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Modulation of Intestinal Microbiota by Supplementation of Fermented Kimchi in Rats

Su Jin An[1†], Jae Young Kim[2†], In Sung Kim[1†], Bishnu Adhikari[3], Da Yoon Yu[1], Jeong A Kim[1], Young Min Kwon[3,4], Sang Suk Lee[5], In Soon Choi[6] and Kwang Keun Cho[1*]

[1]*Department of Animal Resources Technology, Gyeongnam National University of Science and Technology, Jinju 52725, Korea*
[2]*Swine Science and Technology Center, Gyeongnam National University of Science and Technology, Jinju 52725, Korea*
[3]*Department of Poultry Science, University of Arkansas, Fayetteville 72701, USA*
[4]*Cell and Molecular Biology Program, University of Arkansas, Fayetteville 72701, USA*
[5]*Department of Animal Science and Technology, Sunchon National University, Suncheon, Jeonnam 540-742, Korea*
[6]*Department of Life Science, Silla University, Busan 617-736, Korea*

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Intestinal microbiota play a key role in maintaining the host's health, and variety and richness of this microbiota is directly influenced and modulated by the host's diet. Kimchi is a fermented food rich in dietary fibers and lactic acid bacteria (LAB). To investigate the effect of fermented kimchi on the host's response and the composition of intestinal microbiota, 45 male Sprague-Dawley rats six weeks old were divided into three experimental groups that received either a basal diet (CON) or a basal diet supplemented with fermented kimchi (FK) or chitosan-added fermented kimchi (CFK) for four weeks. Body weights and feed intakes were measured weekly, and the intestinal contents were collected aseptically and were used for 16S rRNA gene profiling via pyrosequencing. As compared to the control, FK and CFK groups showed less body weight gain, feed efficiency, and blood triglyceride concentration. The diversity of intestinal microbiota was increased in both FK and CFK as compared to the control. At the phylum level, obesity-associated *Firmicutes* decreased, while leanness-associated *Bacteroidetes* increased. At the genus-level, the genera that consist of LAB, leanness-associated bacteria, and butyric acid - producing bacteria increased in FK and CFK as compared to the control. The overall results suggest that the consumption of fermented kimchi can reduce obesity and promote the host's health through mechanisms involving the modulation of intestinal microbiota.

Key words : Chitosan, Kimchi, microbiota, obesity

Introduction

Fermented Kimchi is a traditional food of South Korea made of mixtures of natural materials containing diverse bioactive substances. Fermented Kimchi is prepared by salting Chinese cabbage (main material) and other vegetables followed by mixing with spices and fermenting the mixture [5]. The types of fermented Kimchi vary depending upon the types of vegetables (Chinese cabbage, Chinese radish, radish, cucumber, etc.) and spices (red pepper, scallion, garlic, ginger, onion, sesame seeds, marine products, etc.) used. Fermented Kimchi is rich in vitamins, dietary fibers, and lactic acid bacteria (LAB). In addition, since fermented kimchi has excellent anti-cancer, anti-oxidative, anti-arteriosclerosis, and anti-obesity effects, it was selected as one of five best health foods in the world in 2006 by *Health Magazine* [3, 4, 29, 30, 32].

Chitosan is a natural biopolymer made from crustaceans such as crabs and shrimps. It has antimicrobial, anticancer, and immune modulation effects [35, 41]. It is in the β-1,4 bound structure of N-acetyl-D-glucosamine, is not digested in the small intestine of human bodies due to the lack of the digestive enzyme, but is fermented by microbes in the large intestine [8]. The addition of chitosan to kimchi fermentation inhibits the growth of *Lactobacillus plantarum* and *Leuconostoc mesenteroides*, thus extending the maturation and preservation period of kimchi [27, 41].

It is well established fact that the intestinal microbiota affects nutrition, growth, and metabolic processes through interactions with the host, and is associated with the onset

†Authors contributed equally.
***Corresponding author**
Tel : +82-55-751-3286, Fax : +82-55-751-3689
E-mail : chotwo2@gntech.ac.kr
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of metabolic diseases such as obesity, arteriosclerosis, and diabetes [11]. In addition, intestinal microbiota affects the host's immune responses [12, 38]. There are a number of factors that affect composition and structure of intestinal microbiota including food, age, and antibiotics; while the effects of food are most significant among them [10, 22, 39].

Researches on fermented Kimchi conducted so far have mostly focused on bioactive actions of fermented Kimchi on the host, and in changes of the fermenting microbes during the process of Kimchi fermentation [1, 23, 28]. However, the study focused on the changes in intestinal microbiota depending on intake of fermented Kimchi remains to be discovered. In our previous study, the changes in intestinal microbiota after fermented Kimchi intake for one week in adult females were investigated. However, due to the short experimental period and inter-individual differences, the results remain to be further studied [13]. The present experiment was conducted using rats as experimental animals in order to characterize the effects of fermented Kimchi (FK) and chitosan-added fermented Kimchi (CFK) on the host response (body weight, feed intake and adiposity indices) as well as the composition and structure of intestinal microbiota.

Materials and Methods

Feed and experimental animal

Fermented Kimchi made by Unlimga Co. (Gwangju, Korea) and chitosan with the molecular weight of 3,500 (Kitto Life, Pyeongtaek, Korea) were used for the preparation of experimental diets. The fermented Kimchi was matured for 12 days at 4℃ and the chitosan-added fermented Kimchi was prepared by adding 1 g of chitosan per 100 g of Chinese cabbage with spices. The chitosan-added fermented Kimchi was matured for 30 days at 4℃ before being used in the experiment. Since chitosan slows down the progression of the fermentation of Kimchi, the fermented Kimchi was matured for 12 days, whereas the chitosan-added fermented Kimchi was matured for 30 days at 4℃[27]. Each fermented Kimchi was freeze-dried, mixed with the basal diet for rat, made into pellets, and then supplied to the experimental animals. The quantity of the fermented Kimchi added to the basal diet was determined according to the daily intake of Kimchi per South Korean adult (100 g for an adult of average body weight 60 kg).

Forty-five male Sprague-Dawley rats of six weeks old were purchased from Samtako (Osan, Korea). With five repetitions of three per cage per group, the experimental animals were divided into three treatment groups that received either basal diet (CON), basal diet supplemented with fermented Kimchi (FK) or chitosan-added fermented Kimchi (CFK) for 4 weeks. The experimental animals were maintained in the controlled environment with 20~25℃ temperature, 50~60% humidity, and 12/12 hr cycle of day/night. Feed and water were provided *ad libitum*. The body weights and feed intakes of the experimental animals were measured once per week during experimental period. The experimental animals were anesthetized after finishing experiment, and their blood was collected to measure blood lipid concentrations. All the animal experimental procedures were approved by the Institutional Animal Care and Use Committees at Gyeongnam National University of Science and Technology (Approval No. 2015-1).

PCR of 16S rRNA genes

The intestinal contents from small intestine, cecum, and large intestine of the experimental animals were collected at the end of the experiment. The contents from the 3 regions were combined for each animal, which was then used to extract genomic DNA using ZR Fecal DNA MiniPrep™ (Zymo Research, USA). Microbiome analysis was conducted through pyrosequencing of the 16S ribosomal RNA (rRNA) genes through ChunLab (Seoul, Korea). Briefly, V1-V3 regions of the 16S rRNA gene was amplified from the genomic DNA samples using primers and PCR conditions as described previously [13]. The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and quantified using a PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA). The amplicons of individual samples in equimolar concentrations were pooled, and sequenced using the Roche/454 FLX system.

Pyrosequencing analysis

The pyrosequencing data of the 16S rRNA gene sequences were processed using the Java-based multi-step bioinformatics pipeline. Unidirectional sequencing reads were distinguished based on the unique barcodes of individual reads. Low-quality sequences shorter than 300 bp were removed from the reads using TBC clustering algorithm [19]. Trimmed sequencing reads were clustered into the sequences with the similarity of 97% or higher [19] to identify operational taxonomic units (OTUs). Representative sequences were selected from the clusters of trimmed sequences for identification of the associated taxonomic groups. Taxonomy was determined for representative sequences by the highest similarities among the top five BLASTN hits in the EzTaxon-e database [14]. The sequences in the EzTaxon-e database that were not identified in the BLASTN searches were classified as non-target sequences and thus were excluded from the further analysis. The similarity of base sequences between the query and candidate species was calculated using the Myers and Miller method [24]. The cladogram was calculated using the TBC clustering algorithm [19]. Overall phylogenetic differences, Shannon index, and heat map analyses of the correlations across three different groups were investigated using the CL community program provided by ChunLab (Seoul, Korea).

Statistical analysis

The statistical analysis of the data was performed using the General Linear Model (GLM) procedure of the SAS (Version 9.1). The pairwise comparison among different groups was performed by Tukey's multiple range tests where the level of significant was considered at 0.05 (p<0.05) unless described otherwise.

Results

Weight gains and feed efficiency

The total body weight gain and average daily body weight gain decreased in both FK and CFK as compared to the control, although the observed differences were not significant (Fig. 1A, Fig. 1C). On the contrary, the total feed intake increased in both FK and CFK as compared to the control, while the increase was significant only for FK (Fig. 1B). The feed efficiency decreased significantly by both FK and CFK groups as compared to the control (Fig. 1D). These results suggest that the supply of the fermented Kimchi or chitosan-added fermented Kimchi effectively suppressed the body weight gain.

Serum lipid concentration

To investigate the effects of the consumption of the fermented Kimchi on the body fat formation, the concentrations of the blood lipid components including low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured. After feeding for 4 weeks, there was no significant difference in serum LDL and HDL cholesterol concentrations among the control, FK, and CFK groups (Fig. 2A, Fig. 2B), but it were higher

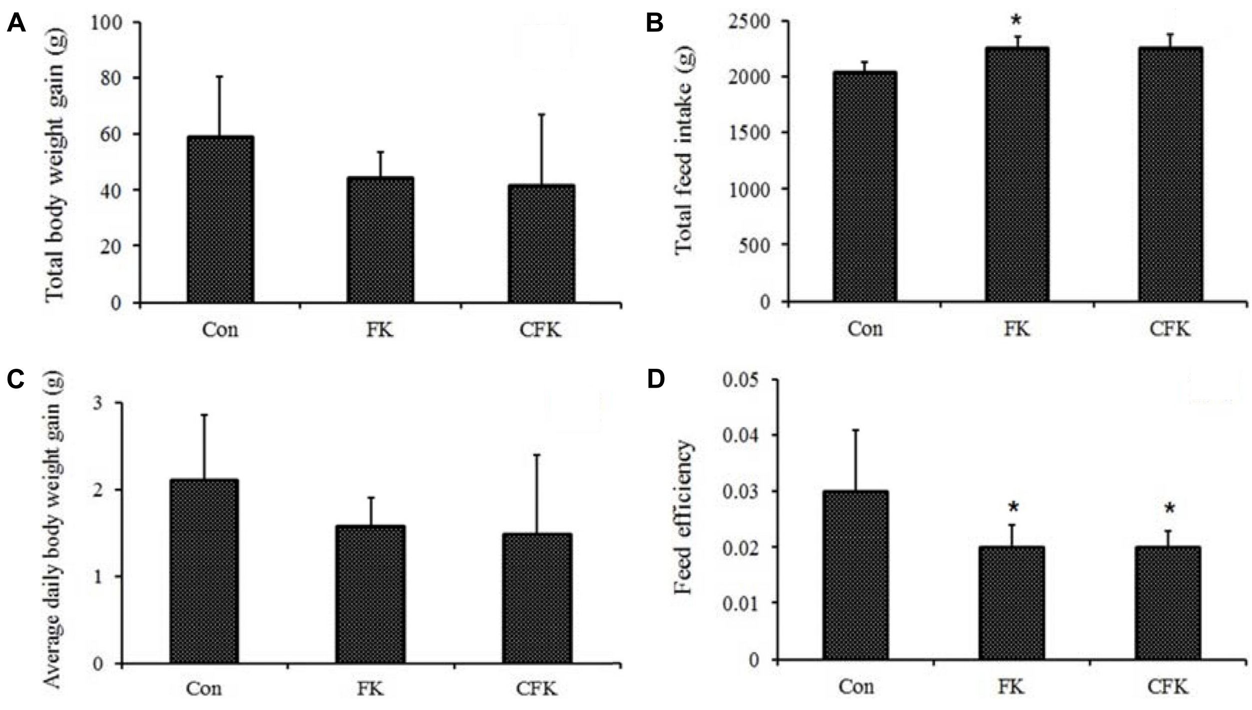

Fig. 1. The effects of the consumption of the diets supplemented with fermented kimchi (FK) and chitosan-added fermented kimchi (CFK) in comparison to the basal diet group (CON) on (A) body weight gain, (B) total feed intake, (C) average daily body weight gain, and (D) feed efficiency of the Sprague-Dawley rats. *; p<0.05.

than that of the control group. However, triglycerides concentrations of FK and CFK groups were 73 mg/dl and 71 mg/dl, respectively, which were significantly lower as compared to the control group (136 mg/dl) (Fig. 2C; $p<0.01$). But there was no significant difference in serum triglycerides concentration between FK and CFK groups. The decrease in serum triglycerides concentrations in both FK and CFK groups show that the fermented Kimchi can suppress body fat accumulation.

Intestinal microbiota analysis

To investigate the effects of fermented Kimchi on the composition and structure of intestinal microbiota, FK and CFK were fed to the experimental rats for 4 weeks. The collected

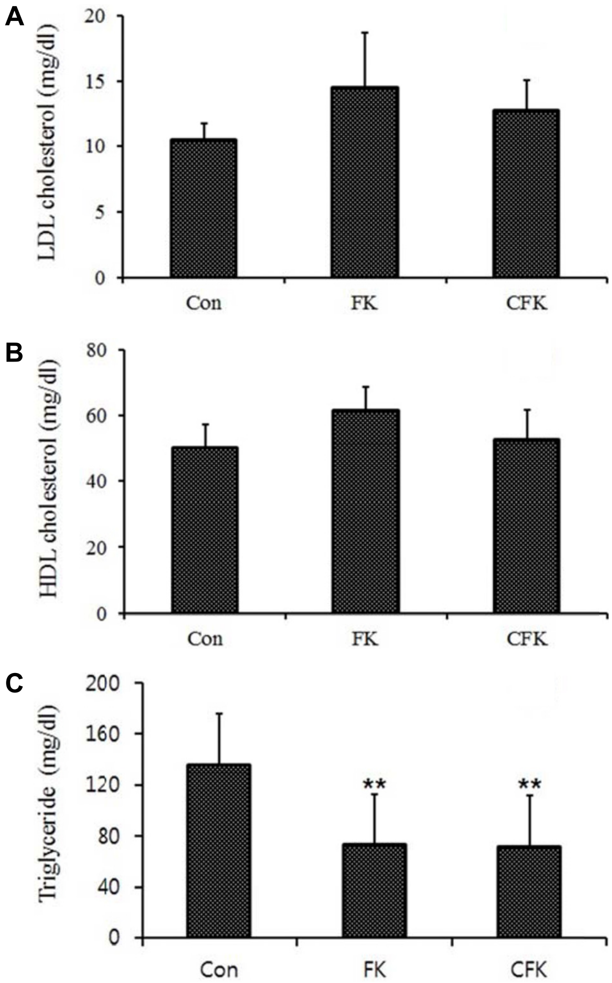

Fig. 2. Effects of the consumption of the diets supplemented with the fermented kimchi (FK) and chitosan-added fermented kimchi (CFK) on fat metabolism of the Sprague-Dawley rats in comparison to the basal diet group (CON). (A) LDL cholesterol, (B) HDL cholesterol, and (C) triglyceride. **; $p<0.01$.

Table 1. The number of different taxonomic groups at different levels of hierarchical classification in the intestinal microbiota of the Sprague-Dawley rats fed with the basal diet (CON), the basal diet with supplemented fermented kimchi (FK) or chitosan-added fermented kimchi (CFK)

	CON	FK	CFK
Phylum	9	9	9
Class	14	17	18
Order	21	23	28
Family	55	60	66
Genus	234	243	250
Species	546	549	579

intestinal contents were combined for each rat, and the PCR products of 16S rRNA genes amplified from the samples were used to analyze the intestinal microbiota through pyrosequencing and bioinformatics analysis. The intestinal bacterial composition at different taxonomic levels was summarized in Table 1. At the phylum level, nine phyla that are common members of rats' intestine were found across all three treatment groups (Fig. 3A). Among the nine phyla, *Firmicutes* and *Bacteroidetes* were the two dominant phyla that accounted for 92.9% (CON), 94.4% (FK), and 93.9% (CFK) of total intestinal microbiota (Fig. 3B). The ratio of *Bacteroidetes* to *Firmicutes,* which has been shown to correlate with the obesity, increased in FK and CFK as compared to the control (Fig. 3B). As indicated in the heat map showing relative abundance of different phyla (Fig. 4), FK and CFK groups were clustered together in comparison to the control group, suggesting that the intestinal microbiota was shifted to form similar microbial communities by the intake of FK and CFK.

At the genus level, altogether 234, 243, and 250 different genera were found in CON, FK, and CFK groups, respectively (Table 1). To understand the functional capacities associated with the changes in intestinal microbiota, the distribution of intestinal microbiota was classified into functional microbial groups of LAB, leanness-associated bacteria, and butyric acid-producing bacteria where the abundance for each of these microbial groups was indicated with the combined number of sequence reads of the corresponding OTUs (Table 2). Regarding LAB, the two genera (*Lactobacillus*, and *Streptococcus*) were present in the control, FK, and CFK groups, while two additional genera (*Bifidobacterium*, and *Leuconostoc*) were present in CFK group. In all three groups, *Lactobacillus*, *Prevotella*, *Alistipes*, and *Butyricicoccus*

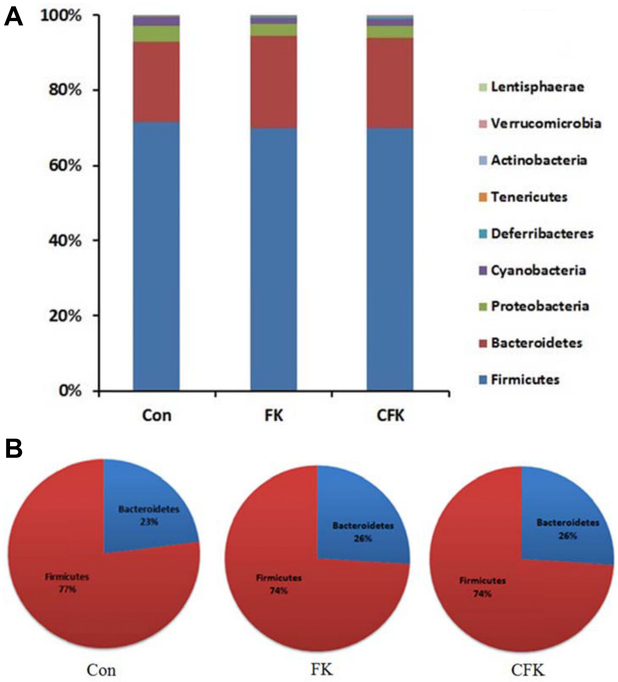

Fig. 3. The relative abundance of different phyla in the intestinal microbiota of the Sprague-Dawley rats fed with the basal diet (CON), the basal diet with supplemented fermented kimchi (FK) or chitosan-added fermented kimchi (CFK). (A) Bar graph showing the relative abundance of 9 phyla, and (B) Pie graph showing only two dominant phyla, *Firmicutes* and *Bacteriodetes*, highlighting the changes in the ratio of the two phyla in FK and CFK in comparison to CON.

Table 2. Relative abundance of the functional bacterial groups in the intestinal microbiota of the Sprague-Dawley rats fed with the basal diet (CON), the basal diet with supplemented fermented kimchi (FK) or chitosan-added fermented kimchi (CFK)

	Con	FK	CFK
Lactic acid bacteria			
Bifidobacterium	0	0	38
Lactobacillus	541	498	2,309
Leuconostoc	0	0	3
Streptococcus	4	9	3
Total number of sequencing reads	545	507	2353
Leanness-associated bacteria			
Prevotella	2,322	3,793	3,673
Alistipes	1,220	984	909
Bacteroides	332	319	504
Total number of sequencing reads	3,874	5,096	5,086
Butyric acid-producing bacteria			
Roseburia	19	49	34
Butyricicoccus	754	1,085	965

Numeric values indicate the numbers of sequence reads assigned to each genus.

were dominant genera. The total portion of LAB was higher in FK and CFK groups as compared to the control group. Moreover, the abundance of LAB in CFK group was nearly two folds higher than the control group. Among the leanness-associated bacterial genera, *Prevotella*, *Alistipes*, and *Bacteroides* were commonly found in all three treatment groups, with *Prevotella* being a dominant genus. Leanness-associated bacteria increased by at least 30% in FK and CFK groups, as compared to the control group. Among the butyric acid-producing bacteria, *Roseburia* and *Butyricoccus* were commonly present in all three treatment groups and was 2.6 and 1.8 times higher in FK and CFK groups, respectively, in comparison to the control group. Clustering analysis of the relative abundance of these functional bacterial groups indicated that FK and CFK groups were clustered together in reference to the control group as shown in the heat map (Fig. 5). The heat map also shows the overall trend that these three functional bacterial groups including LAB, leanness-associated bacteria, and butyric acid-producing bacteria

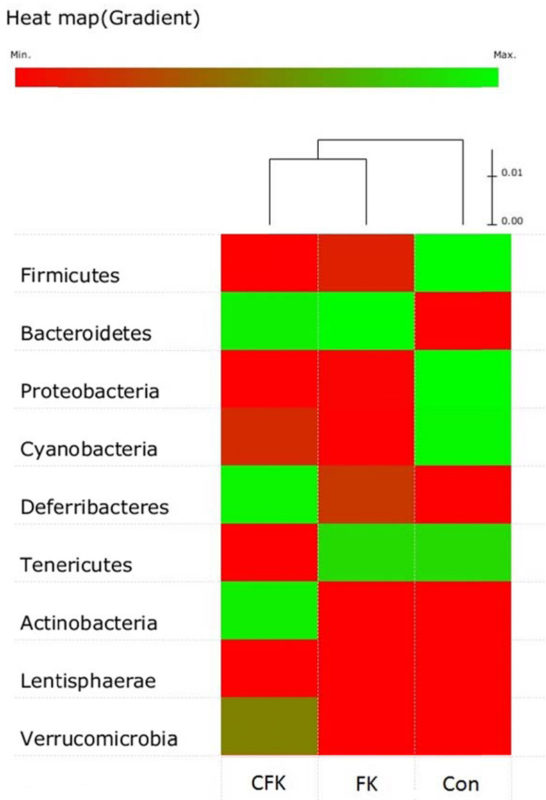

Fig. 4. Heat map showing the relative abundance of 9 phyla in the intestinal microbiota of the Sprague-Dawley rats fed with the basal diet (CON), the basal diet with supplemented fermented kimchi (FK) or chitosan-added fermented kimchi (CFK). Clustering based on the abundance profile is shown on the top of the heat map.

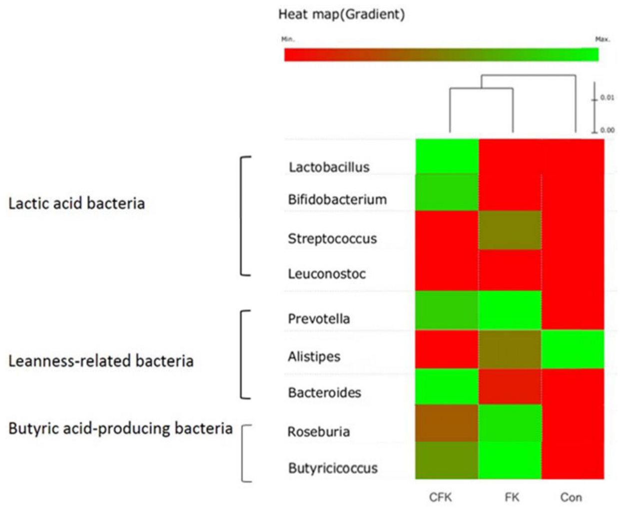

Fig. 5. Heat map showing the relative abundance of genera according to the functional microbial groups in the intestinal microbiota of the Sprague-Dawley rats fed with the basal diet (CON), the basal diet with supplemented fermented kimchi (FK) or chitosan-added fermented kimchi (CFK). Clustering based on the abundance profile is shown on the top of the heat map.

increased by consumption of FK and CFK as compared to the control group.

At species level, 546, 549, and 579 different bacterial species were identified in CON, FK, and CFK groups, respectively (Table 1). In addition, more diverse taxa were recovered from the treated groups as compared to the control at all levels except the phylum. Shannon index was used to measure the alpha diversity whose mean values were 5.88, 6.17, and 6.36 in CON, FK, and CFK groups, respectively. Thus, as compared to the control group, both treatment groups (FK and CFK) showed more diverse microbial communities (Table 1).

Discussion

Although fermented Kimchi which is rich in dietary fibers and LAB was evaluated to improve host's health, available researches on the effects of the fermented Kimchi consumption on intestinal microbiota are insufficient. In our previous study, an experiment was conducted with South Korean adult females to examine the changes in intestinal microbiota following fermented Kimchi consumption for one week [13]. According to the results of the study, intestinal microorganisms beneficial for the host's health were increased with the intake of fermented Kimchi. However, due to the short diet period of fermented Kimchi during previous study, it's difficult to conclude that the intestinal microorganisms settled down stably. During studying by other group where fermented Kimchi was supplied for eight weeks, the composition of intestinal microbiota was clearly different between the first and the eighth week of consumption [9]. In another study that focused on the intestinal microbiota transplantation, the composition of gut microbiota was examined for 4 weeks after transplantation [39]. Thus, the question remained whether the fermented Kimchi intake for one week in our previous experiment was sufficient for intestinal microbes to settle down for the stable intestinal microbiota formation [13]. In addition, although beneficial intestinal bacteria including LAB were significantly increased in the fermented Kimchi groups as compared to the control, there were substancial inter-individual differences within the same treatment group. To solve these problems, in the present study, a feeding period of four weeks was used so that the intestinal microbiota could be stabilized in experimental animals and reduce inter-individual variations. In addition, the effects of chitosan-added fermented Kimchi on intestinal microbes were also examined.

In the present study, after supplying fermented Kimchi or chitosan-added fermented Kimchi for four weeks, the total body weight gain, daily weight gain, total feed intake, and feed efficiency of the experimental animals were measured to investigate the weight gain efficiency of the experimental animals (Fig. 1). Although, as compared to the control group, FK and CFK groups showed tendencies towards decrease in total body weight gain and daily weight gain, and increase in total feed intake, no significant difference was found across the 3 groups (Fig. 1A, Fig. 1B, Fig. 1C). However, the feed efficiency of FK and CFK groups was lower than that of the control group (Fig. 1D). A lower feed efficiency means smaller body weight gain when the same quantity of food is taken. The fact that the fermented Kimchi intake is effective for the suppression of body weight gains is well known. In particular, fermented Kimchi shows the effect of reducing blood triglyceride concentrations and suppressing body fat accumulation [4, 29]. The effect to suppress body fat is due to the dietary fiber and the capsaicin present in the ingredients of Fermented Kimchi. The major ingredient of Fermented Kimchi is Chinese cabbage which is rich in dietary fibers [13]. Water-soluble and viscous dietary fibers increase satiety and lead to decrease in food intake and nutrient absorption in the small intestine [37]. Dietary fibers produce short chain fatty acids (SCFAs) through microbial fermentation in the large intestine and among the SCFAs; butyric acid suppresses fat accumulation [21]. The capsaicin in red pepper that makes the spicy taste of fermented Kimchi stimulates the adrenal sympathetic nerves to increase the secretion of adrenalin, thereby promoting the decomposition and combustion of body fat [2, 16].

Fermented Kimchi reduces plasma triglyceride and LDL cholesterol concentrations, while increasing HDL cholesterol concentrations [17]. However, in the present experiment, no difference in the blood concentrations of LDL- and HDL-cholesterol between the treatments with fermented Kimchi and with chitosan-added fermented Kimchi was observed (Fig. 2A, Fig. 2B). These differences in the experimental results can be attributed to the differences in experimental conditions such as the quantity of supplied fermented Kimchi, feeding period, and high fat diet *vs.* basal diet. Plasma triglyceride concentrations were shown to be lower in the animals supplied with fermented Kimchi or chitosan-added fermented Kimchi (Fig. 2C). Plasma triglyceride concentrations are closely related to abdominal obesity [7]. Therefore, the supply of fermented Kimchi may suppress obesity by reducing plasma triglyceride concentrations.

When high-fat and high-sugar diet, and standard low-fat and high-polysaccharide diet were fed to the mice, those mice taking standard low-fat, high-polysaccharide diet showed a higher diversity of intestinal microbes [39]. This diversity of microorganisms in intestinal microbiota was shown to be closely related to the suppression of obesity. In the present study, when fermented Kimchi or chitosan-added fermented Kimchi was supplied to rats, the rats showed more diverse species of intestinal microorganisms than the animals supplied with the control diet (Table 1).

When intestinal microorganisms from obese mice were transplanted to germ-free mice, the recipient showed remarkable increase in fat accumulation that promotes obesity [39]. The *Firmicutes* and *Bacteroidetes* are dominant bacterial phyla in human intestine and were shown to be associated with obese and lean body types, respectively [34]. Thus, increase in *Firmicutes* and *Bacteroidetes* in the intestine promotes obesity and leanness, respectively. In our study, the *Firmicutes* and *Bacteroidetes* were found to be dominant bacterial phyla in all treatment groups, and accounted for at least 92% of all intestinal bacteria (Fig. 3A). The supply of fermented Kimchi and chitosan-added fermented Kimchi increased *Bacteroidetes* and reduced *Firmicutes* as shown in Fig. 3. This shift in intestinal microbiota in comparison to the control group was similarly observed in both FK and CFK groups as demonstrated in the heat map (Fig. 4). Hence, the intake of fermented Kimchi or chitosan fermented Kimchi promoted the abundance of intestinal bacterial phyla that can suppress the obesity.

LAB affect the immune modulation by secreting bacteriocins which are natural antibiotics that suppress the growth of harmful bacteria, and by promoting the secretion of IgA and gamma-interferon in blood [36]. In addition, *Bifidobacterium longum* and *Lactobacillus acidophilus* reduce the total and LDL cholesterol without changing the concentration of HDL cholesterol [31]. For this reason, *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus* are often used commercially as probiotics. At the genus-level, as compared to the control group, LAB increased in the groups treated with fermented Kimchi and chitosan-added fermented Kimchi (Table 2). Chitosan is a prebiotic that promotes the growth of intestinal *Bifdobacterium* and *Lactobacillus* [18]. Chitosan-added fermented Kimchi group had at least four times more *Lactobacillus* than the control group or the fermented Kimchi group and showed *Bifidobacterium* that did not exist in other treatment groups (Table 2).

The increase in LAB following treatments in the present experiment can be attributed to the dietary fibers and chitosan in fermented Kimchi. *Prevotella*, *Alistipes*, and *Bacteroides* are a group of intestinal bacteria that can suppress obesity and present more in lean persons [6, 25]. Higher intestinal abundance of *Provotella* in mice were correlated with decrease in fat accumulation [26]. These leanness-associated bacteria especially *Prevotella* increased with the supply of fermented Kimchi and chitosan-added fermented Kimchi (Table 2). Indigestible polysaccharides form SCFAs such as acetic acid, propionic acid, and butyric acid through the process of fermentation by intestinal microbes [40]. Butyric acid which is absorbed into blood steam promotes the decomposition of fat into fat cells and regulates the actions of gut hormones and insulin to suppress fat accumulation [15, 20, 33]. Thus, the increase in butyric acid-producing bacteria in the present study might be responsible for the reduction of blood triglycerides concentrations.

The supply of fermented Kimchi and chitosan-added fermented Kimchi reduced feed efficiency and blood triglyceride concentrations. In addition, it promoted the diversity of intestinal microorganisms and increased the abundance of LAB, leanness associated and butyric acid-producing bacteria. Furthermore, LAB increased the most by supplementation of the chitosan-added fermented Kimchi in addition with *Bifidobacterim*. Therefore, the results of the present study support the hypothesis that fermented Kimchi and chitosan-added fermented Kimchi can suppress obesity through mechanism(s) that might involve the modulation of the intestinal microbiota.

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초록 : 발효 김치가 흰쥐의 장내 미생물 형성에 미치는 영향

안수진[1†] · 김재영[2†] · 김인성[1†] · 비슈누 아디카리[3] · 유다윤[1] · 김정아[1] · 권영민[3,4] · 이상석[5] · 최인순[6] · 조광근[1*]
([1]경남과학기술대학교 동물소재공학과, [2]경남과학기술대학교 양돈과학기술센터, [3]아칸소대학교 가금류학과, [4]아칸소대학교 세포 및 분자 생물학 프로그램, [5]순천대학교 동물자원과학과, [6]신라대학교 생명과학과)

장내 미생물은 숙주의 건강을 유지하는 데 중요한 역할을 하며, 식단에 의하여직접적으로 영향을 받아 조절된다. 김치는 식이 섬유와 젖산균(LAB)이 풍부한 발효 식품이다. 발효 김치가 장내 미생물의 구성에 미치는 영향을 조사하기 위하여 6주령의 수컷 Sprague-Dawley 흰쥐 45마리를 대상으로 기본 사료(CON), 발효 김치(FK)와 키토산 첨가 발효 김치(CFK)를 각각 4주간 급여 하였다. 체중과 사료 섭취량을 매주 측정하였으며, 미생물 분석은 장내용물 수집 후 pyrosequencing을 통하여 16S rRNA 유전자 분석으로 확인 하였다. FK 및 CFK군은 대조군에 비해 체중, 사료 효율 및 혈중 triglyceride 농도가 감소한 것으로 나타났다. 장내 미생물의 다양성은 대조군에 비해 FK와 CFK군 모두에서 증가하였다. 비만과 관련된 *Firmicutes* 미생물이 감소한 반면, 체중 감소와 관련된 Bacteroidetes 미생물이 증가하였다. 젖산균과 체중 감소 관련 박테리아 및 butyrate 생산 박테리아는 대조군에 비해 FK 및 CFK군에서 증가하였다. 발효 김치는 비만을 억제하고 장내 유익한 미생물의 성장을 촉진하였다.