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EFFECT OF SUPPLEMENTAL PREBIOTICS, PROBIOTICS AND BIOACTIVE
PROTEINS ON THE MICROBIOME COMPOSITION AND GUT
PHYSIOLOGY IN C57BL6/J MICE

by

Ye Li

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Food Science

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ABSTRACT

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

by

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Utah State University, 2019

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Department: Nutrition, Dietetics, and Food Sciences

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 individually and in combination. Mice were fed the total western diet (TWD) supplemented with prebiotics, probiotics, and Tri-Factor (bioactive proteins) individually and in combination for four weeks. Subsequently, effects on the composition of gut microbiome, gut short chain fatty acids (SCFAs) concentration, gut inflammation and integrity of the mucosal barrier were analyzed. *Ruminococcus gnavus* was increased in mice gut microbiome after feeding prebiotics. *Bifidobacterium longum* was increased after feeding probiotics. Probiotic was associated with higher level of *Clostridium neonatale*. The treatments affected beta-diversity with exception of Tri-Factor, but not alpha diversity of microbiome. All treatments were associated with lower plasma zonulin, compared to the control group, indicating an effect on gut permeability. There were no treatment effects on cecal or fecal SCFAs, and the treatments did not affect gut

inflammation as measured by fecal calprotectin.

(51 pages)

PUBLIC ABSTRACT

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Ye Li

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 individually and in combination. Mice were fed the total western diet (TWD) supplemented with prebiotics, probiotics, and Tri-Factor (bioactive proteins) individually and in combination for four weeks. Subsequently, effects on the composition of gut microbiome, gut short chain fatty acids (SCFAs) concentration, gut inflammation and integrity of the mucosal barrier were analyzed. *Ruminococcus gnavus* was increased in mice gut microbiome after feeding prebiotics. *Bifidobacterium longum* was increased after feeding probiotics. Probiotic was associated with higher level of *Clostridium neonatale*. The treatments affected beta-diversity with exception of Tri-Factor, but not alpha diversity of microbiome. All treatments were associated with lower plasma zonulin, compared to the control group, indicating an effect on gut permeability. There were no treatment effects on cecal or fecal SCFAs, and the treatments did not affect gut inflammation as measured by fecal calprotectin.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations		Symbols	
ANOVA	Analysis of variance	Kg	kilogram
CFU	Colony forming unit	g	gram
COM	Combination group	mg	milligram
DNA	Deoxyribonucleic acid	µg	microgram
ELISA	Enzyme-linked immunosorbent assay	ng	nanogram
FOS	Fructooligosaccharides	ml	milliliter
GOS	Galactooligosaccharides	M	molar
IBD	Inflammatory bowel diseases	mmol	micromolar
OTU	Operational taxonomic unit	d	day
PCR	Polymerase chain reaction	kcal	kilocalorie
PGM	Personal Genome Machine	n	number
PRE	Prebiotic group	°C	degrees Centigrade
PRO	Probiotic group		
SCFAs	Short-chain fatty acids		
SE	Standard error		
TF	Transfer factor group		
TLR	Toll-like receptor		
TWD	Total Western diet		
USU	Utah State University		
XOS	Xylooligosaccharides		

I. 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 (Gibson et al., 2017; Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004). Probiotics are bacteria that improve gut health, and which come predominantly from the *Lactobacillus* and *Bifidobacterium* genera (Colin Hill et al., 2014; C. Hill et al., 2014; Mack, 2005). 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 (Blaut, 2002; Rastall, 2010; Roberfroid, 2007). Probiotics are added as culture of fermented foods like yogurt and kefir or naturally present as starter on vegetable for kimchi and sauerkraut. They are also taken as supplements for human.

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 gut inflammation, decreased susceptibility to food allergy and prevention of cancer (I. Cho & Blaser, 2012; Swennen, Courtin, & Delcour, 2006). 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 (Saez-Lara, Robles-Sanchez, Ruiz-Ojeda, Plaza-Diaz, & Gil, 2016).

In mice, many studies have reported large increases in cecal and fecal SCFAs

with prebiotic inclusion in the diet, which is likely due to the quantity. For example, mouse diets are often supplemented with 5-10% prebiotics (B. S. Hamilton et al., 2017; Murakami et al., 2015a; Nihei et al., 2018; Weitkunat et al., 2015a). 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 (B. S. Hamilton et al., 2017). 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 SCFAs (Nihei et al., 2018). Nihei et al include 5.5% cyclodextrin to a high-fat diet (~5250 kcal/kg) which was associated with an increase in all cecal SCFAs except n-valeric acid. Last, Murakami added 10% epilactose to both low and high fat diets which increase all cecal SCFAs except lactic acid (Murakami et al., 2015b). In the studies above, prebiotics were associated with impressive health benefits. For example, supplementation prevented adiposity development (B. S. Hamilton et al., 2017; Nihei et al., 2018), gut permeability (M. K. Hamilton et al., 2017), improved lipid metabolism (Nihei et al., 2018; Weitkunat et al., 2015b), and increased energy expenditure (Murakami et al., 2015b; Nihei et al., 2018).

In human trials, prebiotics are typically supplemented between 5 and 20 g/d (Childs et al., 2014; Finegold et al., 2014; Holscher et al., 2015; Lecerf et al., 2012; Rajkumar et al., 2015; Vandeputte et al., 2017; Wilms et al., 2016). Fecal SCFAs have been measured in some studies, and to date a clear effect has not been established. No change in fecal SCFAs was determined after 1.4 or 2.8 g/d XOS for 8 weeks (Finegold et al., 2014), or 5 and 7.5 g/d inulin for 21d (Holscher et al., 2015). Conversely, consumption of 5g/d XOS for 4 weeks increased fecal butyrate and decreased acetate, while a mix of 3g/d inulin and 1g/d XOS resulted in an increase in propionate and total

SCFAs (Lecerf et al., 2012). Childs et al provided subjects with 8g/d XOS and 10^9 CFU *Bifidobacterium animalis* subspecies *lactis* Bi-07, singly and in combination for 21d (Childs et al., 2014). Individually, both treatments reduced fecal acetate and butyrate, but the combination did not. In addition, the combination increased fecal iso-valeric acid. At higher intakes, prebiotics supplementation has been shown to increase fecal SCFAs, but is also associated with an increase in gastrointestinal stress. Clarke et al fed subjects either 3×5 g/d of a mixture of inulin and FOS or maltodextrin for 28d (Clarke et al., 2016). The prebiotic supplementation significantly increased total fecal SCFAs, but was associated with significant increases in self-reported GI symptoms and headaches. More concerning, however, is the fact that the 15g/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.

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 10g/kg. For a 70 kg, that translates to 700g of prebiotics per day. However, if nutrient density is used and the prebiotics are normalized to kcal, 2.5g 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 50g/d, which is significantly higher than the Institute of Medicine's recommendation for total dietary fiber, which is 14g/1000 kcal

(Institute_of_Medicine, 2005).

The effects on gut microbiome were observed when administered only probiotics or combined with prebiotics. The changes in gut microbiome can contribute to an increased susceptibility to diseases both within and outside the gut (Cénit, Matzaraki, Tigchelaar, & Zhernakova, 2014). 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 (Carasi et al., 2015; W. Cheng et al., 2017; W Cheng et al., 2018; Cortez-Pinto et al., 2016; Delbes et al., 2018; Foure et al., 2018; Frece et al., 2009; Mariman, Tielen, Koning, & Nagelkerken, 2015; Nihei et al., 2018; Singh et al., 2017; Wang et al., 2012). Changes are typically an increase in abundance of fecal *Bifidobacteria*, *Lactobacilli* and *Alloprevotella* (W. Cheng et al., 2017; W Cheng et al., 2018; Delbes et al., 2018; Frece et al., 2009; M. K. Hamilton et al., 2017; Mischke et al., 2018; Nihei et al., 2018; Singh et al., 2017). The Firmicutes / Bacteroidetes ratio has also been affected by treatments (Foure et al., 2018). An increased ratio of Firmicutes to Bacteroidetes was observed in obese versus lean subjects (Cénit et al., 2014; Turnbaugh et al., 2008).

In general, the effectiveness of probiotics and prebiotics have been more substantial in rodent studies. Several factors may explain the inconsistency in results between mice and humans, 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 rodents, prebiotic supplementation of diets is common at levels between 5.5% and 15% on a mass basis (W. Cheng et al., 2017; Delbes et al., 2018; M. K. Hamilton et

al., 2017; Nihei et al., 2018), 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 given at levels between 5 to 20 g/day (Childs et al., 2014; Clarke et al., 2016; Finegold et al., 2014; Holscher et al., 2015; Lecerf et al., 2012; Rajkumar et al., 2015; Vandeputte et al., 2017; Wilms et al., 2016). 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 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 (Rucker, 2007), 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 than the human studies.

In rodent studies, probiotics have been given in a range of 10^8 and 10^9 CFU/day for mice (Bai et al., 2016; Carasi et al., 2015; Mariman et al., 2015; Wang et al., 2012), and around 10^9 CFU/day for humans (Childs et al., 2014; Gargari et al., 2016; Lee et al., 2017; Rajkumar et al., 2015; Rungsri et al., 2017; Seo et al., 2017; Toscano, De Grandi, Stronati, De Vecchi, & Drago, 2017; van Zanten et al., 2014). If probiotic intakes between rodents and humans are compared on a mass basis (i.e. 10^9 CFU for 75kg human vs. 10^8 CFU for 25g mouse), rodents are typically given ~150X more. When CFUs are

normalized to calorie intake (i.e. 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 (Delbes et al., 2018; Nihei et al., 2018) or administered via oral gavage (Carasi et al., 2015; Foure et al., 2018; Mariman et al., 2015; Singh et al., 2017; Wang et al., 2012). Additionally, probiotics have been added to the drinking water of the rodents (Bai et al., 2016; Umu et al., 2016). The advantage for oral gavage is the precise control of dosage, when compared to inclusion in the food or water.

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 (Szaniszlo et al., 2009).

The overall objective of this study was to evaluate the effect of a human gut health supplement in a mouse model when provided at a physiologically relevant dose. As a control, mice were fed the TWD, a purified rodent diet that matches the average US intake of macro- and micronutrients (Hintze, Benninghoff, Cho, & Ward, 2018). 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), gut inflammation, and plasma zonulin.

The overall hypothesis is that the treatments will increase the diversity of the microbiome and be associated with more SCFAs, less gut inflammation and an improved

mucosal barrier. Specifically, we predict the prebiotics treatment will increase the fecal microbiome diversity, the cecal SFCAs content and decrease fecal calprotectin. We anticipate the probiotics treatment will increase the fecal levels of the probiotics administered and reduce fecal calprotectin. The TF treatment will affect the microbiome composition, and the levels of gut inflammation. Last, the combined treatment is predicted to increase both the fecal microbiome diversity and SCFAs due to the presence of the probiotics and prebiotics, while it will reduce fecal calprotectin.

II. METHOD AND MATERIALS

Diet formulation

The treatment dosages were calculated using a nutrient density approach to convert the dosage of the human supplement (Pre/o Biotics, 4Life, Sandy, UT) to metabolically equivalent doses in mice (Table 1) The supplement, Pre/o Biotics contains 2.5g 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), *Bifidobacterium longum* (BB536) and *Bifidobacterium lactis* (BI-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, a proprietary concentrate of egg yolk and bovine colostrum proteins and peptides (4life, Sandy, UT).

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

	Nutrient intake	Prebiotics	Probiotics	Tri-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 μ g/kcal
	Translated dose	11 mg/d	8.8×10^6 CFU/kcal	0.44 mg/d
Mice	Energy intake (kcal/d)	11	11	11
	Nutrient density	1 mg/kcal	8×10^5 CFU/kcal	40 μ g/kcal
	Actual dose [§]	16.5 mg/d	1.3×10^7 CFU/d	0.66 mg/kcal

[§]The prebiotic and Tri-Factor were increased by 1.5-fold and the probiotics by 3-fold to increase likelihood of measurable effects

To convert the dosages using nutrient density, an average caloric intake of 2,500 kcal/day was used for humans. For mice, 11 kcal was determined according to previous studies. The quantities of prebiotics, probiotics and Tri-Factor in Pre/o Biotics were normalized to an average human caloric intake (i.e. 2.5g 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 (Beserra et al., 2015; Fernandes, do Rosario, Mocellin, Kuntz, & Trindade, 2017). Diet assignments were as follows: 1) TWD: Total Western Diet as control; 2) PRE: prebiotics, 3) PRO: probiotics, 4) TF: 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.

Table 2. Composition of experimental diets

Diet composition	TWD	PRE	PRO	TF	COM
Treatment (g/kg)					
Prebiotic [§]	-	6.75	-	-	6.75
Probiotic	-	-	0.15	-	0.15
Tri-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 [‡]	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

[§]The prebiotics contained equal parts FOS, GOS and XOS.

[‡] Prebiotics, probiotics and Tri-Factor additions were balanced by removing maltodextrin.

Study design

C57BL/6J male mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were randomly assigned by weight to each treatment for 4 weeks. Mice were individually housed in HEPA-filtered micro isolator cages. A 12-hour light/dark cycle was used, and the room temperature was kept between 18-23°C with humidity between 20-50%. 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 Figure 1.

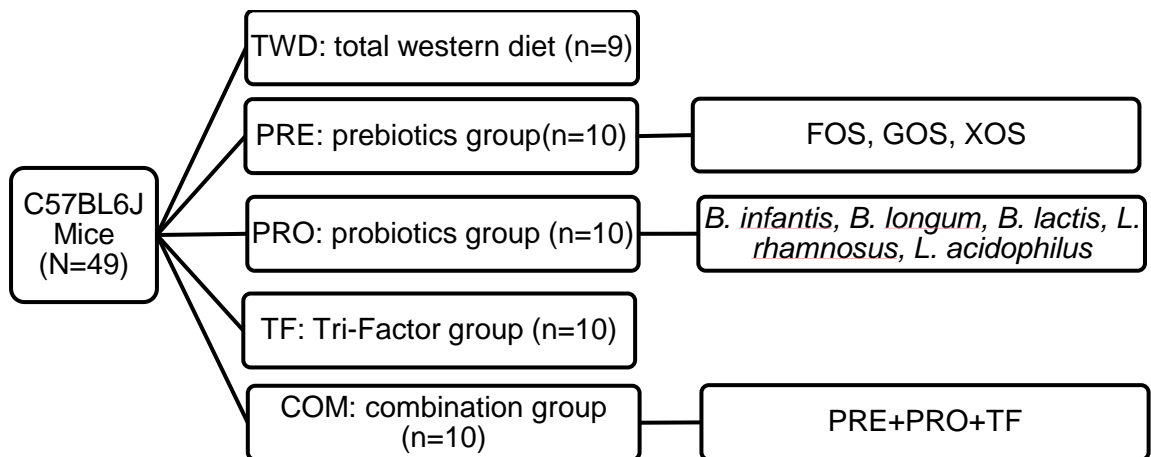


Figure 1. Study design for dietary supplement

Food intake and body weight were measured twice weekly. At the end of intervention, mice were killed by CO₂ 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.

Diet probiotic enumeration

Diet samples were sent to Covance Laboratory (Madison, WI) for Total Probiotic Enumeration using standard plate procedures (Schoeni, 2013).

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 (Ward et al., 2016).

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 16S rRNA (Milani et al., 2013). PCR amplification was performed using the following protocol: 5 minutes at 95°C; 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds; final annealing at 72°C for 10 minutes; 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/ μ L with TE buffer and pooled together into a single tube. Sequencing was performed 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 (Caporaso et al., 2010). Sequences were filtered for quality and assigned operational taxonomic units (OTUs) (Schloss et al., 2009) 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 (DeSantis et al., 2006). Sequences at the highest levels of abundance were chosen as representative sequences, and these were checked for chimeras using uchime61 (Edgar, Haas, Clemente, Quince, & Knight, 2011). 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.

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 CaCl₂, 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).

Plasma zonulin

Plasma samples were analyzed using a commercial ELISA kit according to

manufacturer's directions (MyBioSource, San Diego, CA).

Statistics analysis

Treatment effects and interactions were determined by one way-ANOVA with Dunnett's test. 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.

Because microbiome taxonomy data typically are not normally distributed due to zero-inflation, the effects of diet on relative taxonomic abundance were determined separately as one-way, non-parametric analyses. For these analyses, OTUs were normalized to sequences per million for each sample and then merged by the highest-level classification (to species, if available), family, and phylum taxonomy levels. Single factor comparisons (PRE, PRO, TF, and COM vs TWD) at the phylum, family, and genus level were then made and tested for significance using the Kruskal-Wallis test and results corrected for multiple testing effects by the false discovery rate (FDR) test. Statistical analysis of beta diversity data was performed using the non-parametric PERMANOVA measure, which partitions a distance matrix among sources of variation in order to describe the strength and significance that an experimental variable has in determining variation of distances.

III. RESULTS

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 CFUs in each diet. These numbers were then used to determine the average probiotic intake for each diet (Table 3). In the TWD and TF 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 probiotics, 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 [‡]	$<2.4 \times 10^4$	5.5×10^4	2.6×10^5	$<2.7 \times 10^4$	9×10^6

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

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

Mice consumed significantly 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.

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).

SCFAs

There were very few differences in the SCFAs 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 (Figure 2).

Table 5. SCFAs in cecal and fecal samples for treatments

Cecal SCFAs ($\mu\text{mol/g}$)	TWD	PRE	PRO	TF	COM	P- value
Acetic acid	27.8 \pm 2.1	26.3 \pm 2.5	26.4 \pm 1.7	28.9 \pm 2.1	28.3 \pm 1.3	0.84
Propionic acid	3.87 \pm 0.28	3.88 \pm 0.29	4.58 \pm 0.30	4.11 \pm 0.28	4.61 \pm 0.33	0.22
n-Butyric acid	3.02 \pm 0.30	3.30 \pm 0.43	3.38 \pm 0.23	3.62 \pm 0.35	4.00 \pm 0.35	0.33
iso-Butyric acid	0.48 \pm 0.01	0.44 \pm 0.03	0.47 \pm 0.01	0.44 \pm 0.02	0.47 \pm 0.01	0.47
iso-Valeric acid	0.53 \pm 0.01	0.49 \pm 0.02	0.51 \pm 0.02	0.51 \pm 0.03	0.52 \pm 0.03	0.79
n-Valeric acid	0.51 \pm 0.02	0.48 \pm 0.04	0.49 \pm 0.04	0.52 \pm 0.05	0.52 \pm 0.03	0.86
Caproic acid	0.05 \pm 0.01	0.06 \pm 0.01	0.06 \pm 0.01	0.06 \pm 0.01	0.06 \pm 0.01	0.05
Fecal SCFAs ($\mu\text{mol/g}$)	TWD	PRE	PRO	TF	COM	P- value
Acetic acid	20.4 \pm 2.1	28.0 \pm 2.5	25.5 \pm 4.5	22.8 \pm 1.8	27.2 \pm 1.9	0.27
Propionic acid	2.2 \pm 0.3	2.6 \pm 0.2	2.1 \pm 0.3	2.6 \pm 0.3	2.8 \pm 0.2	0.20
n-Butyric acid	0.70 \pm 0.10	0.97 \pm 0.17	0.87 \pm 0.08	1.05 \pm 0.12	1.03 \pm 0.10	0.26
iso-Butyric acid	0.26 \pm 0.03	0.37 \pm 0.04	0.25 \pm 0.03	0.35 \pm 0.05	0.32 \pm 0.02	0.06
iso-Valeric acid	0.43 \pm 0.05	0.58 \pm 0.05	0.45 \pm 0.03	0.55 \pm 0.07	0.52 \pm 0.02	0.21
n-Valeric acid	0.19 \pm 0.03	0.26 \pm 0.04	0.14 \pm 0.02	0.31 \pm 0.08	0.19 \pm 0.02	0.10
Caproic acid	0.06 \pm 0.01	0.17 \pm 0.06	0.09 \pm 0.02	0.20 \pm 0.10	0.11 \pm 0.03	0.39

SCFAs are expressed as mean \pm SE ($\mu\text{mol/g}$). P-value was calculated by one-way ANOVA.

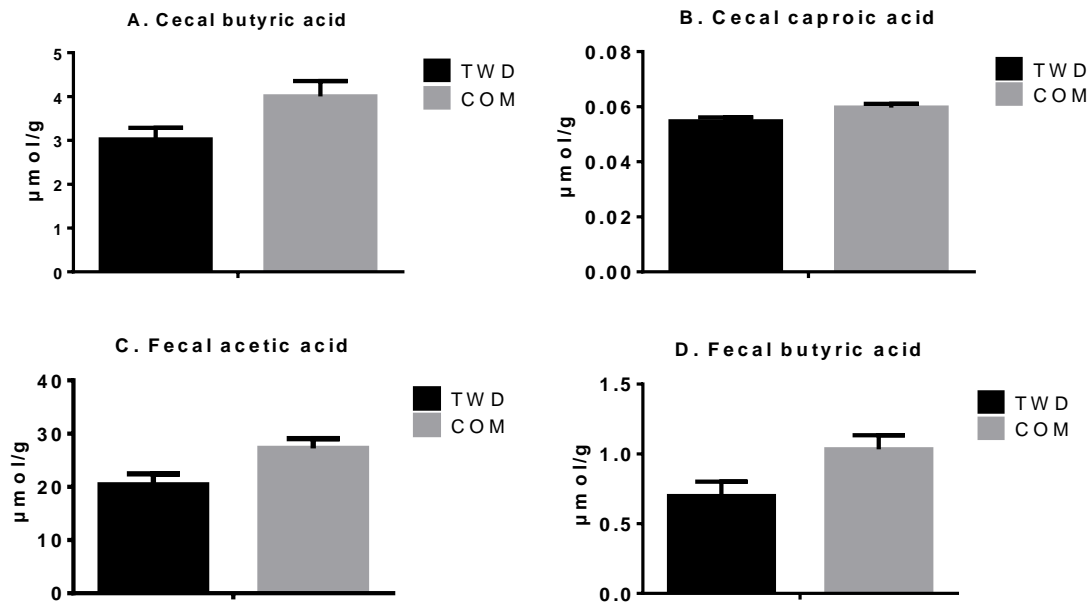


Figure 2. SCFAs analysis, data represent as mean \pm SE ($\mu\text{mol/g}$). A, butyric acid in cecal samples, $p=0.044$. B, caproic acid in cecal samples, $p=0.022$. C, acetic acid in fecal samples, $p=0.025$. D, butyric acid in fecal samples, $p=0.037$.

Microbiome

Taxonomic Summaries

After quality, chimera, and abundance filtering, sequences were assigned to OTUs using the `pick_open_ref_otus` command for an average of 46853 sequences per sample assigned to 1546 OTUs. Compared to diet, PRE, PRO and COM treatments changed the microbiome composition. Figure 3 showed the family level taxonomy.

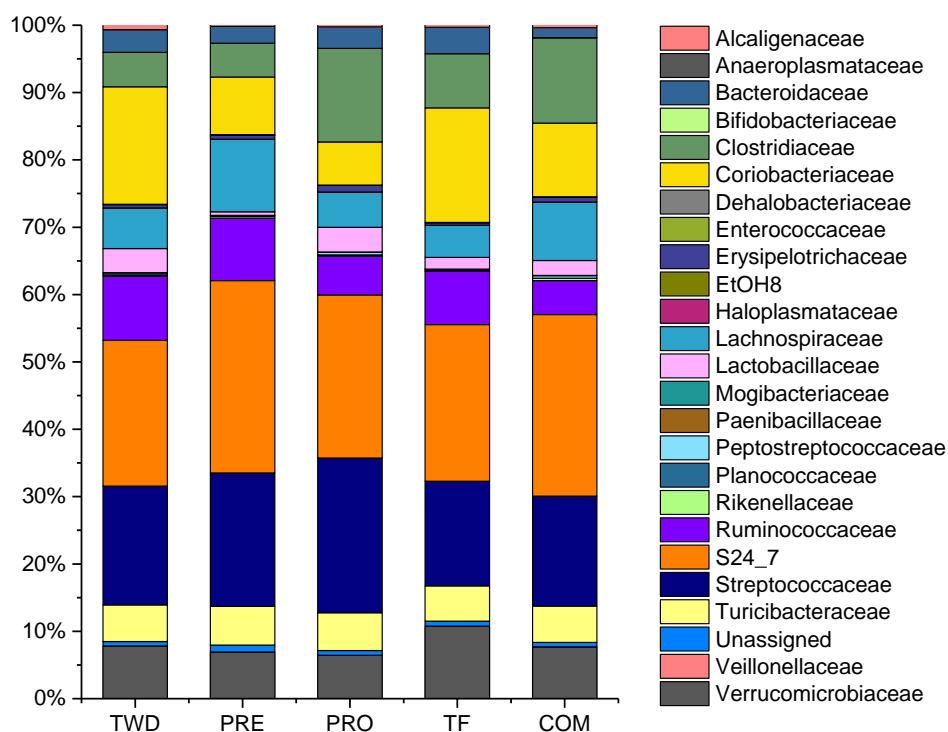


Figure 3. Family level taxonomy, percentage of total OTUs

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.

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	<i>Bifidobacteriaceae</i>	/	0.0078	/	0.0438	PRO, COM
Genus	<i>Ruminococcus</i>	0.0065	/	/	/	PRE
	<i>Bifidobacterium</i>	/	0.0081	/	/	PRO
Species	<i>gnavus</i>	0.0004	/	/	0.0017	PRE, COM
	<i>longum</i>	/	0.0019	/	0.0069	PRO, COM
	<i>neonatale</i>	/	0.0321	/	/	PRO

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

Ruminococcus gnavus was increased after feeding prebiotics. The increasing was observed both in PRE and COM. *Bifidobacterium longum* was increased in mice gut microbiome after feeding prebiotics. The increasing of *Bifidobacteriaceae* was consistent in PRO and COM. Besides, PRO was associated with higher level of *Clostridium neonatale*.

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) (Gering & Crist, 2002). Alpha diversity was determined using Chao1 index. The analyses showed that no significant difference affected by diets in Figure 4. All treatments did not affect alpha-diversity.

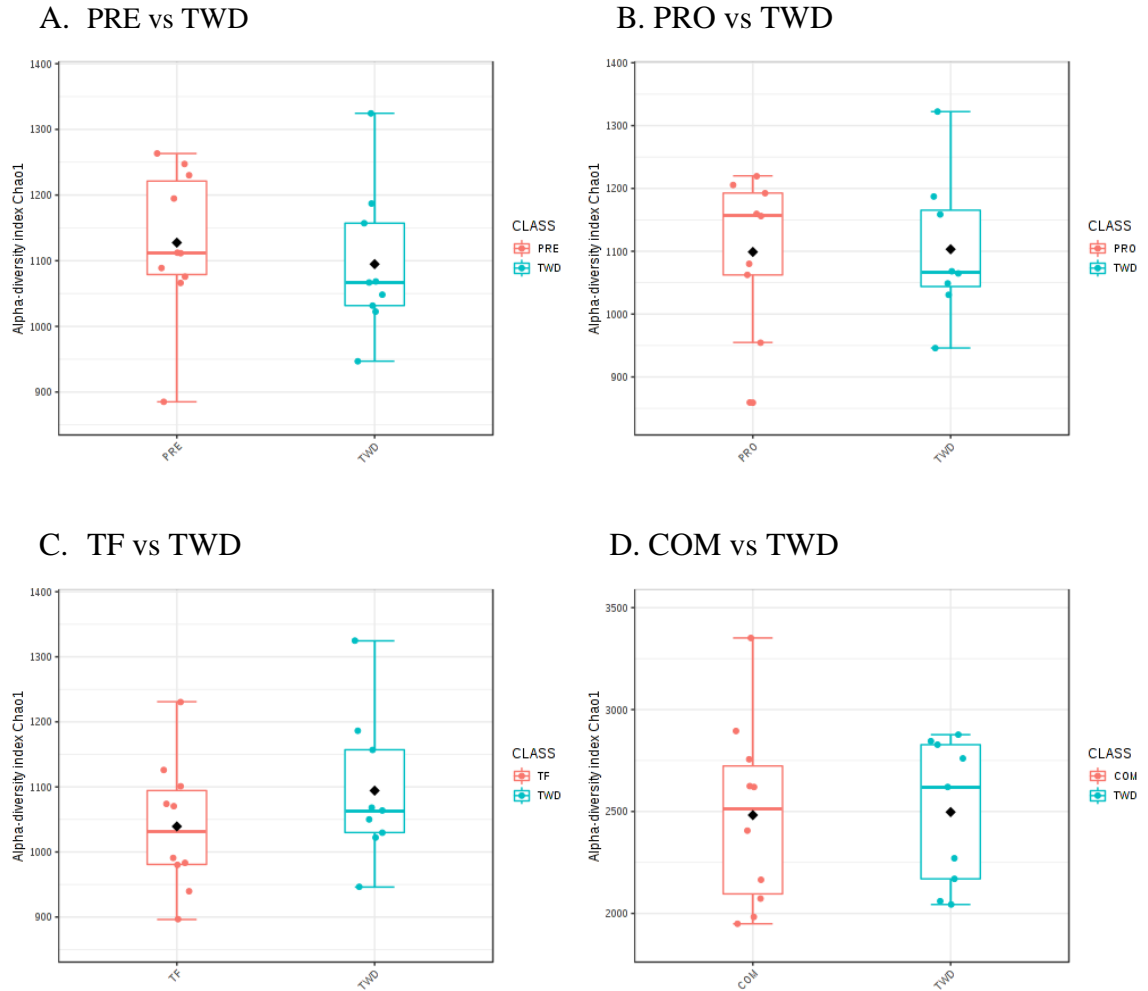


Figure 4. Alpha-diversity of gut microbiome, expressed by OTUs. A, PRE vs TWD, $p=0.243$: B, PRO vs TWD, $p=0.673$: C, TF vs TWD, $p=0.277$: D, COM vs TWD, $p=0.720$

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 (Gering & Crist, 2002). Figure 5 is a spatial representation of beta diversity with Principle Coordinate Analysis. The treatment affected beta-diversity with exception of TF.

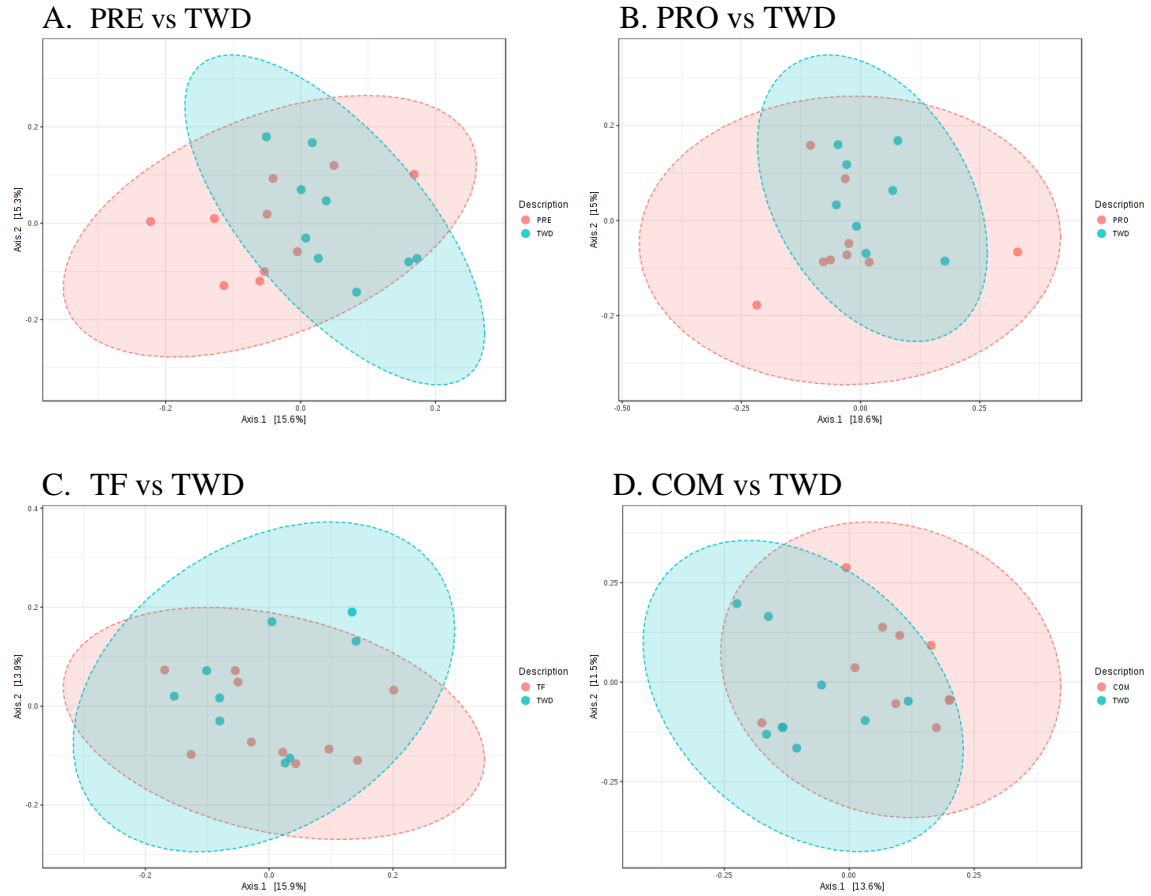


Figure 5. Beta-diversity of gut microbiome, unweighted unifracs distance with non-parametric PERMANOVA test. A, PRE vs TWD, $p=0.021$; B, PRO vs TWD, $p=0.015$; C, TF vs TWD, $p=0.415$; D, COM vs TWD, $p=0.005$

Gut inflammation

The effect of diets on fecal calprotectin is shown in Figure 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 effect among the test diets ($p=0.1355$).

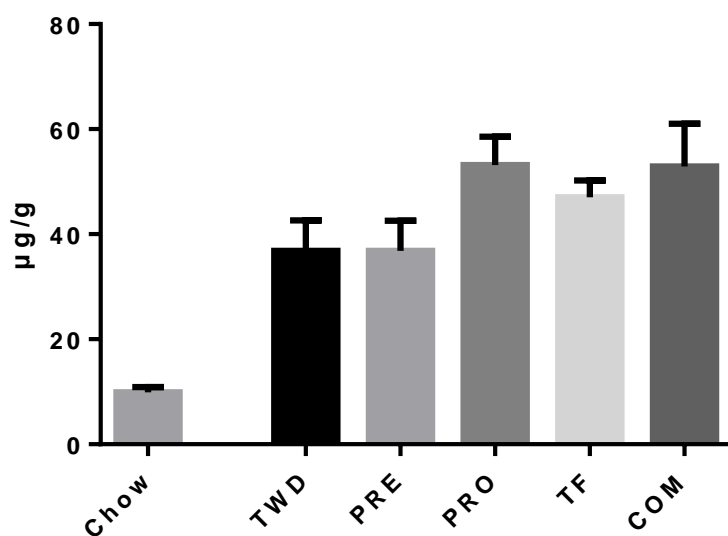


Figure 6. Fecal calprotectin. Data represent mean \pm SE ($\mu\text{g/g}$)

Gut permeability

Zonulin is peptide measured in plasma, and which is associated with an impaired gut barrier. According to Figure 7, plasma zonulin was lower in mice fed the PRE, PRO, TF and COM treatments, compared to the control (TWD) ($p=0.0006$, Figure 7).

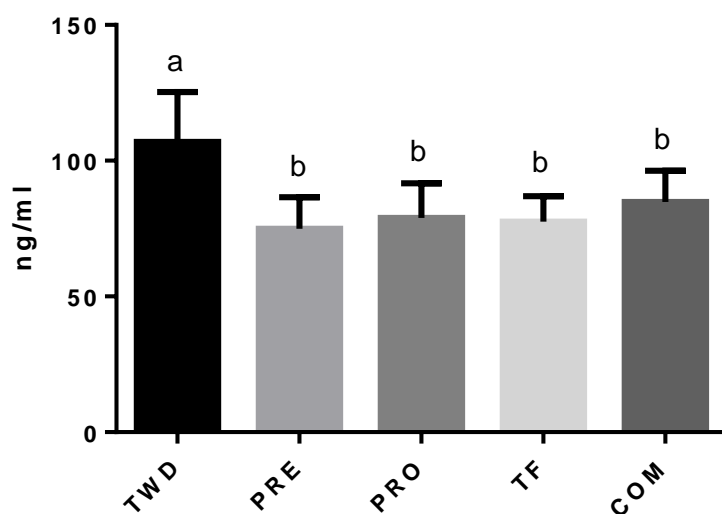


Figure 7. Plasma zonulin. Data represent mean \pm SE (ng/ml). Different letters represent significant difference in ANOVA analysis.

IV. DISCUSSION

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 2). 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 studies (Poutahidis et al., 2014). 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 (Schoeni, 2013).

Mice consuming the PRO diet consumed significantly more calories per day than mice consuming the TWD (Table 4). Yet, the increased calorie consumption was not associated with a greater weight gain, nor metabolic efficiency due to 4-week intervention.

There were few differences in either the cecal or fecal concentration of SCFAs due to inclusion of either the prebiotics, probiotics or both. While compared to previous rodent studies, there were much lower level of treatment which administered and lead to the insignificant results.

Similarly, as actual dosage for mice was much lower than previous animal study, there was no significant difference in phylum level abundance or Firmicutes to Bacteroidetes ratio. Prebiotics and probiotics affected microbiome by increasing *Bifidobacterium longum* and *Ruminococcus gnavus*, respectively. COM 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 in patients with Crohn's disease, an inflammatory bowel disease (IBD) (Scaldaferri et al., 2013). It is suggested that prebiotics might had potential to promote inflammation.

Bifidobacterium longum was one of probiotics supplied in diet in this experiment. Although the dosage for total probiotics was much lower than out expectation, we detected an increasing of *Bifidobacterium longum* in PRO diet compared to TWD, which suggested the effect of supplements was pronounced. Besides, PRO was associated with higher level of *Clostridium neonatale*. It is a strain that digested milk with gas production. Acetic, lactic and butyric acids are detected as metabolic products (Bernard, Burdz, Wiebe, Alfa, & Bernier, 2018).

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, replacement and richness difference within site and among site (Legendre, 2014). Gut microbiome diversity was negatively associated with weight gain, while it was positively correlated with fiber intake. Besides, 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 (Manichanh et al., 2006; Sokol, Lay, Seksik, & Tannock, 2008). When mice supplied with prebiotics or probiotics, enriched microbiome diversity was reported in some studies (W. Cheng et al., 2017; M. K. Hamilton et al., 2017; Park et al., 2013; Umu et al., 2016). But there was controversy of non-affect microbiome diversity with a lower dosage or short intervention supplement (Bai et al., 2016). Human trial reported that microbiome diversity was not affected by prebiotics or probiotics (Finegold et al., 2014; Holscher et al., 2015; Toscano et al., 2017;

van Zanten et al., 2014; Vandeputte et al., 2017). We used the reality dosage for mice rather than megadose, which concluded that the treatment diets did not affect alpha-diversity, but affected beta-diversity with exception of TF treatment.

Calprotectin is a Ca^{2+} binding protein produced by neutrophils (Fallahi et al., 2013) which is bacteriostatic and fungistatic, and which has a minimum inhibitory concentration similar to antibiotics (Bunn et al., 2001). Calprotectin is important for the clearance of infection, as has been shown by the comparison of wild-type and calprotectin-deficient animals (Urban et al., 2009). Calprotectin can be used to predict relapses and detect pouchitis in IBD patients, and is used for IBD in undiagnosed, symptomatic patients (Konikoff & Denson, 2006). Several mice studies showed that megadose of prebiotics and probiotics supplied with high fat diet improved the inflammation situation (Carasi et al., 2015; Garcia, Dogi, de Moreno de LeBlanc, Greco, & Cavaglieri, 2016; Murakami et al., 2015a; Park et al., 2013; Singh et al., 2017). However, a debate was demonstrated from a 2 weeks synbiotics research which showed no effect on gut inflammation and permeability (Wilms et al., 2016). In our case, non-significant difference of calprotectin suggested that treatment did not affect the inflammation situation on healthy mice.

All mice were in Chow diet before intervention. Regular chow is composed of agricultural byproducts. It is a high fiber diet containing complex carbohydrates, with fats from a variety of vegetable sources (Warden & Fisler, 2008). When the mice were assigned a relatively high carbohydrates and high fat diet, TWD and other modified TWD, all treatment showed higher fecal calprotectin compare to Chow diet (Figure 6) after intervention.

Zonulin is a 47 kDa protein, with structural similarity to zonula occludens toxin (ZOT), which is synthesized in the intestine and reversibly regulates the gut barrier (Fasano, 2000; Fasano et al., 2000). Zonulin has been identified as pre haptoglobin-2 (HP2) which is one of two human alleles of the haptoglobin gene (Tripathi et al., 2009). Interestingly, HP2, and thus zonulin, is only found in humans (Levy et al., 2010). Yet, ELISA antibodies to zonulin appear to measure other members of the zonulin family, such as properdin (A. Fasano, personal communication). A synthetic peptide, AT1001, contains the receptor-binding motif and is a zonulin inhibitor. In IL-10 knockout mice, treatment with AT1001 reduces intestinal permeability, and reduces colitis (Arrieta, Madsen, Doyle, & Meddings, 2009). In mice fed high fat diets, plasma zonulin levels are positively correlated with hepatic lipid content (Kwon, Lee, Seo, & Kim, 2019), weight gain and adipose tissue weight (Y. J. Cho, Lee, Seo, Yokoyama, & Kim, 2018). In the current study, all treatments led a significant reduction in plasma zonulin, compared to the control. Yet, the effect was not additive in the COM treatment. Furthermore, the reduction in plasma zonulin was not associated with fecal calprotectin, nor weight gain.

There is no additional effect of Tri-Factor on mice weight and metabolic, gut fermentation, gut microbiome and inflammation. Additive effect was observed on gut microbiome when administered prebiotics and probiotics combinedly.

V. CONCLUSION

Overall, for 1-month intervention, probiotic supplementation increased *Bifidobacterium longum* and *Clostridium neonatale*, 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. All treatments were associated with less plasma zonulin, compared to the control group, indicating an effect on gut permeability. 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 affect gut permeability, but did not affect gut fermentation and inflammation.

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