MASTER'S THESIS

Evaluation of food effects on fecal microbiota *in vitro*

Nakwon Hwang

Department of Biotechnology

GRADUATE SCHOOL

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(Supervised by professor Tatsuya Unno)

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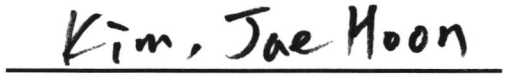

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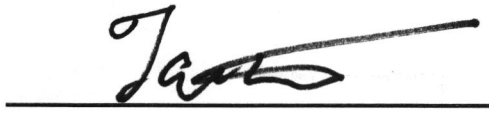

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ABSTRACT

Various microorganisms have been inhabited in the human gastrointestinal tract, bacteria live in the colon are mostly related with both diet and disease. The studies have been continuously conducted to correlate functional food or materials with gut microbiota to cure and prevent the unhealthy state by performing an animal experiment or clinical trials. These studies are often laborious, expensive and may involve ethical issues. Meanwhile, several studies have developed the system to simulate digestion and fermentation *in vitro* using functional substance.

In this study, the digestion and fecal bacterial fermentation using cheonggukjang (CGJ) were performed and the shift of microbiota was investigated to compare food effects both *in vitro* and *in vivo* based on 16S rRNA gene of bacteria. We measured main SCFAs such as acetate, propionate, and butyrate as food effects, while yeast and peptone were removed from basal nutrient media of fermentation because of the usage as an energy source for bacteria. The fermentation time was kept as 2 hours to observe the fermentation effect by CGJ. Different shifts in microbiota in response to CGJ were observed *in vitro* and *in vivo* assays, however, similar alteration of microbiota was shown *in vitro* in some donors. At phylum level, Bacteroidetes were significantly decreased whereas Actinobacteria increased in both tests ($P<0.05$). Several bacteria shown a common increment or decrement pattern at OTU level, while different patterns were observed *in vitro* and *in vivo*. In conclusion, there may involve limitations of *in vivo* reproduction but it worked well to evaluate food effects on microbiota. For further studies, it is suggested to use variety of foods and diverse fecal samples to investigate microbiota shifts.

INTRODUCTION

There are approximately 10^{11} bacterial cells in the human gastrointestinal tract (Sender et al. 2016), the proportion of which depends on dietary habits. Davie et al. reported that animal-based diet generally increased the abundance of *Bacteroides, Bilophila*, and *Alistipes*, while plant-based diet increases the abundance of *Roseburia*, *Ruminococcus bromii*, and *Eubacterium rectale*, the balance of which can be altered within a single day after diet is switched between plant- and animal-based diet. On the other hand, Klimenko et al. had reported that the human gut microbiota was also associated with long-term dietary habits. For example, *Prevotella* and *Bacteroides* were associated with long-term consumption of carbohydrate diet and protein-animal fat diet, respectively (Wu et al. 2011).

Indigestible dietary fibers are fermented in the colon by bacteria (Deehan et al. 2017; Makki et al. 2018), by which those bacteria produce short-chain fatty acids (SCFAs) that are absorbable form in epithelial cells. Three main SCFAs are acetate, propionate, and butyrate, representing 90-95% in the colon (Ríos-Covián et al. 2016). While large portion of colonic butyrate are produced by Firmicutes, the rest of colonic SCFAs are produced by Bacteroidetes (Levy et al. 2016). It has been reported that SCFAs regulated appetite and maintained energy homeostasis of the host (Byrne et al. 2015) as well as immune homeostasis in the intestine (Honda and Littman 2012). In addition, a number of studies have reported the association of gut microbiota with diseases such as atopy (Lee et al. 2018), obesity, diabetes (Baothman et al. 2016), colon cancer (Dahmus et al. 2018), and Irritable Bowel Disease (IBD) (Becker et al. 2015)

Meanwhile, to prevent or treat those unhealthy states, many studies have been consistently investigated that the positive effect of functional food or materials on the gut bacterial community. Functional foods are the products that maintain both health and gut microorganism balance by simply ingesting, thus preferred and produced steadily. Materials as functional foods need to be approved by the Food and Drug Administration (FDA) based on scientific evidence from animal experiments or clinical trials, which are often laborious and costly and sometimes

prohibited due to ethical issues. In addition, effects of functional food often vary depending on the subjects used in trials, which could be due to differences in individual gut microbiota (Dąbrowska and Witkiewicz 2016).

Some other studies developed *in vitro* digestion and fermentation systems to investigate functional materials like batch model (Pompei et al. 2008), continuous model (Duncan et al. 2009), immobilized continuous model (Zihler et al. 2010), multistage continuous model (Maccaferri et al. 2010), and digestion-fermentation-absorption model (Blanquet-Diot et al. 2009). They performed quantitative analysis for certain bacteria during fermentation through specific fluorescent *in situ* hybridization and real-time PCR.

In this study, to reduce the ethical issues and confirm the correlation between functional food and gut microbiota, we simulated *in vitro* gastrointestinal digestion (GID), performed fecal fermentation using human fecal bacteria (FF), and then compared food effect on the microbiota between *in vivo* and *in vitro* assays, based on the V4 region of 16S rRNA gene.

MATERIAL AND METHODS

Sample preparation

Galacto-oligosaccharides powder (GOS) was obtained from CREMAR Inc. (Seoul, South Korea), the product of Cheonggukjang pill (CGJ-P) was purchased in the open market, which is consisted of 100 percent CGJ powder. The developed CGJ products (CGJ-D) were obtained from Microbial Institute for Fermentation industry (Sunchang, South Korea), which was fermented by *Bacillus amyloliquefaciens* isolated from gochujang. To simulating gastrointestinal digestion, GOS and CGJ-P were prepared as original state and water extract of CGJ-D was obtained as performing that it was contained in 100 ㎖ distilled water, boiled for three hours, filtered as cotton cloth and lyophilized.

In vitro gastrointestinal Digestion (GID)

Overall process of GID-FF described in Figure 1. The process of *in vitro* GID was carried out as described previous study (Minekus et al. 2014). α-Amylase from porcine pancreas, pepsin from porcine gastric mucosa powder, and pancreatin from porcine pancreas were purchased from Sigma-Aldrich (St. Louis, MO, USA). The usage for GOS in the first step of GID was 0.1 g (G1) and 0.5 g (G5) (0.1 and 0.5 % W/V) in 100 ㎖ of fermentation process, and CGJ-D and CGJ-P was used 0.1 % (W/V). Digested samples were freeze-dried and then store at -20 ℃.

Fecal sample collection

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional of Jeju National University in Korea (IRB approval number JJNU-IRB-2018-007-002 and JJNU-IRB-2018-040-001) and with the 1964 Helsinki

declaration and its later amendments or comparable ethical standards. Fecal samples were provided from three different healthy male adult volunteers (S1, S2, and S3; Subject) who had not digested antibiotics for at least 1 months before study. To determine the component of media of fecal fermentation and the fermentation time, Feces was collected from only S1. Another fecal sampling was executed from each four subjects before ingesting CGJ-P to perform fecal fermentation. Subsequently, to compare the CGJ effects between *in vitro* (IVT) and *in vivo* (IVV), four subjects ingested 4 g of CGJ-P at every 9 AM and 9 PM for five days, and the fecal samples were collected.

Fecal fermentation using human feces (FF)

For the fecal fermentation processing, the unsealed anaerobic tube containing sterile medium was prepared in triplicate under the same condition the previous day, it excluded peptone and yeast from the previous study (Tzounis et al. 2008), however, these components were added in only energy group (E). The fresh feces of human were collected and mixed with phosphate buffered saline (PBS) (10% W/V) and then sieved as 250 ㎛, 150 ㎛, and 25 ㎛. The extracted fecal bacteria were immediately transferred into an anaerobic chamber (Bactron II, SHEL LAB, USA) and inoculated in the prepared medium (10% V/V), and then a freeze-dried digested powder was also added, but blank group (BLK, B) included only media containing fecal bacteria. The inoculated mixtures were incubated at 37 ℃, 100 rpm for 24 hours to decide the composition of fecal fermentation media and the fermentation time. Fermented samples were collected in triplicate at 0, 2, 4, 6, 8, 12, 18 and 24 hours, immediately froze using liquid nitrogen gas and then stored at - 80℃ until analysis. To compare the shift of *in vivo* and *in vitro* microbiota by CGJ, the fermentation was processed for 2 hours and fermented samples collected in triplicate at 0 and 2 hours.

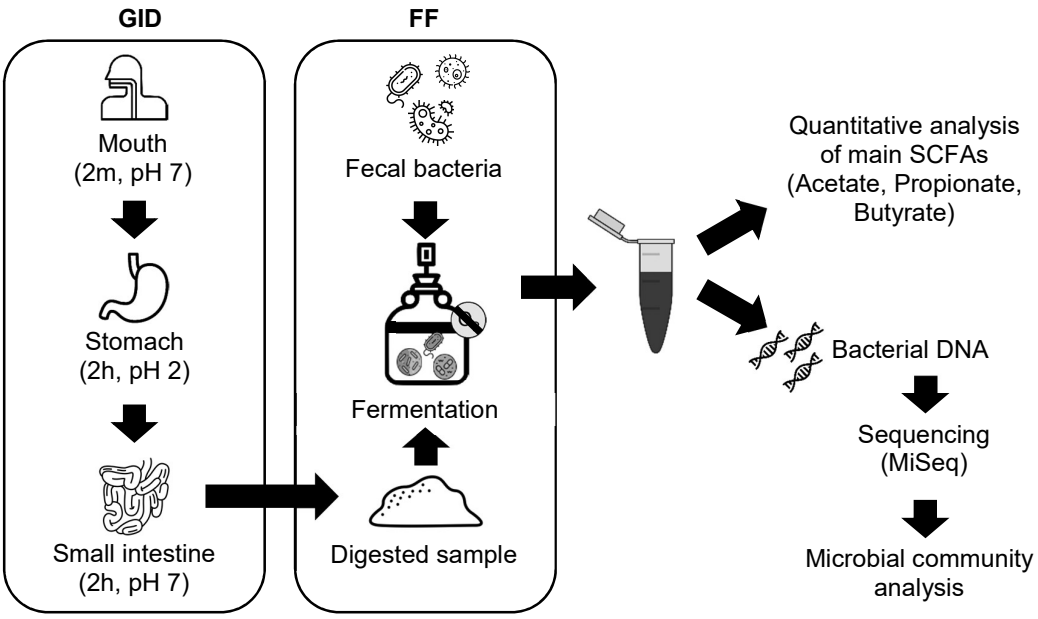


Figure 1. Schema of Gastrointestinal Digestion (GID) and Fecal Fermentation using human feces (FF)

Quantitative analysis of SCFAs

The frozen fermented samples were thaw in ice. 200 ㎕ of fermented sample was added 1㎖ methanol (1:5, V/V) and homogenized 2 min using vortex. The pH was adjusted at 2, incubated for 10 min, and then centrifuge 10,000 rpm at 4 ℃ for 1 min. The supernatant was collected by 1 ㎖ syringe, filtered 0.45 ㎛ of pore size into a new sterile tube, and then frozen and stored in liquid nitrogen gas until analysis. The quantitative analysis of tree main SCFAs was performed by Gas Chromatography mass spectrometry (GC- MS) (QP2010, Shimadzu, Japan) on splitless mode using DB-FFAP column (30m*0.25um*0.25um, Agilent, USA). The temperature injection, ion source, and interface were 230, 230, and 250 ℃, respectively. Sample (1 ㎕) was injected into a column maintained at 60 ℃. After 0.5 min the oven temperature gradually increased up to 100 ℃ at a rate of 40 ℃ min^{-1} and held 0.5 min, and then again raised up to 200 ℃ at a rate of 50 ℃ min^{-1} and finally maintained 1 min.

DNA extraction and 16S rRNA gene sequencing

For bacterial community analysis, DNA was extracted from fermented samples. All of frozen sample was thaw in ice and 1 ㎖ of sample was centrifuged in 10,000 rpm for 10 min at 4 ℃. The supernatant was discarded, the bacterial DNA was extracted from pellet using QIAamp PowerFecal DNA Kit (QIAamp, USA). DNA was concentrated to 5 ng/㎕ and amplicon PCR targeted V4 region of 16S rRNA gene was performed with forward primer (1uM, 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3') and riverse primer (1uM, 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3'), and then a library for Illumina MiSeq was constructed with two-step PCR following Metagenomic Sequencing Library Preparation supported from Illumina Inc.. PCR products for MiSeq were send to Macrogen Inc. (Seoul, South Korea).

Bioinformatical and Statistical analysis

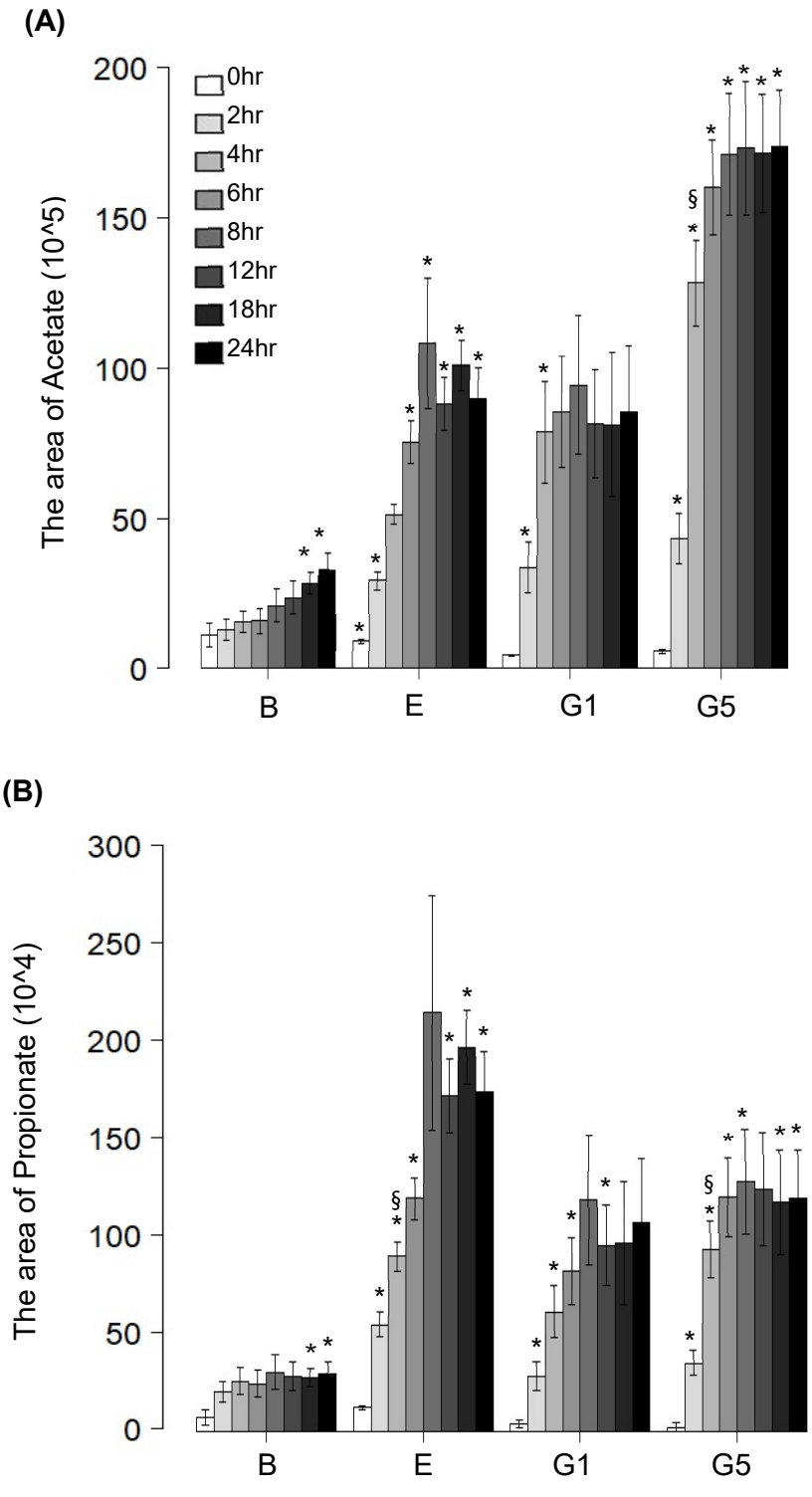
Sequencing data output was analyzed using MOTHUR (Schloss et al. 2009). To compare the shift of microbial communities, non-metric multidimensional scaling (NMDS) (Kruskal 1964) was performed based on Bray-Curtis and the direction of arrows meant which more relevant groups are. Linear discriminant analysis effect size (LEfSe) (Segata et al. 2011) was operated to compare the differential abundance between two treatments. Significant increment and decrement of SCFAs during fermentation was calculated as Student's two-tailed T-test and One-way ANOVA.

RESULTS AND DISCUSSION

The determination of composition of FF media

To decide the components of media for fecal fermentation in this study, we performed *in vitro* FF with four groups (B, E, G1, and G5) and fecal sample collected from S1. Blank group (B) was not contained any material as energy source, peptone and yeast extract were added in basal fermentation media for energy group (E). Digested powder of each 0.1 g and 0.5 g of GOS were used to operate fermentation for G1 and G5 group, respectively. Fermented samples were collected in triplicate from each group at 0, 2, 4, 8, 12, 18, and 24 hours during fecal fermentation, and we extracted three main SCFAs (acetate, propionate, and butyrate) from samples to compare the produced amount resulted from each additional substance (Figure 2). All of SCFAs in B group was statistically increased after 18 hours. Acetate and propionate were significantly increased at 2 hours in E and two GOS groups. Butyrate was also significantly produced at 2 hours in E and G5 group, but after 4 hours in G1 group.

In this study, we observed that SCFAs could be produced when were peptone and yeast extract contained in fecal fermentation media. Previous studies have been performed *in vitro* fermentation including these energy source in the basal medium (Bang et al. 2018; Moon et al. 2016; Sanz et al. 2005; Sarbini et al. 2014; Yang et al. 2013). In general, yeast extract is used as a source of vitamins, has been recognized as a major source of B-complex vitamins in bacterial culture (Ferreira et al. 2010). Also, this material supplies not only vitamins but also proteins, carbohydrates, and some micronutrients as a substrate to microorganism. Peptone distribute nitrogen as a readily assimilable source, they are also used to some extent as carbon and mineral sources in microbial media**.** In accordance with these results, we removed two energy sources in FF media to confirm only food effects on the gut microbiota *in vitro*.



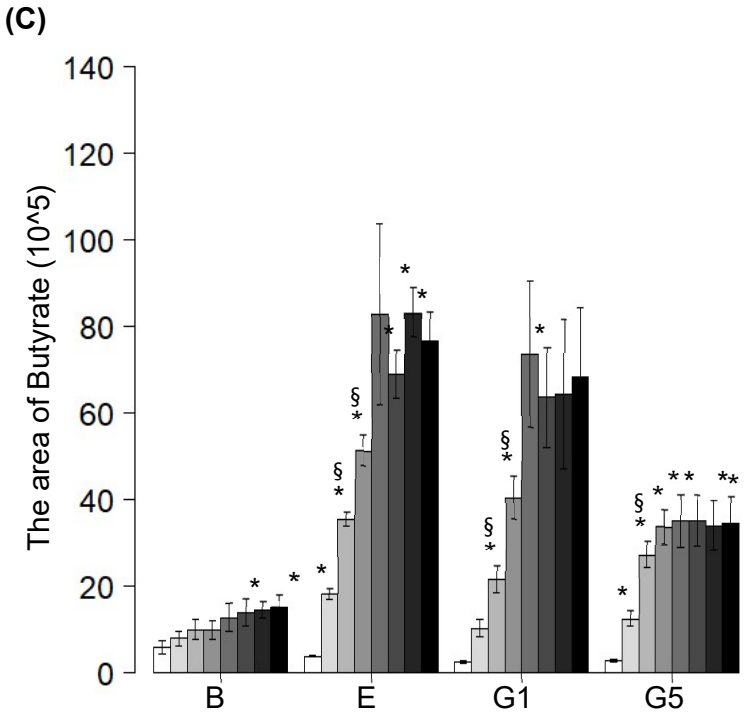


Figure 2. Changes in production amount of acetate (A), propionate (B), and butyrate (C) according to a different energy source during fecal fermentation using human feces.

B : not added any energy source. E : added peptone and yeast extract. G1 ,G5 : added 0.1 and 0.5 galacto-oligosaccharides, respectively. Samples were collected at 0, 2, 4, 6, 8, 12, 18, and 24 hours. *p<0.05 vs 0 hour of each group in two-tailed T-Test.; § p< 0.0.5 vs before sampling time of each group in two-tailed T-Test.

The determination of CGJ fermentation time

Fermentation was performed using digested CGJ-D, and a fecal sample collected from S1 was inoculated in FF. During fermentation, the products were harvested at 0, 2, 4, 6, 8, 12, and 24 hours and used for extracting bacterial DNA. PCR targeted V4 region in 16S rRNA gene was performed, and we obtained total 1,387,092 reads and 1,888 OTUs as the output of sequencing of 39 samples. We subsampled the data as 23,728 reads of minimum value, and then analyzed microbiota, and performed NMDS to confirm shifts of microbiota during fermentation (Figure 3). The microbial communities at 2 hours were tended to move to the right in B group, while to the left at the same time in CGJ-D groups. Interestingly, the microbiota of all groups after 4 hours was seemed to be traveling to a similar tendency like as from the up to the down.

As can be seen in our results, the bacterial communities were altered by only two hours fermentation *in vitro*, and if we took time more for fermenting, it could be showing bacterial incubation effects (Figure 4), not fermentation like as our expectation. Therefore, the fermentation time should be decided as two hours for CGJ. However, since this study was limited to CGJ effects, thus additional researches to determine fermentation time for other food are required.

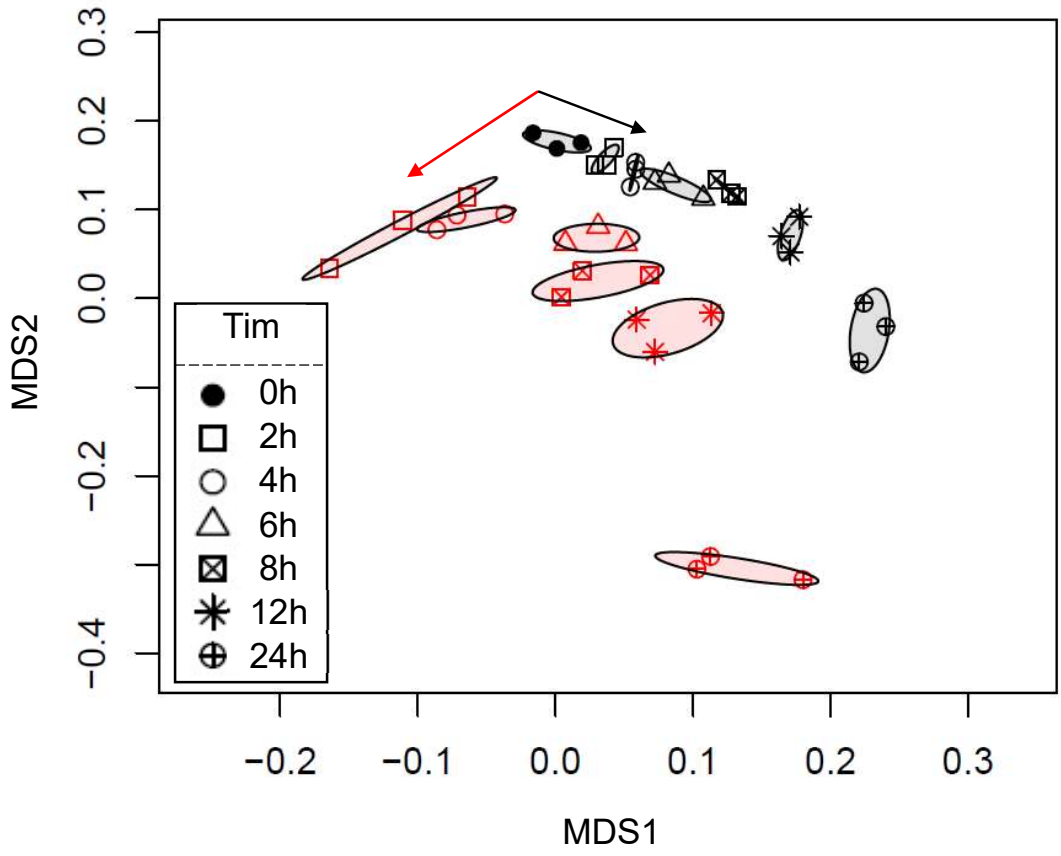
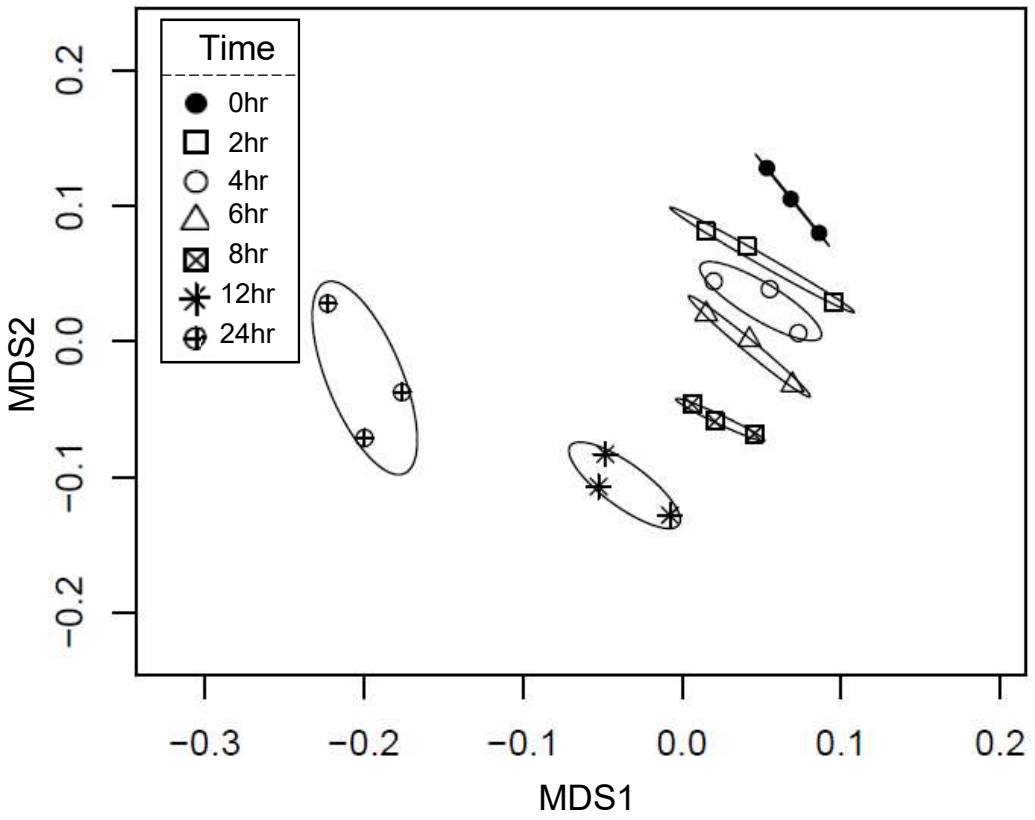


Figure 3. The shift of microbiota during *in vitro* fermentation for 24 hours using human feces and developed cheonggukjang (CGJ-D) on non-metric multidimensional scaling (NMDS).

Fecal Sample were obtained from only Subject 1. All group was tested in triplicate and samples for microbiota analysis were collected at 0, 2, 4, 6, 8, 12, and 24 hours during fermentation. NMDS was plotted based on Bray-Curtis. Black and red symbols indicated blank and CGJ-D group, respectively.

(A)



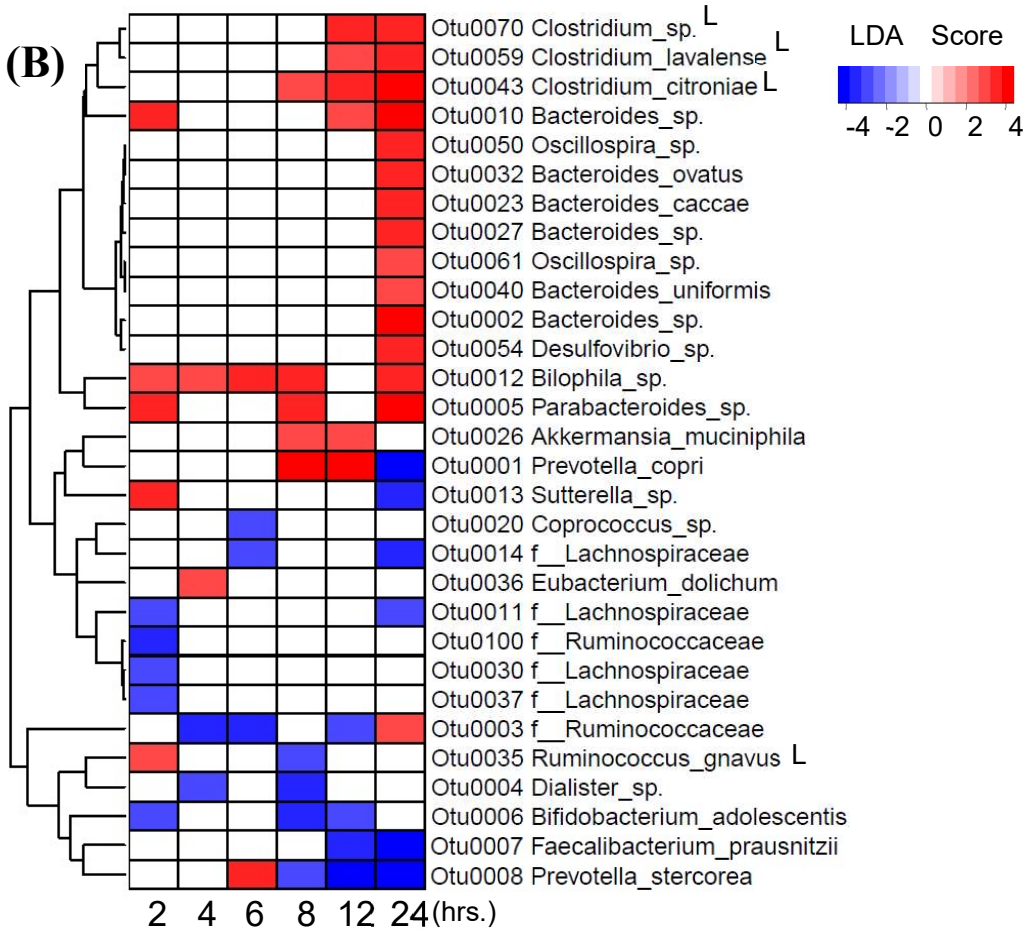


Figure 4. The shift of microbiota of BLK during *in vitro* fermentation for 24 hours.

Fecal Sample was obtained from only Subject 1 and it used for microbiota analysis were collected at 0, 2, 4, 6, 8, 12 and 24 hours in triplicate during fermentation. NMDS (A) was plotted based on Bray-Curtis. The relatively increased (red) or decreased (blue) OTUs (LDA > 3.0) during fermentation by comparing between two sampling time (0h vs 2h, 2h vs 4h, 4h vs 6h, 6h vs 8h, 8h vs 12h, and 12h vs 24h) was shown in (B). 'L' means the species classified from Lachnospiraceae family.

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The effect of CGJ-P on *in vitro* and *in vivo* microbiota

In this study, we analyzed whether altered microbiota during *in vitro* fermentation by food could be shown similar changes *in vivo*. Fecal bacteria were harvested from fresh feces of three subjects before ingesting CKJ-P (BLK group) and after ingesting for 5 days (IVV group). We also performed *in vitro* fermentation for 2 hours using BLK feces and collected fermented samples in triplicate (IVT group). After CGJ fermentation, three main SCFA were measured using GC-MS (Figure 4). All of them was significantly increased in IVT groups of subject 2 ($P<0.001$), while butyrate was not increased in S1 and propionate was not increased in S3. The dissimilar producing tendency of SCFAs may be indicated that the production pathway was different according to the individual gut microbiota.

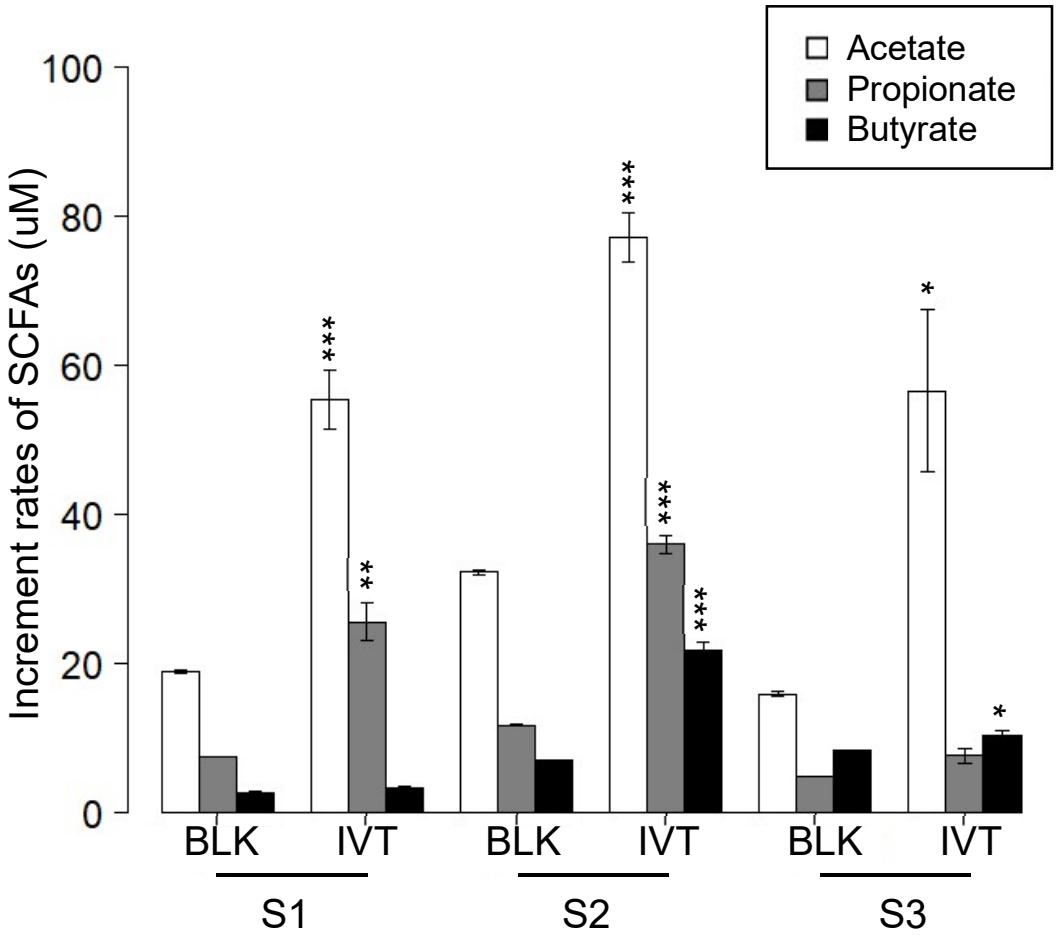


Figure 5. Increment of Three main short chain fatty acids during fecal fermentation using human feces from three volunteer (S1, S2, and S3) and cheonggukjang pill (CGJ-P).

BLK : sample from *in vitro* fecal fermentation without CGJ-P after 2 hours ; IVT : sample from *in vitro* fecal fermentation with CGJ-P after 2 hours. Mean values ± S.D. are shown. One-way ANOVA was used to compare BLK and IVT with the same subject (*p<0.05, **p<0.01, and ***p<0.001).

Subsequently, CGJ-P effects on microbiota *in vitro* and *in vivo* were analyzed. Total 36 samples were run sequencing and outputted 1,042,117 reads and 1,657 OTUs. The data was subsampled as 17,884 reads of minimum value. NMDS plot was shown that CGJ-P led to the shift of microbiota in IVT and IVV (Figure 5). IVT groups of both S2 and S3 were exhibited to the tendency toward diagonal-left down from BLK group (line), and S1 tend to similar direction (left) of them. These alterations were shown a similar trend according to the phylogenic tree (Figure 6). However, this is not shown in all of IVV groups (dotted line).

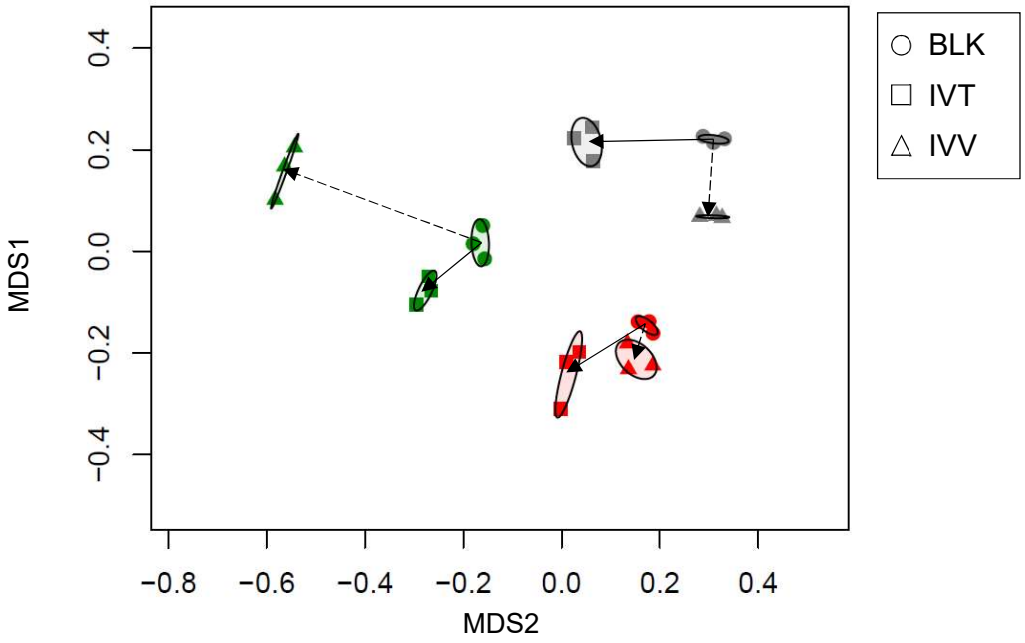


Figure 6. Non-metric multidimensional scaling (NMDS) for comparing the effect of cheonggukjang pill (CGJ-P) *in vitro* and *in vivo* method on the microbiota.

Fecal samples for *in vitro* fermentation were obtained from three volunteer's feces (S1, S2, and S3) before ingesting CGJ-P. In fecal fermentation, all samples were tested in triplicate and collected after 2 hours. All volunteers were ingested CGJ-P at every 9 AM and 9 PM for 5 days, and then another fecal sample were collected. NMDS was plotted based on Bray-Curtis. Arrows were indicated the direction of shift of microbiota from BLK to IVT (line) and IVV (dotted line). Each gray, red, and green spots indicated samples from S1, S2, and S3 respectively. BLK : sample before intake CGJ-P; IVT : sample after fecal fermentation; IVV : sample after intake CGJ-P for five days.

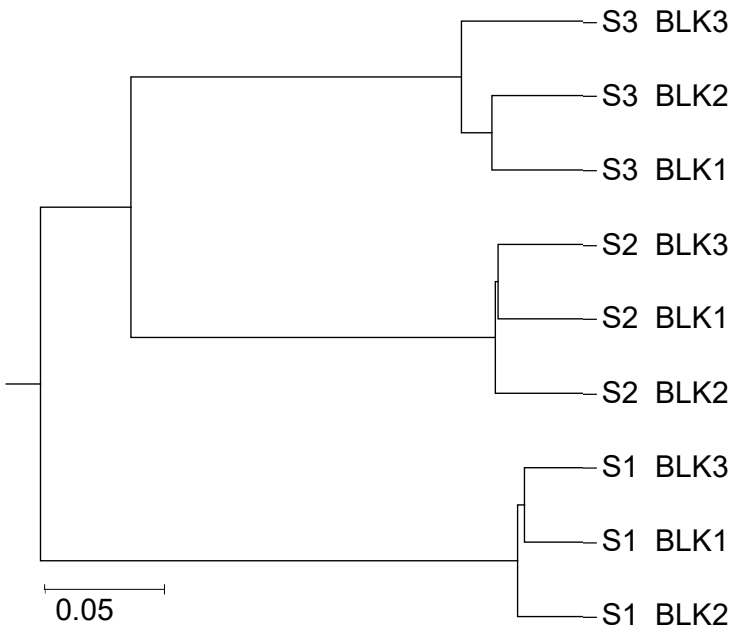


Figure 7. Phylogenetic tree of microbiota from individual feces before ingesting of cheonggukjang pill (CGJ-P).

Fecal samples were obtained from three volunteer's feces (S1, S2, and S3) before ingesting CGJ-P and then tested in triplicate.

The CGJ-P effect on microbiota in phylum level of phylotype was analyzed as comparing each IVT and IVV with BLK in each subject (Table 1). Bacteroidetes significantly decreased in all of IVT groups (LDA>4.3) and also decreased in IVV groups of S2 and S3 (LDA>4.4), while Actinobacteria was increased in all of IVT groups (LDA>4.4) and also increased in IVV groups of S2 and S3 (LDA>4.3). Proteobacteria and Tenericutes were decreased in IVT groups of S2 and S3 (LDA>3.0). Firmicutes was increased in IVT groups of S1 and S2.

According to LEfSe results comparing between BLK and IVT in OTU level, total 149 OTUs were identified as significantly difference ($p<0.05$). The OTUs on heatmap were selected among them as showing LDA > 3.0 (Figure 7). Two OTUs belong to Actinobacteria were detected that *Collinsella aerofaciens* was significantly increased in both S1 and S2, while *Bifidobacterium adolescentis* was increased in all of IVT group. *C. aerofaciens* has been shown to ferment a range of plant and animal origin carbohydrates (Kageyama et al. 1999), and known as the major utilizer of lactic acid (Truchado et al. 2017). *B. adolescentis* identified as inhibiting tumor growth by delivering anticancer genes to the tumor area and stopping angiogenesis (Arunachalam 1999; Li et al. 2003). The abundance of *Bifidobacterium spp.* was also increased in Natto consumption group, which food known as fermented by *Bacillus subtilis.* Natto like cheonggukjang (Fujisawa et al. 2006). In the phylum of Bacteroidetes, *Prevotella copri* were significantly decreased in all of IVT groups. *P. copri* produced succinate from carbohydrate fermentation (De Vadder et al. 2016). Unclassified *Bacteroides spp.* were decreased in all IVT groups, but B.ovatus and B.uniformis was decreased in each S2 and S3, respectively. *Bacteroides sp.* has been associated with production of propionate (Louis and Flint 2017) and *B.uniformis* has been linked to genistein degradation, which is a major isoflavone in soybean (Renouf and Hendrich 2011). *B.uniformis* was also shown increment in both S1 and S2 in this study, thus, they might be involved in degradation of CGJ *in vitro*. The abundance of *Alistipes putredinis* was decreased in only S3, *A.putredinis* has been found as propionate producer from succinate pathway and this bacteria were negatively correlated with host metabolic syndrome parameters (Ke et al. 2019). According to the reduction of both propionate producers (*Bacteroides* and *Alistipes spp.*) in this study, this

metabolite might be decreased in S3 *in vitro*.

In each comparison between BLK vs IVT and BLK vs IVV in each subject, 36 OTUs were selected in LEfSe results in OTU level, showing significantly difference ($P<0.05$), LDA > 3.0 and increment and decrement in both *in vitro* and *in vivo* (Figure 8). Likewise, in Actinobacteria phylum, *Colinsella* and *Bidibacterium spp.* were significantly increased in IVV group as consistent with the result of *in vitro*. The abundance of *A. putredinis* and unclassified *Bacteroides spp.* in Bacteroidetes phylum were also decreased in only IVV of S3 corresponding with the result of IVT group.

The alteration of microbiota by food was shown to similar of several between *in vitro* and *in vivo* in this study, although the other some microorganisms shifted differently i.e. showing opposite effects (Figure 9). This may be due to the limitation to emulation the actual gastrointestinal environment to *in vitro* completely.

Table 1. The shift of gut microbiota in phylum level by CGJ-P *in vitro* and *in vivo*.

Subjects	Phylum (LDA Score)			
	In vitro		*In vivo*	
	Decrease	Increase	Decrease	Increase
S1	Bacteroidetes (4.9)	Actinobacteria (4.8) Firmicutes (4.3)	Not detected	Not detected
S2	Bacteroidetes (4.7) Proteobacteria (3.6) Tenericutes (3.0)	Firmicutes (4.5) Actinobacteria (4.4)	Bacteroidetes (4.4)	Actinobacteria (4.3)
S3	Firmicutes (4.4) Bacteroidetes (4.3) Proteobacteria (3.7) Tenericutes (3.0)	Actinobacteria (4.7)	Bacteroidetes (4.9) Tenericutes (3.4) Verrucomicrobia (3.3)	Firmicutes (4.8) Actinobacteria (4.4)

BLK : sample before intake CGJ-P; IVT : sample after fecal fermentation; IVV : sample after intake CGJ-P for five days; The proportion of phylum > 0.1% and LDA > 3.0

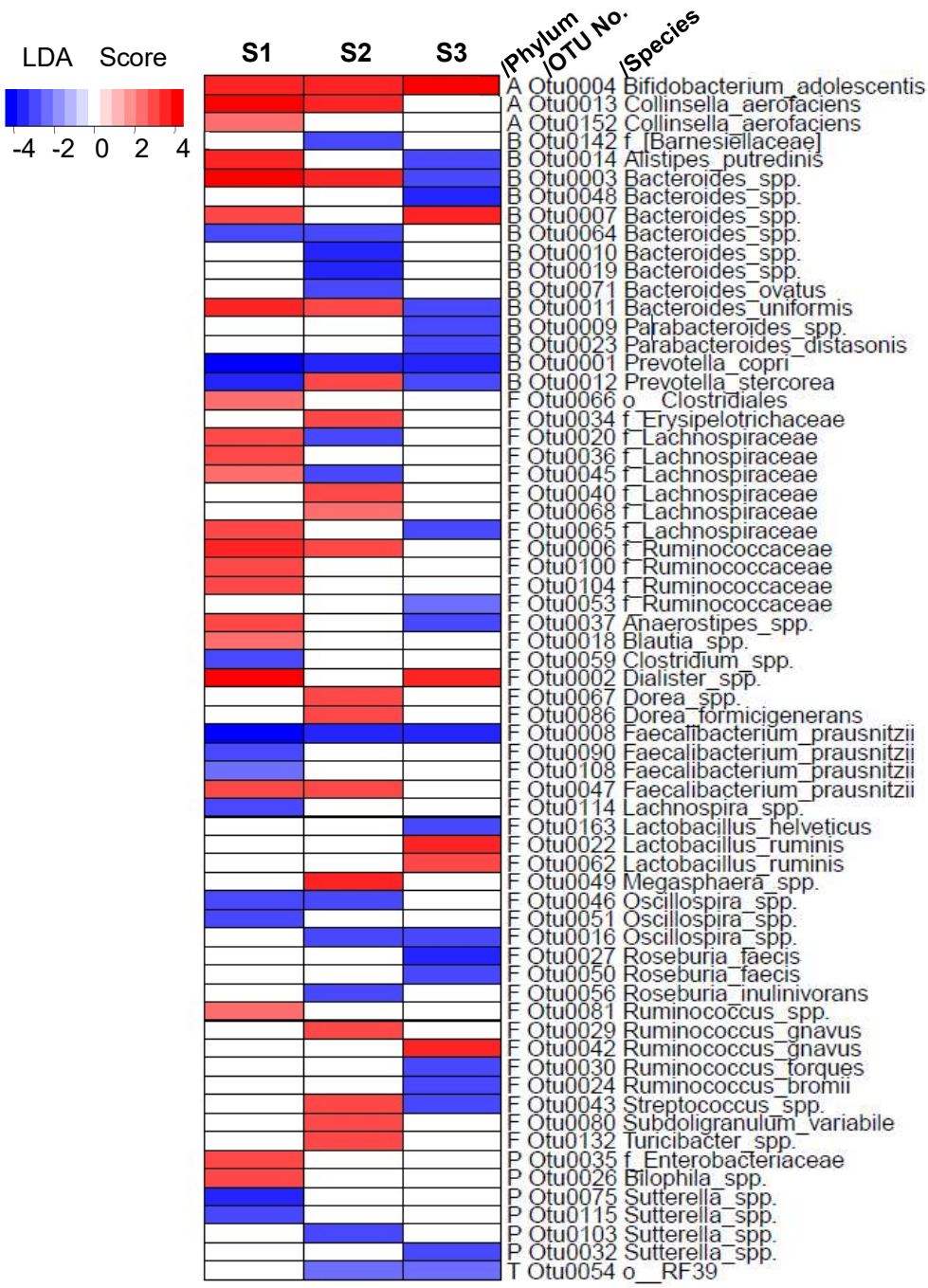
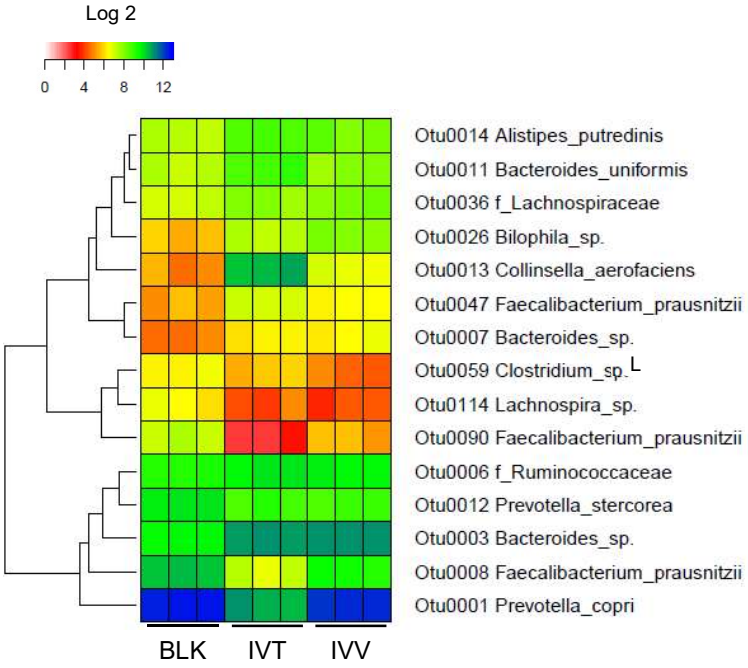


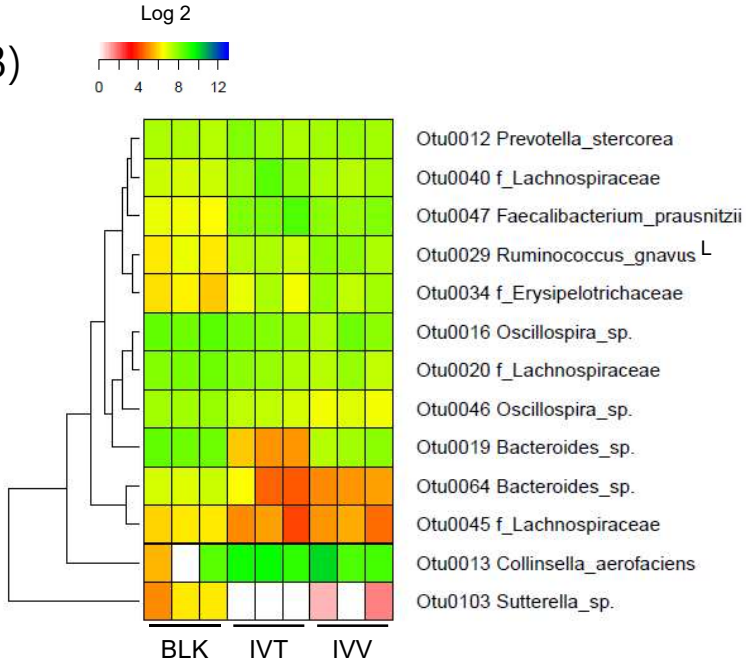
Figure 8. The relatively increased or decreased bacteria in OTU level in the comparison between BLK and IVT.

Each column from left to right on heatmap indicated comparison in S1, S2, and S3. The OTUs showing red color means that increased in IVT than BLK group, while decreased OTUs was shown blue color.

(A)



(B)



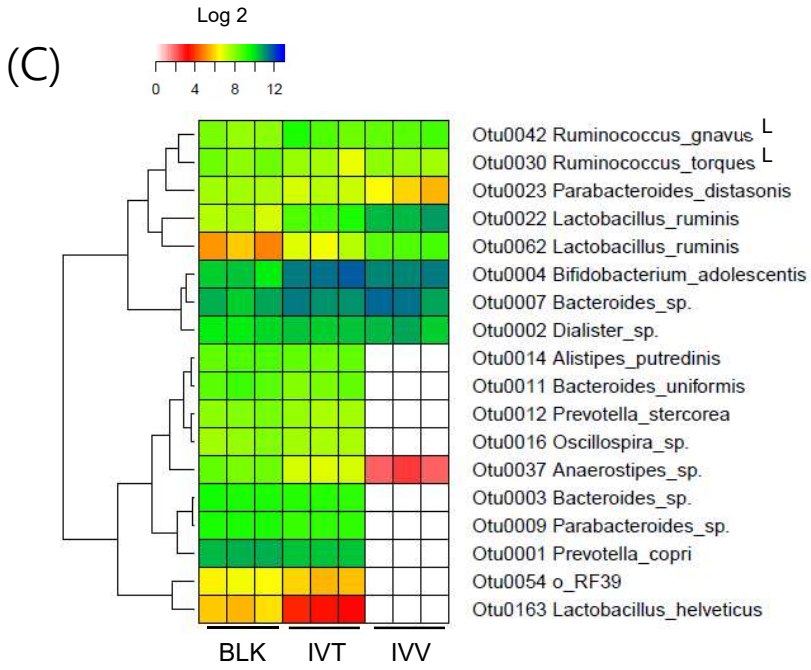
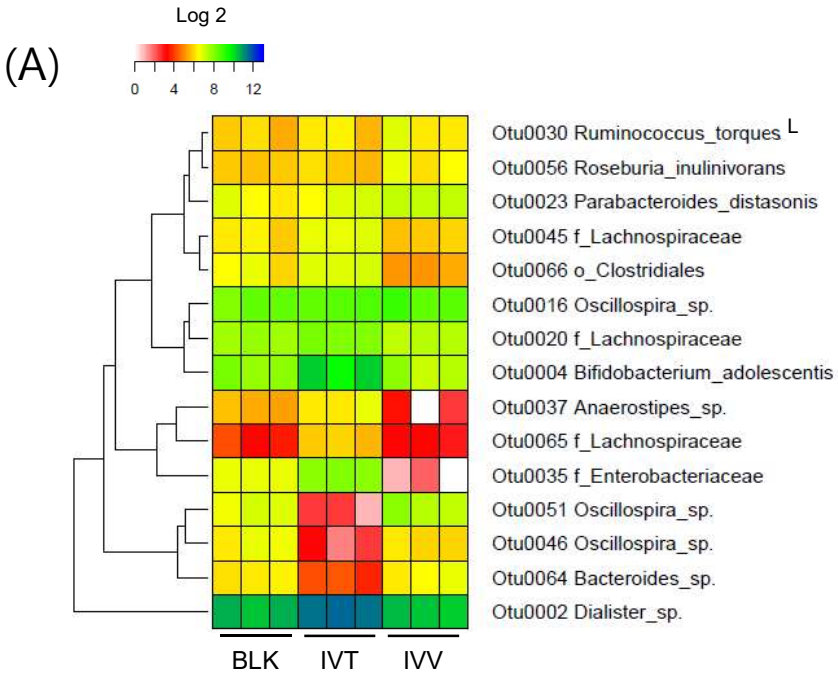
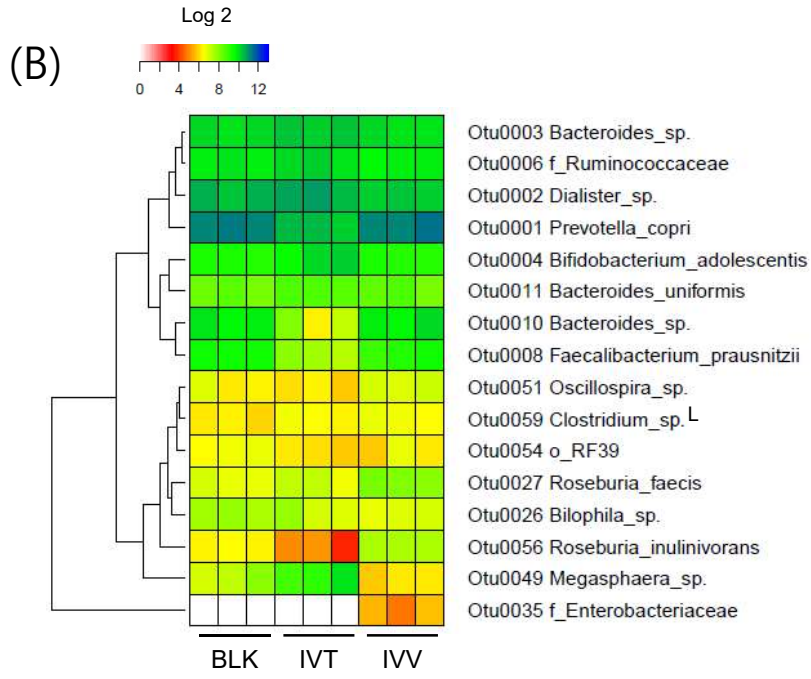


Figure 9. Comparison of relative abundance in OTU level between *in vitro* and *in vivo* evaluation method using fecal bacterial communities of each S1 (A), S2 (B), and S3 (C).

The relative abundance of OTUs was detected from the linear discriminant analysis (LDA) effect size (LEfSe) output. Each OTU was selected showing LDA score>3.0 and then also selected detected in both results of IVT and IVV. Superscripted 'L' mean that the species was classified from Lachnospiraceae family. BLK : sample before intake CGJ-P; IVT : sample after fecal fermentation; IVV : sample after intake CGJ-P for five days.





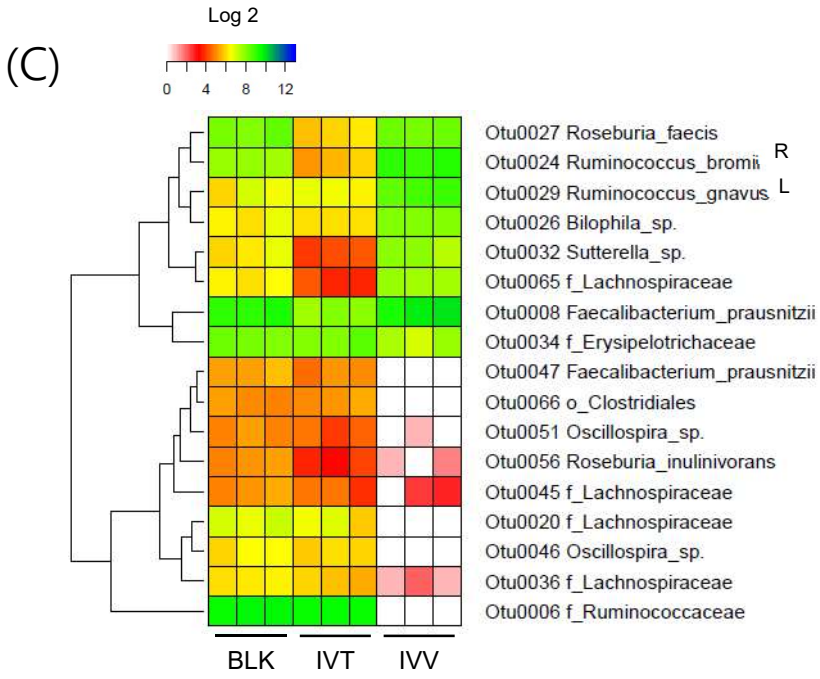


Figure 10. Comparison of relative abundance in OTU level between *in vitro* and *in vivo* evaluation method using fecal bacterial communities of each S1 (A), S2 (B), and S3 (C).

The relative abundance of OTUs was detected from the linear discriminant analysis (LDA) effect size (LEfSe) output. Each OTU was selected showing LDA score>3.0 and then also selected detected in both results of IVT and IVV which were showing the opposite shift in IVT and IVV groups. Superscripted 'L' and 'R': the species was classified from Lachnospiraceae and Ruminococcaceae family, respectively. BLK : sample before intake CGJ-P; IVT : sample after fecal fermentation; IVV : sample after intake CGJ-P for five days.

CONCLUSION

Food digestion was performed *in vitro* as an emulation of the gastrointestinal environment in this study, thus the digested food was used for fermenting with human fecal bacteria. To confirm the only effect of food on microbiota in fecal fermentation, two materials, i.e. yeast and peptone, were removed in basal nutrient media. Also, fermentation time according to food was determined to verify the only effect of fermentation, not bacterial incubation effect. SCFAs were produced differently *in vitro* according to the fecal donor, due to the dissimilar gut microbial community. In other words, the production pathway of SCFAs could be processed differently depending on fecal microbiota. Although the fecal microbiota from each donor before ingest CGJ belonged to similar cluster on phylogenic tree, that was not shown similar alteration *in vitro* and *in vivo*. Nevertheless, several bacteria were observed the comparable effect in both environments, thus, this system is not perfect but shown the possibility to be applicated in the evaluation of food effect on microbiota using more various food and diverse fecal sample.

REFERENCES

Bang S-J et al. (2018) The influence of *in vitro* pectin fermentation on the human fecal microbiome AMB Express 8:98-98 doi:10.1186/s13568-018-0629-9

Baothman OA, Zamzami MA, Taher I, Abubaker J, Abu-Farha M (2016) The role of Gut Microbiota in the development of obesity and Diabetes Lipids Health Dis 15:108-108 doi:10.1186/s12944-016-0278-4

Becker C, Neurath MF, Wirtz S (2015) The Intestinal Microbiota in Inflammatory Bowel Disease ILAR Journal 56:192-204 doi:10.1093/ilar/ilv030

Blanquet-Diot S, Soufi M, Rambeau M, Rock E, Alric M (2009) Digestive stability of xanthophylls exceeds that of carotenes as studied in a dynamic *in vitro* gastrointestinal system The Journal of nutrition 139:876-883 doi:10.3945/jn.108.103655

Byrne CS, Chambers ES, Morrison DJ, Frost G (2015) The role of short chain fatty acids in appetite regulation and energy homeostasis International journal of obesity (2005) 39:1331-1338 doi:10.1038/ijo.2015.84

Dąbrowska K, Witkiewicz W (2016) Correlations of Host Genetics and Gut Microbiome Composition Frontiers in Microbiology 7 doi:10.3389/fmicb.2016.01357

Dahmus JD, Kotler DL, Kastenberg DM, Kistler CA (2018) The gut microbiome and colorectal cancer: a review of bacterial pathogenesis J Gastrointest Oncol 9:769-777 doi:10.21037/jgo.2018.04.07

David LA et al. (2014) Diet rapidly and reproducibly alters the human gut microbiome Nature 505:559-563 doi:10.1038/nature12820

De Vadder F, Kovatcheva-Datchary P, Zitoun C, Duchampt A, Bäckhed F, Mithieux G (2016) Microbiota-Produced Succinate Improves Glucose Homeostasis via Intestinal Gluconeogenesis Cell Metabolism 24:151-157 doi:https://doi.org/10.1016/j.cmet.2016.06.013

Deehan EC, Duar RM, Armet AM, Perez-Muñoz ME, Jin M, Walter J (2017) Modulation of the Gastrointestinal Microbiome with Nondigestible Fermentable Carbohydrates To Improve Human Health Microbiol Spectr 5 doi:10.1128/microbiolspec.bad-0019-2017

Duncan SH, Louis P, Thomson JM, Flint HJ (2009) The role of pH in determining the species composition of the human colonic microbiota Environ Microbiol 11:2112-2122 doi:10.1111/j.1462-2920.2009.01931.x

Ferreira IMPLVO, Pinho O, Vieira E, Tavarela JG (2010) Brewer's Saccharomyces yeast biomass: characteristics and potential applications Trends in Food Science & Technology 21:77-84 doi:https://doi.org/10.1016/j.tifs.2009.10.008

Fujisawa T, Shinohara K, Kishimoto Y, Terada A (2006) Effect of miso soup containing Natto on the composition and metabolic activity of the human faecal flora Microbial Ecology in Health and Disease 18:79-84 doi:10.1080/08910600600931942

Honda K, Littman DR (2012) The microbiome in infectious disease and inflammation Annual review of immunology 30:759-795 doi:10.1146/annurev-immunol-020711-074937

Klimenko NS et al. (2018) Microbiome Responses to an Uncontrolled Short-Term Diet Intervention in the Frame of the Citizen Science Project Nutrients 10:576 doi:10.3390/nu10050576

Kruskal JB (1964) Nonmetric multidimensional scaling: A numerical method Psychometrika 29:115-129 doi:10.1007/BF02289694

Levy M, Thaiss CA, Elinav E (2016) Metabolites: messengers between the microbiota and the immune system Genes & development 30:1589-1597 doi:10.1101/gad.284091.116

Maccaferri S et al. (2010) Rifaximin modulates the colonic microbiota of patients with Crohn's disease: an *in vitro* approach using a continuous culture colonic model system The Journal of antimicrobial chemotherapy 65:2556-2565 doi:10.1093/jac/dkq345

Makki K, Deehan EC, Walter J, Backhed F (2018) The Impact of Dietary Fiber on Gut Microbiota in Host Health and Disease Cell Host Microbe 23:705-715 doi:10.1016/j.chom.2018.05.012

Minekus M et al. (2014) A standardised static *in vitro* digestion method suitable for food - an international consensus Food Funct 5:1113-1124 doi:10.1039/c3fo60702j

Moon JS, Joo W, Ling L, Choi HS, Han NS (2016*) In vitro* digestion and fermentation of sialyllactoses by infant gut microflora Journal of Functional Foods 21:497-506 doi:https://doi.org/10.1016/j.jff.2015.12.002

Pompei A, Cordisco L, Raimondi S, Amaretti A, Pagnoni UM, Matteuzzi D, Rossi M (2008) *In vitro* comparison of the prebiotic effects of two inulin-type fructans Anaerobe 14:280-286 doi:https://doi.org/10.1016/j.anaerobe.2008.07.002

Ríos-Covián D, Ruas-Madiedo P, Margolles A, Gueimonde M, de los Reyes-Gavilán CG, Salazar N (2016) Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health Frontiers in Microbiology 7 doi:10.3389/fmicb.2016.00185

Sanz ML, Gibson GR, Rastall RA (2005) Influence of disaccharide structure on prebiotic selectivity *in vitro* Journal of agricultural and food chemistry 53:5192-5199 doi:10.1021/jf050276w

Sarbini SR, Kolida S, Deaville ER, Gibson GR, Rastall RA (2014) Potential of novel dextran oligosaccharides as prebiotics for obesity management through *in vitro* experimentation British Journal of Nutrition 112:1303-1314 doi:10.1017/S0007114514002177

Schloss PD et al. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities Appl Environ Microbiol 75:7537-7541 doi:10.1128/AEM.01541-09

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C (2011) Metagenomic biomarker discovery and explanation Genome biology 12:R60 doi:10.1186/gb-2011-12-6-r60

Sender R, Fuchs S, Milo R (2016) Revised Estimates for the Number of Human and Bacteria Cells in the Body PLoS Biol 14:e1002533 doi:10.1371/journal.pbio.1002533

Tzounis X et al. (2008) Flavanol monomer-induced changes to the human faecal microflora Br J Nutr 99:782-792 doi:10.1017/S0007114507853384

Wu GD et al. (2011) Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes Science 334:105-108 doi:10.1126/science.1208344

Yang J, Martínez I, Walter J, Keshavarzian A, Rose DJ (2013) *In vitro* characterization of the impact of selected dietary fibers on fecal microbiota composition and short chain fatty acid production Anaerobe 23:74-81 doi:https://doi.org/10.1016/j.anaerobe.2013.06.012

Zihler A, Gagnon M, Chassard C, Hegland A, Stevens MJ, Braegger CP, Lacroix C (2010) Unexpected consequences of administering bacteriocinogenic probiotic strains for Salmonella populations, revealed by an *in vitro* colonic model of the child gut Microbiology 156:3342-3353 doi:10.1099/mic.0.042036-0