increased ratio of Firmicutes to Bacteroidetes was observed in obese versus lean subjects [68,73]. In our experiment, as actual dosage for mice was much lower than previous animal studies, there was no significant difference in phylum level abundance or Firmicutes to Bacteroidetes ratio.

Prebiotics and probiotics affected microbiome by increasing *Ruminococcus gnavus* and, *Bifidobacterium longum* respectively. The COM treatment showed the same effect of modification as PRE and PRO due to it combined both prebiotics and probiotics. An increasing of *Ruminococcus gnavus* was found in fecal microbiome in patients with Crohn's disease, an inflammatory bowel disease (IBD) [74].

Bifidobacterium longum was one of probiotics supplied in diet in this experiment. Although the dosage for total probiotics was much lower than our expectation, we detected an increasing of *Bifidobacterium longum* in PRO diet compared to TWD, which suggested the effect of supplements was pronounced.

Alpha-diversity and beta-diversity are often used to evaluate the variation of microbiome composition. The diversity analysis would give a better understanding of similarity, and richness within the cecal and fecal contents [75]. Gut microbiome diversity has often been negatively associated with weight gain, and positively correlated with fiber intake [76]. In addition, inflammatory bowel

diseases (IBD) patients show an overall decreased gut bacteria diversity with a reduction of the dominant Firmicutes and Bacteroidetes compared to healthy people [77,78]. When mice are supplied with prebiotics and/or probiotics, an enrichment in microbiome diversity has been reported in some studies [15,16,38,79]. Yet this is sometimes not the case with a lower dosage or shorter intervention time [28]. In human trials, microbiome diversity is often not affected by prebiotics or probiotics [17,19,20,33,35]. A novel aspect of the current study is that the treatment dosages were determined for mice based on human intakes translated for mice using nutrient density. The treatment diets did not affect alpha-diversity, but affected beta-diversity with exception of TF treatment. These results indicate the treatments did not cause an increase in the number of different microbes detected in the cecal and fecal samples, but did have a significant effect on the relative abundance between diet treatments.

Calprotectin is a Ca^{2+} binding protein produced by neutrophils [80] which is bacteriostatic and fungistatic, and which has a minimum inhibitory concentration similar to antibiotics [81]. Calprotectin is important for the clearance of infection, as has been shown by the comparison of wild-type and calprotectin-deficient animals [82]. Calprotectin can be used to predict relapses and detect pouchitis in IBD patients, and is used for IBD in undiagnosed, symptomatic patients [83]. Several mice studies have reported that large intakes of prebiotics and probiotics supplied with high fat diet improved the inflammatory response [26,37,64,79,84]. Conversely, a two week trial with a synbiotic showed no effect on gut inflammation and permeability [22]. The calprotectin levels of the fecal samples all significantly increased when mice were transitioned from chow to the semi-purified diets (Fig. 6). All mice consumed a basic lab chow diet before the intervention. Chow is composed of agricultural byproducts, and is a high fiber diet containing complex carbohydrates, with fats from a variety of vegetable sources [85]. This suggests that some factor in chow reduces fecal calprotectin, and a likely candidate is the fiber driving SCFA production. In addition to lower fecal calprotecin, mice fed chow had 70% more fecal acetate, 42% more butyrate and 90% more propionate than mice fed the TWD diet (data not shown). These data raise the possibility that the treatments did not affect fecal calprotectin as they did not sufficiently increase fecal SCFA. Futher studies are being designed to test this finding.

5. Conclusions

Overall, for 1-month intervention, probiotic supplementation increased *Bifidobacterium longum*, while the prebiotic supplementation increased *Ruminococcus gnavus*. Combined prebiotic and probiotic administration increased *Ruminococcus gnavus* and *Bifidobacterium longum* as additive effect. The treatments did not affect alpha diversity, but affected beta-diversity with exception of Tri-Factor. There were no treatment effects on cecal or fecal short chain fatty acid levels, and the treatments did not affect gut inflammation as measured by fecal calprotectin. Physiologically relevant doses of dietary supplements for mice modified gut microbiome and affected plasma levels of haptoglobin, but did not affect gut fermentation or a measure of gut inflammation.

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