TITLE PAGE
- Food Science of Animal Resources -
Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title	Effect of Probiotic-Fortified Infant Formula on Infant Gut Health and Microbiota Modulation
Running Title (within 10 words)	Gut microbiota modulation by infant formula
Author	Ju Young Eor[a], Chul Sang Lee[a,b], Sung Ho Moon[a], Ju Young Cheon[a], Duleepa Pathiraja[a], Byeonghyeok Park[a], Min Jae Shin[a], Jae-Young Kim[a,b], Sangjong Kim[c], Youngbae Noh[c], Yunhan Kim[c], In-Geol Choi[a,b*], Sae Hun Kim[a,b*]
Affiliation	[a]*College of Life Sciences and Biotechnology, Korea University, Seoul 02841, South Korea* [b]*Institute of Life Sciences and Natural Resources, Korea University, Seoul 02841, South Korea* [c]*Lotte R&D Center, Seoul 07594, South Korea*
Special remarks – if authors have additional information to inform the editorial office	
ORCID (All authors must have ORCID) https://orcid.org	Ju Young Eor (https://orcid.org/0000-0002-3764-3339) Chul Sang Lee (https://orcid.org/0000-0001-5371-5366) Sung Ho Moon (https://orcid.org/0000-0002-4502-3607) Ju Young Cheon (https://orcid.org/0000-0001-7899-7611) Duleepa Pathiraja (https://orcid.org/0000-0001-6239-5958) Byeonghyeok Park (https://orcid.org/0000-0002-8187-7650) Min Jae Shin (https://orcid.org/0000-0003-1719-4246) Jae-Young Kim (https://orcid.org/0000-0003-1937-9535) Sangjong Kim (https://orcid.org/0000-0003-0281-3577) Youngbae Noh (https://orcid.org/0000-0002-1181-4099) Yunhan Kim (https://orcid.org/0000-0001-5120-3295) Sae Hun Kim (https://orcid.org/0000-0002-0990-2268) In-Geol Choi (https://orcid.org/0000-0001-7403-6274)
Conflicts of interest List any present or potential conflict s of interest for all authors. (This field may be published.)	The authors declare no potential conflict of interest.
Acknowledgements State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available. (This field may be published.)	This work was supported by the Ministry of Education of the Republic of Korea and the National Research Foundation of Korea (2021R1A6A3A01086566), Korea University Grant, and Lotte Foods Co. Ltd.
Author contributions (This field may be published.)	Conceptualization: Eor JY, Lee CS, Moon SH, Cheon JY Data curation: Eor JY, Lee CS, Moon SH, Cheon JY, Pathiraja D, Park B Formal analysis: Moon SH, Cheon JY, Pathiraja D, Park B Methodology: Moon SH, Cheon JY, Pathiraja D, Park B, Kim S, Noh Y, Kim Y Software: Eor JY, Lee CS Validation: Eor JY, Lee CS Investigation: Eor JY, Lee CS Writing - original draft: Eor JY, Lee CS, Shin MJ, Kim JY, Kim SH, Choi IG Writing - review & editing: Eor JY, Lee CS, Moon SH, Cheon JY, Pathiraja D, Park B, Shin MJ, Kim JY, Kim S, Noh Y, Kim Y, Kim SH, Choi IG
Ethics approval (IRB/IACUC) (This field may be published.)	The study was approved by the Korea University Institutional Review Board (IRB, 1040548-KU-IRB-17-251-A-2, and KUIRB-2019-0136-01).

CORRESPONDING AUTHOR CONTACT INFORMATION

ACCEPTED

For the corresponding author (responsible for correspondence, proofreading, and reprints)	**Fill in information in each box below**
First name, middle initial, last name	Sae Hun Kim
Email address – this is where your proofs will be sent	saehkim@korea.ac.kr
Secondary Email address	Saehkim@gmail.com
Postal address	College of Life Sciences and Biotechnology, Korea University, Seoul 02841, South Korea
Cell phone number	010-9071-3055
Office phone number	02-3290-3491
Fax number	

7

8

ACCEPTED

Effect of Probiotic-Fortified Infant Formula on Infant Gut Health and Microbiota Modulation

Ju Young Eor[a,b], Chul Sang Lee[a,b], Sung Ho Moon[a], Ju Young Cheon[a], Duleepa Pathiraja[a], Byeonghyeok Park[a], Min Jae Shin[a], Jae-Young Kim[a,b], Sangjong Kim[c], Youngbae Noh[c], Yunhan Kim[c], In-Geol Choi[a,b]*, Sae Hun Kim[a,b]*

Running title: Gut microbiota modulation by infant formula

[a]*College of Life Sciences and Biotechnology, Korea University, Seoul 02841, South Korea*

[b]*Institute of Life Sciences and Natural Resources, Korea University, Seoul 02841, South Korea*

[c]*Lotte R&D Center, Seoul 07594, South Korea*

*Corresponding authors:

Prof. Sae Hun Kim, Ph.D.; Email address: saehkim@korea.ac.kr

Prof. In-Geol Choi, Ph.D.; Email address: igchoi@korea.ac.kr

ACCEPTED

Abstract

Compared to infant formula, breast milk is the best source of nutrition for infants; it not only improves the neonatal intestinal function, but also regulates the immune system and gut microbiota composition. However, probiotic-fortified infant formula may further enhance the infant gut environment by overcoming the limitations of traditional infant formula. We investigated the probiotic formula administration for one month by comparing 118 Korean infants into the following three groups: infants in each group fed with breast milk (50), probiotic formula (35), or placebo formula-fed group (33). Probiotic formula improved stool consistency and defecation frequency compared to placebo formula-fed group. The probiotic formula helped maintaining the level of secretory immunoglobulin A (sIgA), which had remarkably decreased over time in placebo formula-fed infants (compared to weeks 0 and 4). Moreover, probiotic formula decreased the acidity of stool and considerably increased the butyrate concentration. Furthermore, the fecal microbiota of each group was evaluated at weeks 0 and 4. The microbial composition was distinct between each groups, and the abundance of health-promoting bacteria increased in the probiotic formula compared to the placebo formula-fed group. In summary, supplementation of probiotic infant formula can help optimize the infant gut environment, microbial composition, and metabolic activity of the microbiota, mimicking those of breast milk.

Keywords: Probiotics, microbiota, infant formula, short-chain fatty acids, gut health

ACCEPTED

Introduction

Infant formula (IF) is defined as an infant diet in a powdered form that mimics human breast milk (HBM), and therefore can be used as a substitute of HBM (FDA., 2019). HBM consists of 87% water, 3.8% fat, 1.0% protein, and 7% lactose (Martin et al., 2016). IF is usually prepared using a bovine milk base, which is known to have higher protein and lower carbohydrate levels than HBM (Wargo and Wayne., 2016). Carbohydrates are the major source of energy for humans when converted in glucose by intestinal digestion. Carbohydrates are important because of their necessity in childhood growth (Kalhan and Kilic., 1999). These compositional differences hamper infant growth and lead to the development of certain childhood illnesses such as sore throat, ear pain and skin infections (Boyd et al., 2007). Boué et al. (2018) suggested that HBM oligosaccharides might lower the risk of developing gastrointestinal (GI) infections and respiratory tract infections by inhibiting the adherence of pathogens to the gut mucosa. Neurodevelopment is also enhanced by HBM feeding, possibly by DHA transmission, contributing to structural changes in the brain through FADS2 gene regulation (Horta and Victora.,2013). Fortification of HBM using IF, improved growth parameters such as weight gain and head growth in low-birth-weight infants (Gupta et al., 2020).

To narrow the compositional and functional gap between HBM and IF, IF fortification has been implemented by supplementation with additives such as micronutrients, prebiotics, and probiotics. Consumption of iron-containing fortified milk for a year reduced anemia and iron deficiency in 12-to 30-month-old children (Brown et al., 2007). Consumption of IF supplemented with prebiotics, galactooligosaccharides (GOS), and fructooligosaccharides (FOS), increased bifidobacterial colonization in the infant GI tract (Borewicz et al., 2019). *Bifidobacterium* is a predominant bacterium found in HBM-fed infants and confers protection to the host against pathogens by enhancing mucosal immune system functions (Makino., 2018).

ACCEPTED

Probiotic-enriched infant formula, containing *B. animalis*, altered the gut microbiota (GM) pattern, fecal IgA content, and stool pH to resemble those in HBM-fed infants (Radke et al., 2017).

The GM, which is a complex microbial community of bacteria, archaea, fungi, viruses, and phages living in the gut, reportedly controls various host pathways (Derrien et al., 2019). Infant GM regulates infant metabolism, nutrient absorption, immune system maturation, and pathogen colonization (Belkaid and Hand., 2014). The development of GM is shaped by exterior factors such as diet, environment, and lifestyle; therefore, there exists a unique gut microbial composition specific to each individual (Derrien et al., 2019).

Infant diets contribute to the formation of distinct GM composition. HBM feeding promotes the growth of a bifidobacteria-dominated gut microbiome, which exerts a major effect on immune functions (Bäckhed et al., 2015; Stewart et al., 2018; Vandenplas et al., 2020). IF feeding results in the formation of a GM highly diverse but unstable, with overrepresentation of *Clostridium difficile* (Azad et al., 2013; Jost et al., 2015; Tannock et al., 2013). To mimic the HBM-fed gut microbiome and its beneficial functions, attempts of IF probiotic supplementation have been performed. *Lactobacillus rhamnosus* GG supplementation in HBM or IF for a month in eight- to ten-month-old infants lowers the proportion of *C. histolyticum* type bacteria to the total bacterial count (Pärtty et al., 2013). *C. histolyticum* increases intestinal dysbiosis, which can regulate GI peptide production correlated with satiety, and subsequently increases food intake and the risk of developing obesity (Gomes et al., 2018). *B. lactis* BB-12 supplementation increases Bifidobacterial abundance, lowers *Enterobacteriaceae* and *Clostridium* spp. counts, and exerts no effect on colonization of antibiotic-resistant organisms (Mohan et al., 2006). The presence of *Enterobacteriaceae* is a hallmark of dysbiosis (Sassone-Corsi et al., 2016), and both *Enterobacteriaceae* and *Clostridium* spp. are frequently isolated in cancer patients (Touchefeu et al., 2014).

ACCEPTED

However, the results of controlling GM by probiotic supplementation remain controversial. Probiotic supplementation in infant formula failed to exert any considerable effect on growth, stool consistency, incidence of diarrhea, crying, or vomiting (Mugambi et al., 2012). The European Food Safety Authority (EFSA) states that more scientific evidence is necessary to prove the existence of a relationship between probiotic product consumption and their health-related beneficial effects (Rijkers et al., 2011) Thus, more clinical evidence is warranted to elucidate the effects of probiotic supplementation on infant growth and GM composition.

Therefore, our study aimed to improve infant gut health and growth performance by providing probiotic-fortified infant formula (PFIF) and by determining its effects on infant GM. Infant participants (n = 118) were allocated into the following three groups: breast milk-fed group (BF), probiotic-fortified formula-fed group (PF), and placebo formula-fed group (FF). Beginning from 30 days postnatal, each designated diet was provided for a period of 4 weeks, and during this period, fecal samples were collected three times a week. Effects of the diet were determined by analyzing fecal consistency, pH, short-chain fatty acid (SCFA) levels, secretory immunoglobulin A (sIgA) levels, and fecal microbiota composition by next-generation sequencing (NGS).

Materials and methods

Infant cohort study

To conduct the present study, participating mothers and infants were recruited from postnatal care centers (Seoul, South Korea). The aims, protocol, and outcomes of the present study were explained to all participants, and they were inquired about their willingness to participate. Exclusion criteria were as follows: severe disorder of the liver, heart, respiratory organs, endocrine glands, or metabolism; and use of antibiotic medication at the time of the clinical

ACCEPTED

trial. A total of 118 participants were recruited for this study. The study was approved by the Korea University Institutional Review Board (IRB, 1040548-KU-IRB-17-251-A-2, and KUIRB-2019-0136-01), which is independent of the Lotte Food Co. Ltd.. Written inf ormed consent was obtained from all participants.

Probiotic-enriched infant formula preparation

The infant formula and bacterial strains used in this study were obtained from the Dairy Department of Lotte R&D Center (Seoul, Korea). *Lactobacillus rhamnosus* GG, *L. fermentum* LC40, *L. reuteri* DSM 17938, and *Bifidobacterium lactis* BB12 were selected based on their acid, bile tolerance, and safety assays. Strains were cultured in Man Rogosa Sharpe broth (MRS; Difco, USA) for 18 h at 37 ℃. The strains were subcultured three times prior to the conduction of each experiment. All strains were prepared using 50 % (v/v) glycerol as a cryoprotectant and maintained at -80 ℃ until use. Infants consumed approximately 1.34×10^7 CFU/mL probiotics in formula without the presence of any other bacterial species.

Treatment protocol

This randomized, double-blind, placebo-controlled study was performed from January 1, 2018, to June 1, 2020. Randomization was conducted using a computer program, and participants were assigned into the following three groups: breast milk (BF), probiotic formula (PF), and placebo formula-fed group (FF). Individual differences in gestational period and infant body weight were minimized among the three groups. Each feeding was practiced for a period of 4 weeks, beginning from 30 days postnatally. Participants received stool containers with a spatula placed inside (SPL Life Science, Pocheon, Korea), together with a sanitary plastic bag, instruction form, and a questionnaire. Participants collected stool samples from the diaper into the collection tube and sent it directly to the microbiology lab (Seoul, Korea) via an express

ACCEPTED

158 courier. Participants were recommended to collect and send samples early in the morning or
159 late evening to avoid delays.
160
161 **Infant weight and height measurement**
162 Both the weight and height of all infants were measured on the following three designated days:
163 1st, day of birth, 2nd; second, 1 month after the birth; and 3rd, last day of the experiment. The
164 measurements of both weight and height were conducted in triplicate.
165
166 **Fecal defecation frequency determination and fecal sample collection**
167 Infant fecal samples were collected by their parents immediately after defecation. Concurrently,
168 the defecation frequency was determined following the instructions provided in a paper format.
169 The samples were collected on three different days in one week. Collected samples were then
170 stored at 80 ℃ until analysis in the microbiology lab (Seoul, Korea).
171
172 **Fecal consistency analysis**
173 Following the protocol prescribed by the Bristol stool chart, three of our lab members
174 performed fecal consistency analysis. The thawed fecal samples were randomly distributed to
175 all participating members at one time, and the samples were scored.
176
177 **Fecal pH measurement**
178 Fecal pH was measured to determine whether the treatments could change the acidity of the
179 feces in the gut. The weight of each sample was measured to 1 g prior to the conduction of
180 measurements, and the samples were then placed into 15-mL tubes (SPL Life Science, Pocheon,
181 Korea) with saline. The pH of the mixed fecal samples was measured using a pH meter (Agilent
182 Technologies, CA, USA).

ACCEPTED

183

184 **Fecal SCFA concentration assessment**

185 Fecal samples were weighed individually in an Eppendorf tube according to the methods
186 reported by Eor et al. (2020); 10 mg fecal samples in Eppendorf tubes were included for
187 analysis individually. The samples were then homogenized after addition of 100 µL crotonic
188 acid, 50 µL HCl, and 200 µL ether. Homogenates were then centrifuged at 1,000 × *g* for 10
189 min. After centrifugation, the top ether layer was transferred to glass vials. The collected ether
190 was then mixed with 16 µL N-tert-butyldimethylsilyl-N-methylttrifluoroacetamide
191 (MTBSTFA), and the vials were sealed with ParafilmM (Sigma-Aldrich, MO, USA). The
192 samples were heated at 80 ℃ for 20 min in a water bath and then incubated for 48 h. The
193 samples were then placed into the 6890N Network GC system (Agilent Technologies Inc.,
194 Wilmington, DE, USA) with an HP-5MS column (0.25 330m 30.25 mm) and 5973 Network
195 Mass Selective Detector (Agilent Technologies Inc., Wilmington, DE). Helium (purity
196 99.9999%) was used as a delivery gas and the carrier gas flow rate was 1.2 mL per min. The
197 head pressure was 97 kPa and split at 20:1. The inlet and temperature of the transfer line were
198 250 ℃ and 260 ℃, respectively. The following temperature program was used: 60 ℃ (3 min),
199 60–120 ℃ (5 ℃/min), and 120–300 ℃ (20 ℃/min). One microliter of the sample was injected
200 (run time: 30 min). SCFA concentrations were then determined by comparing their peak areas
201 with the standards.

202

203 **Fecal secretory immunoglobulin A (s-IgA) analysis**

204 S-IgA was quantified using the Secretory IgA ELISA kit (ImmuChrom GmbH). All procedures
205 were performed according to the manufacturer's instructions. Stool samples (100 mg) were
206 mixed with 5 mL of wash buffer on a vortex mixer until a homogenous mixture was obtained.
207 One milliliter of the mixture was then transferred into an "Eppendorf" reaction vial and

ACCEPTED

centrifuged for 10 min at 2000 ×*g*. The supernatant was diluted 1:250 with wash buffer (4 µL + 996 µL wash buffer). One hundred microliters of the dilution was used per well. The sample preparation method was performed for a total of four times for each sample.

DNA extraction from fecal samples, library preparation, and sequencing

To determine the intestinal microbiota composition, genomic DNA (gDNA) was extracted from the stool samples of each group using the QIAamp DNA stool kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, 200 mg of fecal sample was mixed with 1.4 mL ASL buffer in a 2-mL tube and vortexed until the sample was thoroughly homogenized. Samples were subsequently heated for 5 min at 95 ℃, vortexed for 15 s, and centrifuged at 196768 g for 1 min to pellet the stool particles. After 1.2 mL of the supernatant was transferred into a new 2-mL tube, 1 InhibitEX Tablet was added, and the tube was vortexed immediately until the tablet was completely suspended. The tube was incubated for 1 min at 25℃, centrifuged for 3 min at full speed, and 200 µL of supernatant was transferred into a new 1.5-mL tube. After 15 µL proteinase K and 200 µL Buffer AL were added and the mixture was vortexed, the tube containing the mixture was incubated at 70 ℃ for 10 min. An additional 200 µL of ethanol was added to the lysate and mixed by vortexing. The complete lysate was transferred to a QIAamp spin column with a 2-mL collection tube and centrifuged for 1 min. 500 µL Buffer AW1 was added, and the column was centrifuged at full speed for 1 min. The collection tube containing the filtrate was then discarded. The same process was conducted with 500 µL of AW2. Finally, the QIAamp spin column was transferred into a new 1.5-mL tube, and 200 µL of Buffer AE was directly pipetted onto the QIAamp membrane. All eluted DNA samples were acquired from the collection tube after 1 min of centrifugation.

Small subunit (16S) ribosomal DNA amplicon sequencing was performed to analyze the microbial community structure. The hyper variable V3-V4 regions of 16S rDNA were

ACCEPTED

amplified using the following universal primer set (forward 5′-CCT-ACG-GGN-GGC-WGC-AG-3′, reverse 5′-GAC-TAC-HVG-GGT-ATC-TAA-TCC-3′). The resulting DNA amplicon of ~460 bp was purified using the Agencourt AMPure XP kit (Beckman Coulter, USA). The second PCR was performed to attach Illumina universal p5/p7 overhang sequences and sample-specific barcodes. A sequencing library of ~550 bp was purified using the Agencourt AMPure XP kit (Beckman Coulter, USA). Sequencing was conducted using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using the MiSeq Reagent kit V3 (2 × 300 PE) (Illumina, San Diego, CA, USA). Control experiments for DNA extraction, library preparation, and sequencing were performed as follows. The mock DNA library was prepared using the ZymoBiomics microbial community DNA standard (Zymo Research, Irvine, CA, USA) using the DNA amplicon library preparation procedure described above. Negative control was established by following the same DNA extraction procedure without feces. The final elute of the DNA extraction negative control was used as the template for PCR negative control, following the same PCR conditions described above. We confirmed that the samples were not contaminated during DNA extraction or library preparation.

Bioinformatics analysis of sequencing data

Sequencing reads were processed using the DADA2 pipeline (Ver 1.12.1). Reads were trimmed and filtered, paired reads were merged, and chimeric sequences were removed. The SILVA ribosomal RNA reference database (Release 128) was used for taxonomic assignment of the amplicon sequence variants (ASV). Data on low-quality samples with <10,000 observed OTUs were removed from the sample set. Among the 1,984 total OTUs, only 762 OTUs with a total count of more than 100 were considered. The *microbiome* R/Bioconductor package was used to calculate the alpha diversity. The community structure was illustrated using an in-house R script based on the *phyloseq* R/Bionconductor package.

ACCEPTED

Statistical analyses

IBM SPSS Statistics software (version 25.0; IBM Corp, Armonk, NY, USA) was used to analyze the data. One-way analysis of variance was used to compare the sample means. Multiple comparisons of means were performed using the Duncan's multiple range test and Tukey's post-hoc test. $P < 0.05$ was considered statistically significant.

Results

Effect of PFIF of infant body weight and height

A slight change in body weight was observed after each treatment. The initial body weights of BF, PF, and FF at 0 weeks were 4.74, 4.65, and 4.06 kg. After 4 weeks of treatment, the body weights of three groups were increased to 5.75, 5.97, and 5.18 kg. Although the difference between the treatment periods were 1.01, 1.11, and 1.13 kg. Significantly increased body weight was observed in the 4^{th} week of PF treatment compared to FF ($P < 0.05$) (**Fig. 1A**). Moreover, similar to the observations with body weight increase, the heights of PF groups was also significantly higher than FF treated groups ($P < 0.05$), which indicates the increased growth performance of PF group (**Fig. 1B**). The heights in groups BF, PF, and FF at 0 weeks were 54.4, 53.9, and 51.2 cm. However, in the 4^{th} week, the heights were 56.8, 59.1, and 55.3 cm, with a variance (VAR) within 4 weeks showing 2.4, 5.3, and 4.1 cm, respectively.

Effect of PFIF on stool condition

The four types of assessment of stool conditions are illustrated in **Fig. 2**. First, the results of stool pH analysis showed significantly lower levels in the BF groups compared to other groups

ACCEPTED

($P < 0.05$) (**Fig. 2A**). Second, stool consistency was analyzed by scoring the condition of the stool samples using the Bristol chart method (**Fig. 2B**). The results suggested that stool consistency did not considerably changed in the BF, PF, and FF groups throughout the study period. However, the average consistency of FF showed a significantly decreased score rate compared to BF and PF groups since 1st week to the study period (**Fig. 2B**). The frequency of defecation showed the same trend from the 1st week to the 3rd week. However, the 4th week results indicated that FF showed a significant decrease defecation frequency score ($P < 0.05$) compared to both the BF and PF groups (**Fig. 2C**). Finally, the stool IgA levels in each treatment throughout the study period did not show any significant difference (**Fig. 2D**).

Effect of PFIF on stool SCFA concentration

Stool SCFAs were analyzed before and after the conduction of each treatment. The concentration of acetate showed a significant increase only in the PF group ($P < 0.05$) (**Fig. 3A**). Additionally, both the BF and PF groups showed significant increases ($P < 0.05$) in terms of both propionate and butyrate concentration after 4 weeks of treatment (**Fig. 3B** and **3D**). However, the iso-butyrate concentration showed no considerable difference after 4 weeks of treatment (**Fig. 3C**).

Alpha diversity index in fecal microbiota of infants

Fecal microbiota alpha diversity was calculated using the observed operational taxonomic units (OTUs), Chao1, Shannon, and Simpson (**Fig. 4**). Overall, there were remarkable differences between the BF and PF groups after 4 weeks of each treatment in terms of the OTUs (**Fig. 4A**), Chao1 (**Fig. 4B**), Shannon (**Fig. 4C**), and Simpson (**Fig. 4D**) indices. The alpha diversity changes in each treatment group showed no significant differences between 0 and 4 weeks after treatment, except in the PF group (**Fig. 4A and 4B**). Furthermore, the Shannon and Simpson

ACCEPTED

indices indicated significant increased diversity between BF and PF after 4 weeks of treatment ($P < 0.05$) (**Fig. 4C and 4D**). The results indicate that PF and FF treatment are capable of increasing the diversity of observed species in infant gut.

Microbiome composition changes in fecal samples of infants

At various taxonomic levels, gut microbiome composition was compared between the 0- and 24-week fecal samples in each treatment group. Overall, there were no substantial differences in the microbiome composition changes at various levels in the BF group (Fig. 5). At the phylum level, Firmicutes showed significantly less ($P < 0.05$) abundance in the gut microbiome in the 4-week samples than that in the 0-day samples in the PF and FF groups. However, the abundance of Actinobacteria and Proteobacteria increased after 4 weeks of treatment (Fig. 5A). At the class level, *Bacilli* showed significantly less ($P < 0.05$) abundance in the GM at 4 weeks in the PF and FF groups. However, the relative abundances of Gamma proteobacteria and Actinobacteria increased after 4 weeks (Fig. 5B). At the order level, the abundance of *Lactobacillales* was reduced in the 4-week samples in the PF and FF groups. However, *Enterobacteriales* expanded after 4 weeks of treatment (Fig. 5C). At the family level, *Lactobacillaceae* abundance was reduced after 4 weeks of treatment in the PF and FF groups. However, the relative abundances of *Enterobacteriaceae* and *Bifidobacteriaceae* increased significantly ($P < 0.05$) at 4 weeks (Fig. 5D). At the genus level, *Lactobacillus* abundance decreased after 4 weeks of treatment in the PF and FF groups. Notably, *Streptococcus* abundance reduced in the 4^{th} week samples in the FF group. However, *Escherichia*, *Shigella*, and *Bifidobacterium* abundances increased significantly ($P < 0.05$) after 4 weeks of treatment (Fig. 5E).

ACCEPTED

Discussion

A considerable height change in PF and FF was observed in the 4th week of treatment. Importantly, the variance of height showed an increase in both PF and FF compared to BF. The PFIF showed remarkable differences in height and weight compared to the BF and FF groups. This result was consistent with the results reported by a previous study conducted by Vendt et al. (2006).

The difference in stool pH shows an alteration in the GI environment, and a lower pH of stool may suggest a more enhanced state of the GI tract occurring due to pathogens. (Henrick et al., 2018). Therefore, the BF group reveals the most protective effect conferred against viral infection and demonstrates the effect exerted on the immune system of newborn babies against infection among the group (Moore et al., 2021; Toscano et al., 2017). Stool consistency, which indicates the state of stools expelled by the body, showed a significant difference ($P < 0.05$) occurring since the 1st week, and PF restored the decrease and maintained almost the same results as BF. The stool defecation frequency indicates the motility and healthiness of the gut. The results showed a significant difference ($P < 0.05$) occurring since the 1st week to the 4th week without critical changes. These results were similar to those reported by previous studies conducted using probiotics and prebiotics, respectively (Vandenplas et al., 2015; Vendt et al., 2006). Stool IgA concentration did not change in each week. These results were similar to those reported by a previous study (Bakker-Zierikzee et al., 2006).

Based on the GM modulation, changes in each level indicated that dietary differences might induce microbiota changes. At the phylum level, we identified the relative abundances of Actinobacteria, Proteobacteria, Firmicutes, Verrucomicrobia, and Bacteroidetes. In our study, the lower abundance of Firmicutes, Verrucomicrobia, Bacteroidetes, and higher abundance of Proteobacteria and Actinobacteria were associated with the treatment effects of probiotics and the formulation. In comparison, data obtained from human and animal studies showed that the

ACCEPTED

abundance of Firmicutes and Actinobacteria increased in obesity models and tended to increase with weight gain. Moreover, the proportions of Bacteroidetes and Verrucomicrobia are decreased in obese subjects and the rate of obesity continues to increase (Chakraborti., 2015; Zhang et al., 2021). However, the results of the present study were different from those of previous studies conducted on body weight and height. Furthermore, the chief butyrate-producing bacteria in the human gut belong to the phylum Firmicutes (Louis and Flint., 2009; Louis and Flint., 2017). Butyrate is a SCFA derived from the microbial fermentation of dietary fibers in the colon and it is recognized for its potential to act on secondary chemoprevention by slowing growth and activating apoptosis in colon cancer cells (Canani et al., 2012). Actinobacteria, Bacteroidetes, and Proteobacteria are potential butyrate producers (Vital et al., 2014). Additionally, apart from butyrate, the production of other SCFAs is mediated by bacterial members belonging to Actinobacteria that produce acetate and lactate during carbohydrate fermentation (Rivière et al., 2016). Also, the mucin-degrading bacteria phylum Verrucomicrobia produces both propionate and acetate (Derrien et al., 2004). In this study, we revealed that the relative abundances of Actinobacteria and Proteobacteria were significantly increased ($P < 0.05$) in the probiotics and formula-fed groups. Our results are consistent with those reported by previous studies. In stool consistency and defecation frequency analyses, several studies have reported that Bacteroidetes to Firmicutes ratio is higher in the subjects with a small amount of defecation than in those with a considerable amount of defecation, and at the genus level, *Bifidobacterium* is less abundant in the subjects with a small amount of defecation (Kwon et al., 2019). Moreover, remarkable increase in the relative abundance of Firmicutes in the gut microbiome of group presenting with loose stool in comparison with the group presenting with normal stool consistency (Bregg et al., 2020). In this study, the results of stool consistency were similar to those reported by previous studies. The stool consistency and stool defecation frequency rates were significantly higher ($P < 0.05$) in BF fed group

ACCEPTED

compared to FF group, which can indicated the gut health of the infants. This result is similar with the study reported by Vandeputte et al. and the colleges which have shown that increased gut health was correlated with the increased diversity of GM (Vandeputte et al., 2016). Compared to FF group, BF and PF fed groups have shown the increased diversity of GM in the genus level was observed in our study. Moreover, at the genus level, we discovered that the relative abundances of *Bifidobacterium*, *Escherichia*/*Shigella*, *Streptococcus*, *Klebsiella*, and *Bacteroides* were considerably different in the fecal microbiota of the probiotic and formula-fed groups. Notably, previous studies indicated that a decreased abundance of *Bacteroides* was associated with the development of several diseases, such as obesity and diabetes (Bervoets et al., 2013; Zhang et al., 2013). In our study, the results are similar to those reported by previous studies in terms of the probiotic formula-fed group with body weight gain. Moreover, the relative abundance of the genus *Bacteroides* demonstrates a high capacity for the production of SCFAs such as butyrate (Ohira et al., 2017).

Conclusion

The present study showed overall fecal microbiota modulation induced by dietary changes in infants. Although the results from BF intestinal health promoting effect showed the better than FF groups, probiotic strain fortified FF showed improved effect on gut health. Based on the effects, we analyzed GM from the fecal samples. The GM composition and the abundance of health-promoting bacteria increased in the probiotic formula group compared to the placebo formula-fed group. Supplementation with probiotics can help optimize the infant gut environment, composition, and metabolic activity of the microbiota, mimicking those of breast milk. Despite this limitation exist, we tried to find overall meaning from changed genera that have previously been reported. Additionally, the findings from this study would provide

ACCEPTED

strategy or reference who wants to investigate on manufacturing fortified infant formula for infants with novel GM modulation.

Conflicts of Interest

The authors declare no potential conflicts of interest.

Acknowledgements

This work was supported by the Ministry of Education of the Republic of Korea and the National Research Foundation of Korea (2021R1A6A3A01086566), Korea University Grant, and Lotte Foods Co. Ltd.

Author Contributions

Conceptualization: Eor JY, Lee CS, Moon SH, Cheon JY. Data curation: Eor JY, Lee CS, Moon SH, Cheon JY, Pathiraja D, Park B. Formal analysis: Moon SH, Cheon JY, Pathiraja D, Park B. Methodology: Moon SH, Cheon JY, Pathiraja D, Park B, S Kim, YB Noh, Y Kim. Software: Eor JY, Lee CS. Validation: Eor JY, Lee CS. Investigation: Eor JY, Lee CS. Writing – original draft: Eor JY, Lee CS, Shin MJ, Kim J-Y, Kim SH, Choi I-G. Writing –review & editing : Eor JY, Lee CS, Moon SH, Cheon JY, Pathiraja D, Park B, Shin MJ, Kim J-Y, S Kim, YB Noh, Y Kim, Kim SH, Choi I-G

Ethics Approval

The study was approved by the Korea University Institutional Review Board (IRB, 1040548-KU-IRB-17-251-A-2, and KUIRB-2019-0136-01).

ACCEPTED

References

Azad, M.B., Konya, T., Maughan, H., Guttman, D.S., Field, C.J., Chari, R.S., Sears, M.R., Becker, A.B., Scott, J.A. and Kozyrskyj, A.L. (2013) Gut microbiota of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months. *CMAJ* **185**, 385-394.

Bakker-Zierikzee, A.M., Van, Tol, E.A.F., Kroes, H., Alles, M.S., Kok, F.J. and Bindels, J.G. (2006) Faecal SIgA secretion in infants fed on pre-or probiotic infant formula. *Pediatr Allergy Immunol* **17**, 134-140.

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., Li, Y., Xia, Y., Xie, H., Zhong, H., Muhammad, T.K., Jianfeng, Z., Junhua, L., Liang, X., Jumana, A.A., Dongya, Z., Ying, S.L., Dorota, K., Camilla C., Valentina, T., Ye, Y., Stefan, B., Xun, X., Lise, M., Karsten, K., Jovanna, D. and Jun, W. (2015) Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* **17**, 690-703. Belkaid, Y. and Hand, T.W. (2014) Role of the microbiota in immunity and inflammation. *Cell* **157**, 121-141.

Bervoets, L., Van, Hoorenbeeck, K., Kortleven, I., Van, Noten, C., Hens, N., Vael, C., Goossens, H., Desager, K.N. and Vankerckhoven, V. (2013) Differences in gut microbiota composition between obese and lean children: a cross-sectional study. *Gut Pathog* **5**, 1-10.

Borewicz, K., Suarez-Diez, M., Hechler, C., Beijers, R., de Weerth, C., Arts, I., John, P., Carel, T., Arjen, N., Cordula, L., Ellen, V.L., Elaine, E.V. and Hauke, S. (2019) The effect of prebiotic fortified infant formulas on microbiota composition and dynamics in early life. *Sci Rep* **9**, 1-13.

Boué, G., Cummins, E., Guillou, S., Antignac, J.P., Le, Bizec, B. and Membré, J.M. (2018) Public health risks and benefits associated with breast milk and infant formula consumption. Crit. *Rev. Food Sci Nutr* **58**, 126-145.

ACCEPTED

Bragg, M., Freeman, E.W., Lim, H.C., Songsasen, N. and Muletz-Wolz, C.R. (2020) Gut Microbiomes Differ Among Dietary Types and Stool Consistency in the Captive Red Wolf (Canis rufus). *Front Microbiol* **11**, 27-77.

Brown, K.H., de Romaña, D.L., Arsenault, J.E., Peerson, J.M. and Penny, M.E. (2007) Comparison of the effects of zinc delivered in a fortified food or a liquid supplement on the growth, morbidity, and plasma zinc concentrations of young Peruvian children. *Am J Clin Nutr* **85**, 538-547.

Boyd, C.A., Quigley, M.A. and Brocklehurst, P. (2007) Donor breast milk versus infant formula for preterm infants: systematic review and meta-analysis. *Arch Dis Child* **92**, F169-F175.

Chakraborti, C.K. (2015) New-found link between microbiota and obesity. *World J Gastrointest Pathophysiol* **6**, 110.

Derrien, M., Alvarez, A.S. and de Vos, W.M. (2019) The gut microbiota in the first decade of life. *Trends Microbiol* **27**, 997-1010.

Derrien, M., Vaughan, E.E., Plugge, C.M. and de Vos, W.M. (2004) Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *Int J Syst Evol Microbiol* **54**, 1469-1476.

FDA (2019) Preparation of Food Contact Notifications for Food Contact Substances in Contact with Infant Formula and/or Human Milk: Guidance for Industry.

Gomes, A.C., Hoffmann, C. and Mota, J.F. (2018) The human gut microbiota: Metabolism and perspective in obesity. *Gut Microbes* **9**, 308-325.

Gupta, V., Rebekah, G., Sudhakar, Y., Santhanam, S., Kumar, M. and Thomas, N. (2020) A randomized controlled trial comparing the effect of fortification of human milk with an infant formula powder versus unfortified human milk on the growth of preterm very low birth weight infants. *J Matern-Fetal Neonatal Med* **33**, 2507-2515.

ACCEPTED

Henrick, B.M., Hutton, A.A., Palumbo, M.C., Casaburi, G., Mitchell, R.D., Underwood, M.A., Smilowitz, J.T. and Frese, S.A. (2018) Elevated fecal pH indicates a profound change in the breastfed infant gut microbiome due to reduction of Bifidobacterium over the past century. *MSphere* **3**.

Horta, B.L. and Victora, C.G. (2013) World Health Organization. Short-term effects of breastfeeding: a systematic review on the benefits of breastfeeding on diarrhoea and pneumonia mortality.

Jost, T., Lacroix, C., Braegger, C. and Chassard, C. (2015) Impact of human milk bacteria and oligosaccharides on neonatal gut microbiota establishment and gut health. *Nutrition reviews* **73**, 426-437.

Kalhan, S. C., & Kilic, I. (1999). Carbohydrate as nutrient in the infant and child: range of acceptable intake. *Eur J Clin Nutr*, **53**, 94-100.

Kwon, H.J., Lim, J.H., Kang, D., Lim, S., Park, S.J. and Kim, J.H. (2019) Is stool frequency associated with the richness and community composition of gut microbiota?. *Intest Res* **17**, 419.

Louis, P. and Flint, H.J. (2009) Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS microbiology letters* **294**, 1-8.

Louis, P. and Flint, H.J. (2017) Formation of propionate and butyrate by the human colonic microbiota. *Environ Microbiol* **19**, 29-41.

Makino, H. (2018) Bifidobacterial strains in the intestines of newborns originate from their mothers. *Biosci Microbiota Food Health*. **37**, 79-85.

Martin, C.R., Ling, P.R. and Blackburn, G.L. (2016) Review of infant feeding: key features of breast milk and infant formula. *Nutrients* **8**, 279.

Mohan, R., Koebnick, C., Schildt, J., Schmidt, S., Mueller, M., Possner, M., Radke, M. and Blaut, M. (2006) Effects of Bifidobacterium lactis Bb12 supplementation on intestinal

ACCEPTED

503 microbiota of preterm infants: a double-blind, placebo-controlled, randomized study. *J Clin*
504 *Microbiol* **44**, 4025-4031.
505 Moore, R.E., Xu, L.L. and Townsend, S.D. (2021) Prospecting Human Milk Oligosaccharides
506 as a Defense Against Viral Infections. *ACS Infect Dis* **7**, 254-263.
507 Mugambi, M.N., Musekiwa, A., Lombard, M., Young, T. and Blaauw, R. (2012) Synbiotics,
508 probiotics or prebiotics in infant formula for full term infants: a systematic review. *Nutr J* **11**,
509 81.
510 Ohira, H., Tsutsui, W. and Fujioka, Y. (2017) Are short chain fatty acids in gut microbiota
511 defensive players for inflammation and atherosclerosis?. *J Atheroscler Thromb* **24**, 660-672.
512 Pärtty, A., Luoto, R., Kalliomäki, M., Salminen, S. and Isolauri, E. (2013) Effects of early
513 prebiotic and probiotic supplementation on development of gut microbiota and fussing and
514 crying in preterm infants: a randomized, double-blind, placebo-controlled trial. *J Pediatr* **163**,
515 1272-1277.
516 Radke, M., Picaud, J.C., Loui, A., Cambonie, G., Faas, D., Lafeber, H.N., de Groot, N., Pecquet,
517 S.S., Steenhout, P.G. and Hascoet, J.M. (2017) Starter formula enriched in prebiotics and
518 probiotics ensures normal growth of infants and promotes gut health: a randomized clinical
519 trial. *Pediatric Res* **81**, 622-631.
520 Rijkers, G.T., De Vos, W.M., Brummer, R.J., Morelli, L., Corthier, G. and Marteau, P. (2011)
521 Health benefits and health claims of probiotics: bridging science and marketing. *Br J Nutr* **106**,
522 1291-1296.
523 Rivière, A., Selak, M., Lantin, D., Leroy, F. and De Vuyst, L. (2016) Bifidobacteria and
524 butyrate-producing colon bacteria: importance and strategies for their stimulation in the human
525 gut. *Front Microbiol* **7**, 979.

ACCEPTED

Sassone-Corsi, M., Nuccio, S.P., Liu, H., Hernandez, D., Vu, C.T., Takahashi, A.A. and Raffatellu, M. (2016) Microcins mediate competition among Enterobacteriaceae in the inflamed gut. *Nature* **540**, 280-283.

Stewart, C.J., Ajami, N.J., O'Brien, J.L., Hutchinson, D.S., Smith, D.P., Wong, M.C., Ross, M.C., Lloyd, R.E., Doddapaneni, H., Metcalf, G.A., Donna Muzny, Richard, A.G.,Tommi, V., Curtis, H., Ramnik, J.X., Marian, R., William, H., Jorma, T., Anette-G, Z., Jin-Xiong, S.,Beena, A., Ake, L., Heikki, H., Kendra, V., Jeffrey, P.K. and Joseph, F.P. (2018) Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature* **562**, 583-588.

Tannock, G.W., Lawley, B., Munro, K., Pathmanathan, S.G., Zhou, S.J., Makrides, M., Gibson, R.A., Sullivan, T., Prosser, C.G., Lowry, D. and Alison, J.H. (2013) Comparison of the compositions of the stool microbiotas of infants fed goat milk formula, cow milk-based formula, or breast milk. *Appl Environ Microbiol* **79**, 3040-3048.

Toscano, M., De Grandi, R., Grossi, E. and Drago, L. (2017) Role of the human breast milk-associated microbiota on the newborns' immune system: A mini review. *Front Microbiol* **8**, 2100.

Touchefeu, Y., Montassier, E., Nieman, K., Gastinne, T., Potel, G., Bruley, des Varannes, S., Le Vacon, F. and de La Cochetière, M.F. (2014) Systematic review: the role of the gut microbiota in chemotherapy-or radiation-induced gastrointestinal mucositis–current evidence and potential clinical applications. *Aliment Pharmacol Ther* **40**, 409-421.

Vandenplas, Y., Carnielli, V.P., Ksiazyk, J., Luna, M.S., Migacheva, N., Mosselmans, J.M., Picaud, J.C., Possner, M., Singhal, A. and Wabitsch, M. (2020) Factors affecting early-life intestinal microbiota development. *Nutrition* **78**, 110812.

Vandenplas, Y., Zakharova, I. and Dmitrieva, Y. (2015) Oligosaccharides in infant formula: more evidence to validate the role of prebiotics. *Br J Nutr* **113**, 1339-1344.

551 Vandeputte, D., Falony, G., Vieira-Silva, S., Tito, R. Y., Joossens, M., & Raes, J. (2016). Stool
552 consistency is strongly associated with gut microbiota richness and composition, enterotypes
553 and bacterial growth rates. *Gut*, **65**, 57-62.
554 Vendt, N., Grünberg, H., Tuure, T., Malminiemi, O., Wuolijoki, E., Tillmann, V., Sepp, E. and
555 Korpela, R. (2006) Growth during the first 6 months of life in infants using formula enriched
556 with *Lactobacillus rhamnosus* GG: double-blind, randomized trial. *J Hum Nutr Diet* **19**, 51-58.
557 Vital, M., Howe, A.C., and Tiedje, J.M. (2014) Revealing the bacterial butyrate synthesis
558 pathways by analyzing (meta) genomic data. *MBio* **5**.
559 Wargo and Wayne, F. (2016) The history of infant formula: quality, safety, and standard
560 methods. *J AOAC Int* **99**, 7-11.

ACCEPTED

Figure Legends

Figure. 1. Effect of PFIF on **(A)** body weight and **(B)** height of each infant group throughout the experimental period. Results are expressed as mean ± SE (*n* = Breast milk (BF): 50; Probiotic formula (PF): 35; Placebo formula (FF): 33). Lowercase superscript letters ($^{a, b}$) denotes significantly different mean values in the same series ($P < 0.05$)

Figure. 2. Effect of PFIF on stool **(A)** pH, **(B)** consistency**, (C)** defecation frequency, and **(D)** sIgA concentration of each infant group throughout the experimental period. Results are expressed as mean ± SE (*n* = Breast milk (BF): 50; Probiotic formula (PF): 35; Placebo formula (FF): 33). Lowercase superscript letters ($^{a, b}$) denotes significantly different mean values in the same series ($P < 0.05$)

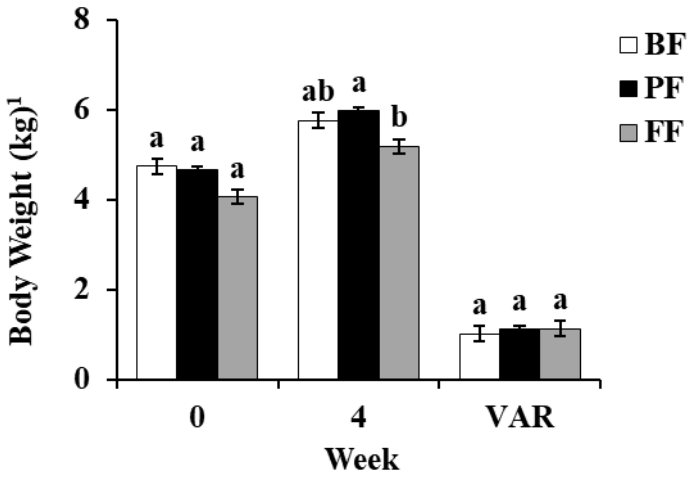
Figure. 3. Effect of PFIF on stool SCFAs; **(A)** acetate, **(B)** propionate**, (C)** iso-butyrate, and **(D)** butyrate concentration of each infant group throughout the experimental period. Results are expressed as mean ± SE (*n* = Breast milk (BF): 50; Probiotic formula (PF): 35; Placebo formula (FF): 33). Lowercase superscript letters ($^{a, b}$) denotes significantly different mean values in the same series ($P < 0.05$)

Figure. 4. Alpha diversity index changes after 4 weeks of each treatment; **(A)** observed OTUs, **(B)** Chao1**, (C)** Shannon, and **(D)** Simpson. Results are expressed as mean ± SE (*n* = Breast milk (BF): 50; Probiotic formula (PF): 35; Placebo formula (FF): 33). Lowercase superscript letters ($^{a, b}$) denotes significantly different mean values in the same series ($P < 0.05$)

Figure. 5. Effect of each treatment for 4 weeks on fecal microbiota compositional changes in the level of **(A)** phylum, and **(B)** genus. Results are relative abundance ratio of each group determined using OTU at the 95% identity level and the data on low-quality samples with <10,000 observed OTUs were removed from the sample set.

ACCEPTED

A.



B.

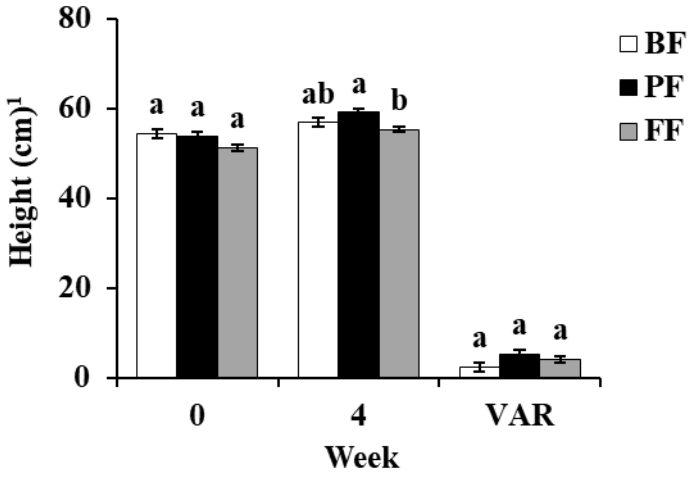
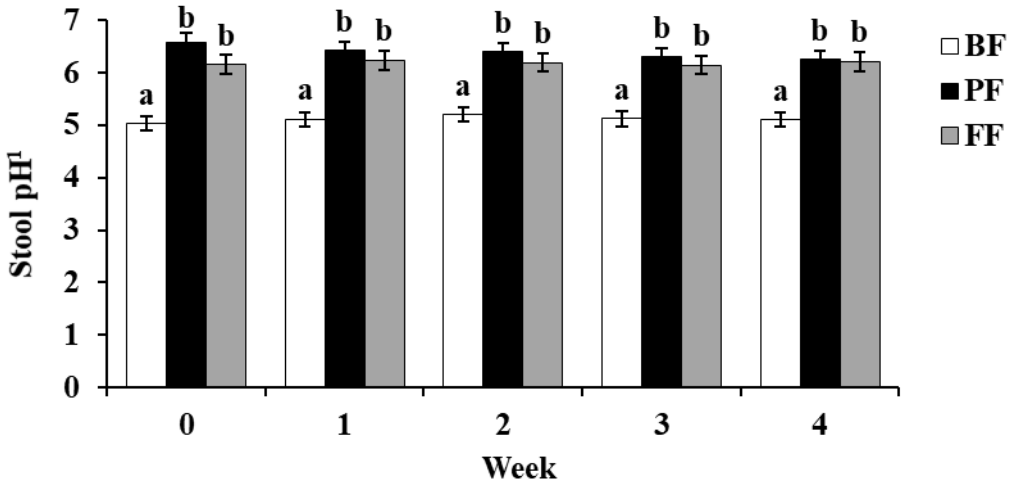


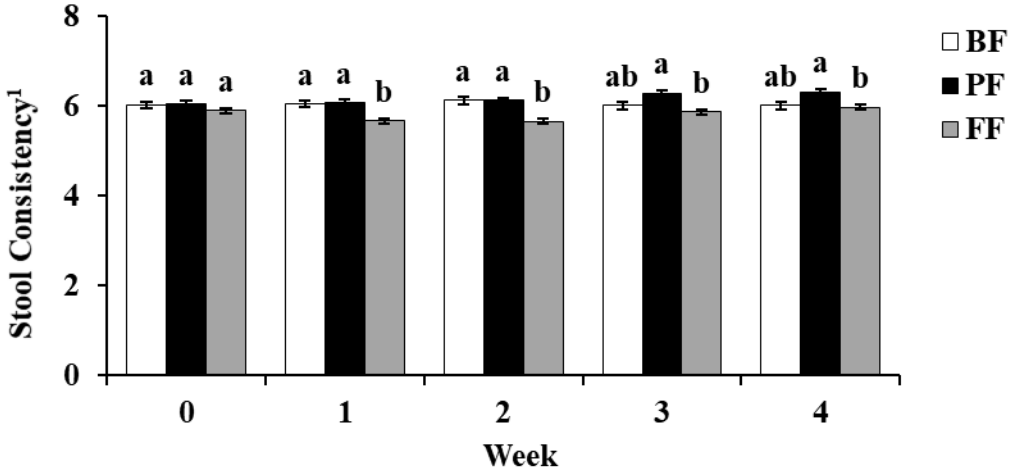
Figure 1.

ACCEPTED

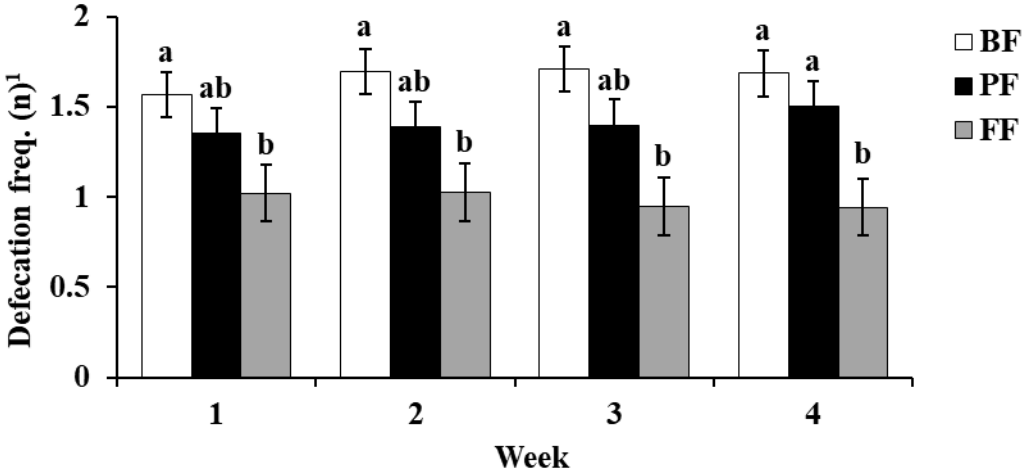
A.


Stool pH¹
7
6
5
4
3
2
1
0
a b b
a b b
a b b
a b b
a b b
0
1
2
3
4
Week
BF
PF
FF

B.


Stool Consistency¹
8
6
4
2
0
a a a
a a b
a a b
ab a b
ab a b
0
1
2
3
4
Week
BF
PF
FF

C.


Defecation freq. (n)[1]
2
1.5
1
0.5
0
a
ab
b
a
ab
b
a
ab
b
a
a
b
1
2
3
4
Week
BF
PF
FF

D.

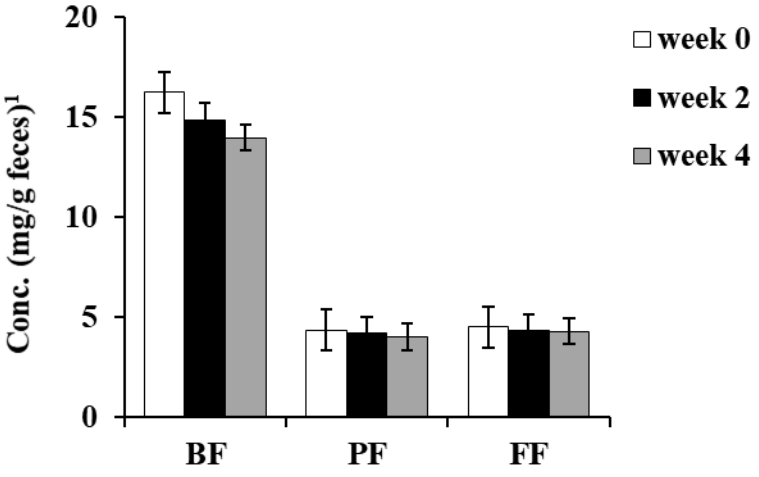
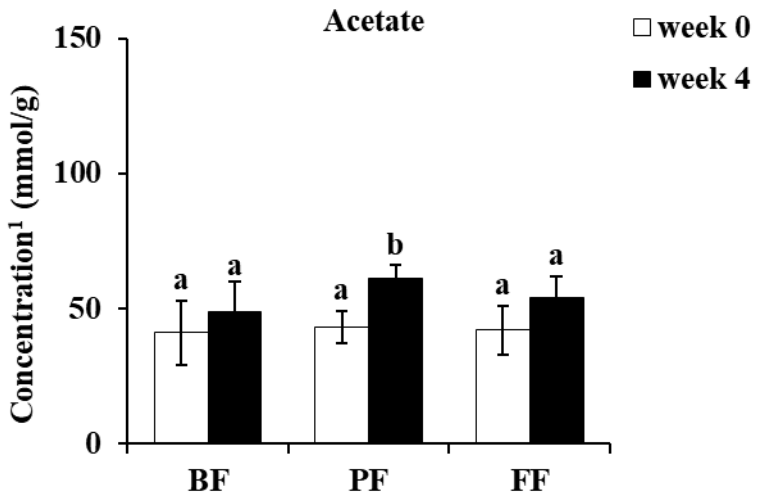
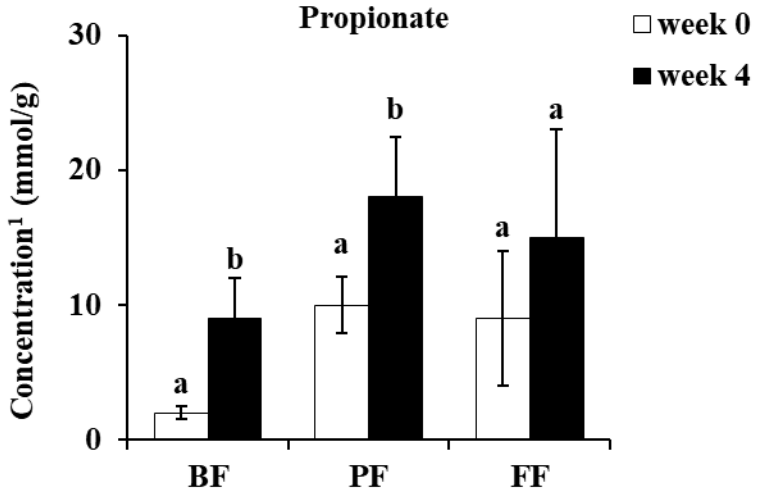

Conc. (mg/g feces)[1]
20
15
10
5
0
BF
PF
FF
week 0
week 2
week 4

Figure 2.

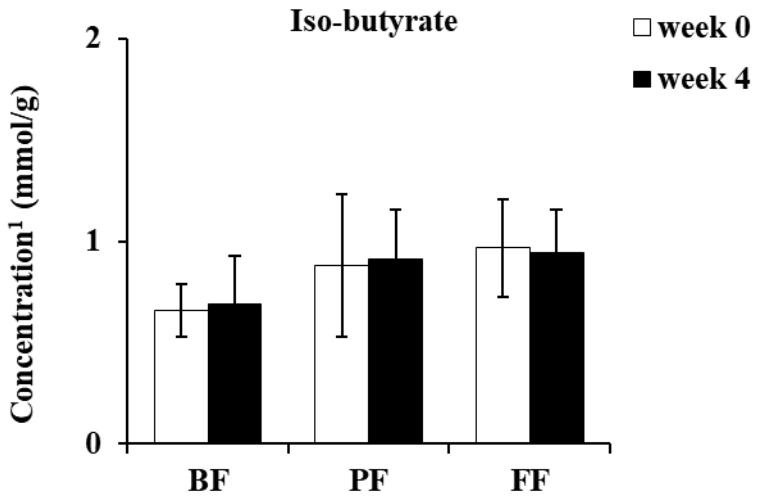
A.


Acetate
week 0
week 4
Concentration[1] (mmol/g)
150
100
50
0
a
a
a
b
a
a
BF
PF
FF

B.


Propionate
week 0
week 4
Concentration[1] (mmol/g)
30
20
10
0
a
b
a
b
a
a
BF
PF
FF

C.



D.

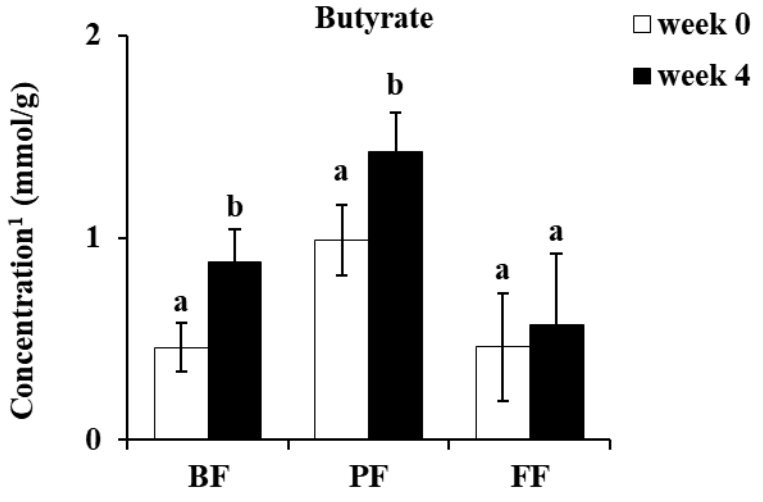
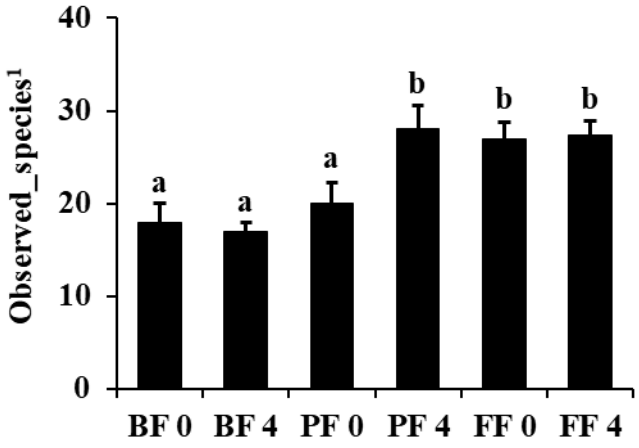


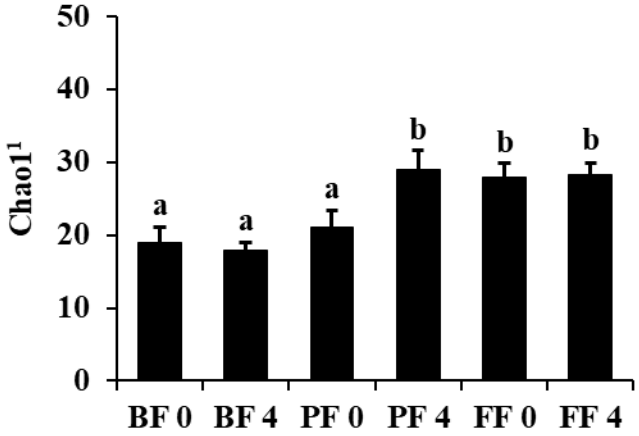
Figure 3.

ACCEPTED

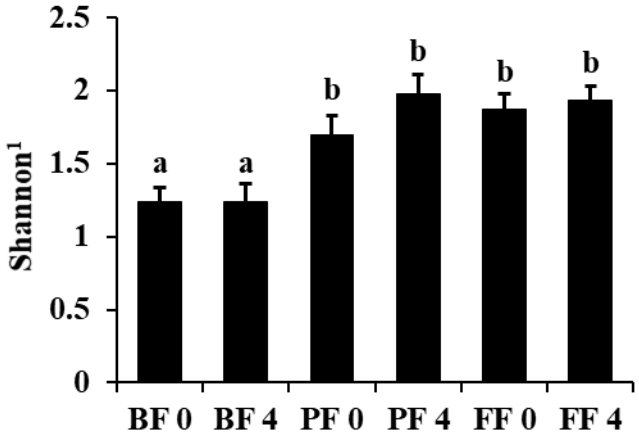
A.



B.



C.



ACCEPTED

D.

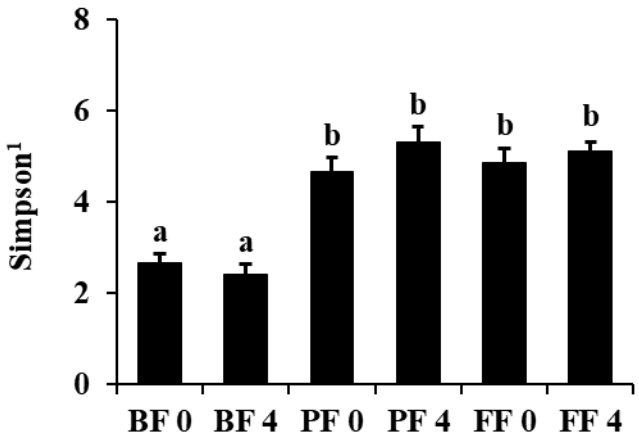


Figure 4.

ACCEPTED

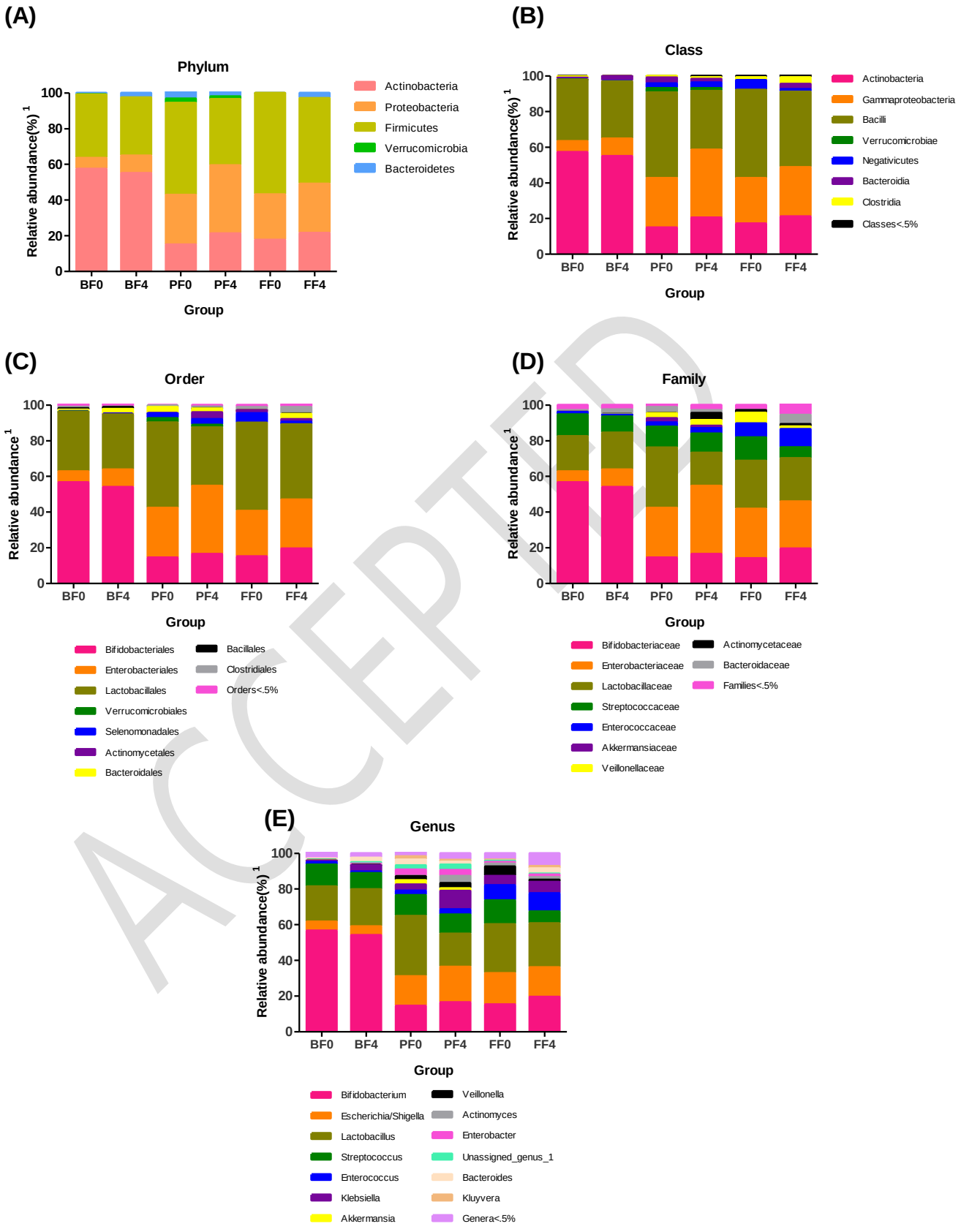


Figure 5.

ACCEPTED