

## Changes in Korean Adult Females' Intestinal Microbiota Resulting from Kimchi Intake

Kim JY<sup>1</sup>, Choi EY<sup>2</sup>, Hong YH<sup>3</sup>, Song YO<sup>4</sup>, Han JS<sup>4</sup>, Lee SS<sup>5</sup>, Han ES<sup>6</sup>, Kim TW<sup>6</sup>, Choi IS<sup>2</sup> and Cho KK<sup>3\*</sup>

<sup>1</sup>Swine Science and Technology Center, Gyeongsang National University of Science and Technology, Jinju, Gyeongsang, Republic of Korea

<sup>2</sup>Department of Biological Sciences, Silla University, Busan, Republic of Korea

<sup>3</sup>Department of Animal Resources Technology, Gyeongsang National University of Science and Technology, Jinju, Gyeongsang, Republic of Korea

<sup>4</sup>Department of Food Science and Nutrition, Pusan National University, Busan, Republic of Korea

<sup>5</sup>Department of Animal Science and Technology, Suncheon National University, Suncheon Republic of Korea

<sup>6</sup>World Institute of Kimchi, Kwang Ju, Republic of Korea

\*Corresponding author: Cho KK, Department of Animal Resources Technology, Gyeongsang National University of Science and Technology, Chinju 660 - 758, Republic of Korea, Tel: +82-55-751-3286; Fax: +82-55-751-3689; E-mail: [chotwo2@gntech.ac.kr](mailto:chotwo2@gntech.ac.kr)

Received date: Feb 22, 2016; Accepted date: Mar 28, 2016; Published date: Mar 31, 2016

Copyright: © 2016 Kim JY, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

### Abstract

Intestinal microbiota can be changed by diet and its composition is directly related to an individual's health. In the present study, the effects of kimchi as a synbiotics on adult females' intestinal microbiota were examined.

One hundred male and female study applicants were invited to apply; of those 12 females met the study criteria and were chosen as study participants. The 12 females were divided into a low kimchi intake group (15 g/day, 15% of the average Korean daily kimchi intake) and a high kimchi intake group (150 g/day) based on the amount kimchi intake, and they were provided with boarding for 7 days.

To analyze the intestinal microorganisms, feces samples from 12 female participants were obtained. The 16S ribosomal RNA genes of the microorganisms in those samples were examined using customized microarray chips to identify 702 species of intestinal microorganisms in 17 phyla. In the high kimchi group, the percentage of 16 species of microorganisms, including Gammaproteobacteria containing many pathogenic microorganisms decreased to less than half the percentage and the percentage of 18 species microorganisms, including 6 species of kimchi-dominant fermenting microorganisms, such as *Leuconostoc mesenteroides*, increased to at least twice. Changes in the intestinal microbiota based on kimchi intake were examined hourly for 7 days using quantitative real-time PCR. Among the 12 species of kimchi-dominant microorganisms, 3 species of lactic acid bacteria, including *Leuconostoc mesenteroides*, increased in the high intake group.

These study results indicate that kimchi intake affected the formation of intestinal microbiota. Although personal differences were observed in the individual participants, kimchi was shown to affect the formation of intestinal microbiota and to be beneficial as a healthy synbiotics.

**Keywords:** Kimchi; Intestinal microbiota; Microarray chip; Synbiotics

### Introduction

Kimchi is a traditional Korean lactic acid bacteria fermented food that is made by mixing natural ingredients containing physiologically diverse bioactive substances. The types of kimchi vary dependently on the types of vegetables (Chinese cabbage, Chinese radish, cucumber, etc.) and condiments (chili, scallion, garlic, ginger, onion, sesame seeds, marine products, etc.) used [1]. Kimchi is distinguished from pickles in the West, which are salted foods, kimchi in Japan, pàocài which are fermented vegetables in China, and sauerkraut which is fermented cabbage in the West. Kimchi is defined as a food made by salting the main material, which is often Chinese cabbage or another vegetable, then mixing that ingredient with condiments, and fermenting it [2]. The main materials and condiments used to make kimchi are rich in many nutritive components, including vitamins and

dietary fibers as well as health enhancing functional materials. Therefore, kimchi has excellent anti-cancer, anti-oxidative, anti-arteriosclerotic, and anti-obesity effects and, in 2006, it was selected as one of five top healthy foods by the 'Health', a US magazine specializing in health (<http://www.health.com/>) [3-6].

Prebiotics as food components should not be absorbed or hydrolyzed in the intestines. The prebiotics should stimulate the growth of potentially beneficial commensal bacteria in the intestines, and should promote the host health following the formation of intestinal microbiota [7]. Kimchi contains rich dietary fibers because its main ingredient is vegetables. Since dietary fibers promote the growth of certain probiotic bacteria *in vitro*, they are considered to act as prebiotics [8,9]. Kimchi fermentation occurs due to the microorganisms that are found in the materials used to make it. Lactic acid bacteria (LAB) are one of the main microorganism species that plays important roles in kimchi fermentation processes [10,11]. In the early stages of kimchi fermentation, many types of microorganisms that originate from the ingredients form the microbiota in kimchi. As

fermentation progresses, the dominant microbiota is formed by which fall under the order Lactobacillales such as the bacterial species *Welsella*, *Leuconostoc*, and *lactobacillus* [1]. After ingesting fermented kimchi, LAB, which has strong acid resistance, moves into the large intestine through the stomach so that the kimchi act as a probiotics.

The analytical methods of the 16S ribosomal RNA (rRNA) genes of fecal microorganisms can reveal the diversity of human intestinal microorganisms to the species level and identify the microorganism's phyletic evolution [12]. As for the phyletic speciation of intestinal microorganisms, although a limited number of dominant microorganism species commonly reside in the gut [13]; individual differences are an important factor for the formation of certain types of microbiota [14]. The microorganisms that form the dominant species in the large intestine are affected by the types of food ingested and these dominant species are directly related to personal health [15,16]. Through their interactions with hosts, intestinal microorganisms are involved in nutrition, metabolic processes, resistance to pathogenic microorganisms, and immune response regulation [17-20]. The formation of intestinal dominant bacteria is affected by not only foods but also by age, antibiotics, probiotics, and prebiotics [21-23]. Intestinal health is determined by the dominant bacteria and it is connected to the overall quality of human health.

Although many studies have reported on the changes in the microbiota that are involved in the process of kimchi fermentation after the kimchi has been manufactured, few studies have reported on the changes in human intestinal microbiota following the intake of kimchi as a synbiotic food. Therefore, the present study will examine the changes that kimchi intake causes in the formation of human intestinal microbiota using microarray chips of 16S rRNA genes of the microorganisms.

## Materials and Methods

### Neutral detergent fiber (NDF) analysis

One gram of the kimchi sample was pulverized using a 1 mm screen and then it was placed into a Berzelius beaker. Next, 100 ml of neutral detergent solution, 2 ml of decaline, and 2 g of sodium sulfite were put into the beaker and the beaker was heated and maintained at boiling point temperature for 60 min. The heated mixture was washed three times using 80°C - 90°C distilled water and then it was filtered. Thereafter, the mixture was again washed twice using acetone and then it was filtered. Finally, the mixture was dried and weighed. The neutral detergent fiber percentage (%) was obtained using the following equation [24].

$$\text{NDF (\%)} = [(\text{crucible after treatment} + \text{sample weight}) - \text{crucible weight}] / \text{sample weight} \times 100$$

### Acid detergent fiber (ADF) analysis

One gram of the kimchi sample was placed into a beaker, and then 100 ml of acid detergent solution and 2 ml of decaline were added. The beaker was then heated and maintained at boiling point temperature for 60 min. The heated mixture was washed three times using 80°C - 90°C distilled water and then it was filtered. Thereafter, the mixture was washed twice using acetone and then it was filtered. The mixture was completely filtered using hexane and the dried weight was measured. The acid detergent fiber percentage (%) was obtained using the following equation [24].

$$\text{ADF (\%)} = [(\text{residual quantity after drying} + \text{crucible weight}) - \text{crucible weight}] / \text{sample weight} \times 100$$

### Clinical trial

One hundred Korean females and males at Pusan National University, ranging in age from 20 to 30, were recruited to participate in the study through posters and e-mails. All of the recruited individuals were informed about the content of the experiment and written consent for their participation was received. The clinical trial was conducted after being approved by the Institutional Review Board (IRB) of Pusan National University Hospital (approval number 2011076). As part of the study, the participants that met the inclusion criteria (12 females) were provided with boarding in the same facility for 7 days. The selective criteria of volunteers are non-alcohol drinking and non-smoking people who did not have antibiotics within 1 month of starting this experiment. Those participants came to the dormitory with an empty stomach on the day the experiment began and they were divided into a low kimchi intake group (15 g/day, 15% of the average Korean kimchi intake) and a high kimchi intake group (150 g/day, 150% of the average Korean kimchi intake) based on the amount of kimchi ingested with restricting exercise and other dietary intake. The participants were supplied with Chinese cabbage kimchi, which was made by mixing Chinese cabbage as the main ingredient with seasoning (dried red pepper powder, onion, garlic, salted seafood, etc.) and fermenting it for 14-16 days at 4°C.

### Separation of the genomic DNA of the microorganisms in the feces and 16S rRNA gene amplification

The twelve females who met the inclusion criteria were selected and divided into two groups: six in the low kimchi intake group and six in the high kimchi intake group. Feces samples were taken from these 12 study participants once a day, for a total of six times during the seven day boarding periods. The genomic DNA (gDNA) of the intestinal microorganisms was extracted from the feces samples using a ZR Fecal DNA MiniPrep™ kit (Zymo Research, Irvine, CA, USA) in the method described in product manual provided by the manufacturer. PCR was performed using the gDNA to amplify the 16S rRNA genes.

Universal primers 27F (5'-AGAGTTTGTATCMTGGCTCAG-3') and T7 (5'-TCTAATACGACTCACTATAGGGGGYTACCTTGTTACGACTT-3') were used. For the PCR conditions, 100 ng of gDNA was denatured for 5 min at 95°C, then it underwent 30 cycles of 30 sec each at 94°C, followed by 30 cycles of 30 sec each at 58°C, and then 30 cycles of 90 sec each at 72°C, after which it was elongated for 5 min at 72°C.

### Microarray chip production

Genome-Sequencer FLX (454 Life Sciences, Roche, Switzerland) was implemented to secure the 16S rRNA genes of the general intestinal microorganisms, LAB, and harmful microorganisms through the Ribosomal RNA Database Project (RDP-II), National Center for Biotechnology Information (NCBI) GenBank database. Based on the secured 16S rRNA data, probes were designed for the genes of a total 751 species of microorganisms, including 583 species of general intestinal microorganisms, 100 species of LAB, 10 species of pathogenic microorganisms, and 58 species of other microorganisms (Supplementary Table 1). The microarray chips were purchased from MacroGen (Seoul, Korea).

## Microarray analysis

The biotin-labeled RNAs were made from 200 ng of the PCR products using MEGAScript T7 *in vitro* transcription kits (Ambion, Austin, TX, USA), Biotin-11-CTP, and Biotin-11-UTP. The biotin-labeled RNAs were purified using MEGAclean™ kits (Ambion, Austin, TX, USA), and the concentration was identified using NanoDrops (NanoDrop 2000, Thermo Scientific, USA). The microarray chips were analyzed using CustomArray™ 4X2K Microarrays (CombiMatrix Corp., CA, USA) and the experiments were conducted according to the instructions provided by the manufacture. That method is as follows. The chips were pre-heated for 10 min at 65°C and made to react in a pre-hybridization solution (6X SSPE, 0.05% Tween20, 20 mM EDTA, 5X Denhardt's solution, 100 ng/L denatured salmon sperm DNA, 0.05% sodium dodecyl sulfate) for 30 min. Then, 1 µg of the RNA fragmentation was dissolved in a hybridization solution (6X SSPE, 0.05% Tween20, 20 mM EDTA, 25% formamide, 100 ng/µl salmon sperm DNA, 0.04% SDS). Denatured for 5 min at 95°C, and hybridized for 16 hrs at 45°C in order to wash the RNA fragmentation for 5 min. Next, the 6X SSPET solutions (6X SSPE, 0.05% Tween20) were pre-heated to 45°C in order to wash the RNA fragmentation for 5 min. The RNA fragmentation was then washed with the 3X SSPET solutions for 1 min at room temperature, with the 0.5X SSPET solutions for 1 min at room temperature, and then it was washed with PBST (2X phosphate buffer, 0.1% Tween20) for 1 min at room temperature. Thereafter, the RNA fragmentation was left unattended in a biotin blocking solution (2X PBS, 0.1% Tween20, 1% acetylated BSA) at room temperature. Then, 1 mg/ml of Fluorolink™Cy5<sup>2</sup>-labeled streptavidin was diluted in the biotin blocking solution at 1:1000 for biotin labeling at room temperature. Next, the RNA fragmentation was washed with PBST (2X phosphate buffer, 0.1% Tween20) and PBS twice respectively at room temperature. Using an Axon Genepix 4200A microarray scanner (Axon Instruments Inc., Union City, CA, USA), the color was scanned at PMT 200-300 and a resolution of 5 mm. The DNA chip data were analyzed using a microarray imager (CombiMatrix, Irvine, CA, USA) (Supplementary Figure 1).

## Quantitative real-time PCR

To check changes in the LAB found in the intestinal microbiota over time, quantitative real-time PCR (qPCR) was conducted with microorganism gDNA taken from the feces samples that were obtained from the participants for 6 days [the samples on the last day of the study period (day 7) were not examined]. Specific primers for 12 species of LAB, including *Leuconostoc mesenteroides*, *Leuconostoc citreum*, and *Weissella cibaria* were used; a universal primer was used as an internal control (Supplementary Table 2). The number of cycles for the non-saturating PCR condition was determined through a preliminary experiment. The PCR was conducted as follows: 1 cycle for 2 min at 98°C and 45 cycles for 5 sec at 98°C and for 30 sec at 60°C.

## Statistical analysis

All of the experimental results were analyzed using the General Linear Model of SAS (1999), and t-tests and Tukey's multiple-range test were conducted for comparison between the averages of the treatments.

## Results

### Analysis of fiber components of kimchi

To analyze the effects of kimchi intake on the formation of intestinal microbiota, the water content, water soluble carbohydrate content, and fiber content of the kimchi used in the present experiment were examined. Among the possible types of fibers, NDF and ADF were analyzed. Water (87%) was the largest component in kimchi and the water soluble carbohydrates (WSC) that were present were mainly monosaccharides, such as glucose, sucrose, fructose, maltose, and lactose. The percentage of the fiber content in the kimchi was: neutral detergent insoluble fibers (28%) and acid detergent insoluble fibers (17%), (Table 1).

Analysis	Mean (%)	Standard error
WSC <sup>1</sup>	4.65	0.05
NDF <sup>2</sup>	28.2	2.204
ADF <sup>3</sup>	17.12	1.073
Moisture	87.61	0.098

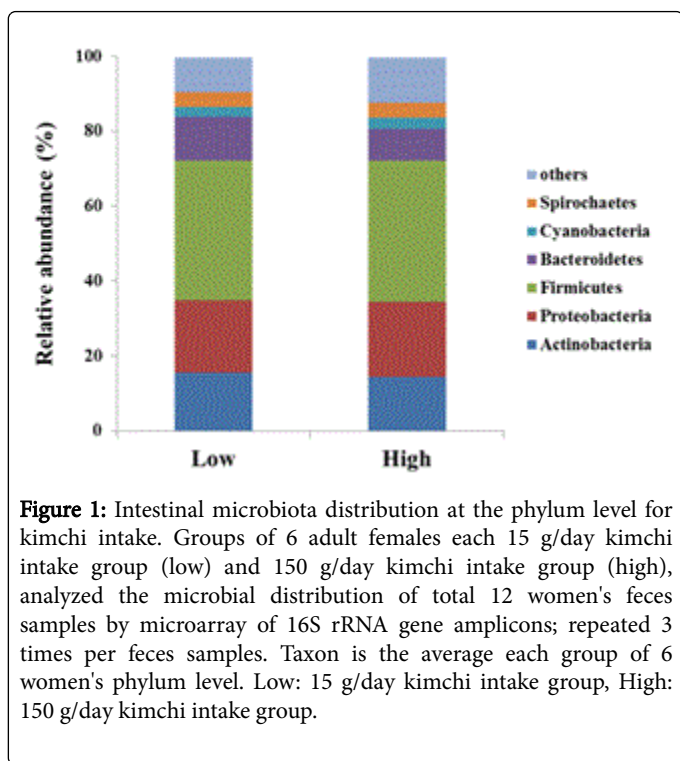
**Table 1:** Fiber components of kimchi (Unit: %), <sup>1</sup>WSC: Water-soluble carbohydrate, <sup>2</sup>NDF: Neutral Detergent Fiber, <sup>3</sup>ADF: Acid Detergent Fiber, repeated four times.

The NDFs consist of cellulose, hemicellulose, and lignin. The ADFs do not contain hemicellulose, but they do consist of 10% cellulose and 18% lignin. Those fibers as NDF and ADFs are not non-digestible substances and they affect the formation of microbiota in the large intestine as prebiotics used by the intestinal microorganisms.

### Analysis of the microarray chips of intestinal microbiota according to kimchi intake levels

The feces samples were collected sixth day of the experiment and differences in the intestinal microorganisms between the two types of kimchi intake levels were analyzed. Six of the females were from the low kimchi intake group and six were from the high kimchi intake group.

Among the 751 species in the microarray chips, 702 species in 17 phyla (Figure 1) of intestinal microorganisms were identified through the feces samples taken from both the low kimchi intake group and the high kimchi intake group (Supplementary Table 3). The diversity of the microorganism species in the low kimchi intake group and the high kimchi intake group was the same; the samples from both treatment groups contained 301 species of Firmicutes, which was the largest of species in the samples, followed by 197 species of Proteobacteria, 105 species of Bacteroidetes, 58 species of Actinobacteria, and 41 species of other intestinal microorganisms. With regard to the percentages of the microorganisms in the feces samples, Actinobacteria, Proteobacteria, Firmicutes, and Bacteroidetes comprised the majority of the intestinal microorganisms in both treatment groups. Firmicutes and Bacteroidetes, which are related to obesity, accounted for 48% of the microorganisms in the low kimchi intake group and 45% of the microorganisms in the high intake group.



As the amount of kimchi intake increased, the percentage of Firmicutes did not change, but the percentage of Bacteroidetes decreased from 11.6% to 8.5%, which shows a difference in the microorganism distribution percentages between the two treatment groups (Supplementary Table 4).

To examine show the amount of kimchi intake impacts the distribution of beneficial or harmful intestinal microorganisms; the phylotypes in the genus (the hybridization signals of which were identified in the microarray chip) were classified into beneficial microorganisms and harmful microorganisms (Table 2).

Items	Species	Low (%)	High (%)
Harmful or beneficial	<i>Bacteroides</i>	6.174 <sup>a</sup>	2.805
	<i>Eubacterium</i>	0.778	0.778
	<i>Enterococcus</i>	0.671	1.077
Potentially Harmful	<i>Listeria</i>	5.187	3.916
	<i>Clostridium</i>	5.718	5.385
	<i>Pseudomonas</i>	1.129	1.33

	<i>Salmonella</i>	0.125	0.152
	<i>Enterobacter</i>	1.39	0.979
	<i>Prevotella</i>	1.47	0.782
	<i>Shigella</i>	2.822	2.672
	<i>Staphylococcus</i>	0.105	0.072
Potentially beneficial	<i>Bifidobacterium</i>	0.016	0.02
	<i>Lactobacillus</i>	10.616	11.55
	<i>Leuconostoc</i>	0.074	0.171
	<i>Streptococcus</i>	1.481	1.181
	<i>Pediococcus</i>	0.233	0.223

**Table 2:** Analysis of the genus level of beneficial or harmful microorganisms in the feces sample by kimchi intake level. <sup>a</sup>Combined percent contribution of phylo-species in each bacterial genus to the total hybridization signal measured by microbiota microarray. Low; 15 g/day kimchi intake group (n = 6), High; 150 g/day kimchi intake group (n = 6).

The percentage of *Listeria*, *Clostridium*, *Enterobacter*, *Prevotella*, and *Shigella*, which are classified as harmful microorganisms, decreased in the high intake group compared to the low intake group. Increases in the amount of kimchi intake suppressed the colonization of harmful intestinal microorganisms. The percentage of *Enterococcus*, which could be beneficial or harmful, increased as the amount of kimchi intake increased while the percentage of *Bacteroides* decreased and the percentage of *Eubacterium* did not change. No differences in the percentage of microorganisms that are classified as being beneficial were found between the two levels of kimchi intake.

Statistical analyses were conducted using t-tests to analyze the differences in the changes in the percentage of intestinal microorganisms following changes in the kimchi intake levels between the low intake group and the high intake group. A total of 34 species of intestinal microorganisms showed significant differences in terms of changes in their percentages (increasing / decreasing) between the two groups (p < 0.05). Among the 34 species of microorganisms, the percentage of 16 species decreased to less than half of the percentage of the species found in the high kimchi intake group and, among them, Gammaproteobacteria, which contain many pathogenic microorganisms, accounted for 37% of the 16 species of microorganisms (Table 3).

Class	Genus	Species	Low <sup>1</sup>	High <sup>1</sup>	High / Low
Alphaproteobacteria	<i>Rhodovibrio</i>	<i>Rhodovibrio salinarum</i>	11.33	10.3	-2.040*
Bacilli	<i>Streptococcus</i>	<i>Streptococcus parauberis</i>	9.965	8.629	-2.525*
Bacteroidetes	<i>Bacteroides</i>	<i>Bacteroides nordii</i>	9.722	8.601	-2.174*
Bacteroidetes	<i>Alistipes</i>	<i>Alistipes onderdonkii</i>	8.796	7.661	-2.196*
Bacteroidetes	<i>Bacteroides</i>	<i>Bacteroides pectinophilus</i>	9.099	8.082	-2.025*

Bacteroidetes	<i>Bacteroides</i>	<i>Bacteroides fragilis</i>	12.86	11.75	-2.157*
Bacteroidetes	<i>Bacteroides</i>	<i>Bacteroides stercoris</i>	11.65	10.55	-2.142*
Clostridia	<i>Phascolarctobacterium</i>	<i>Phascolarctobacterium faecium</i>	7.504	6.228	-2.422*
Epsilonproteobacteria	<i>Arcobacter</i>	<i>Arcobacter halophilus</i>	8.559	7.445	-2.165*
Gammaproteobacteria	<i>Enterbacteriaceae</i>	<i>Enterobacter asburiae</i>	8.137	7.025	-2.162*
Gammaproteobacteria	<i>Enterbacteriaceae</i>	<i>Enterobacter amnigenus</i>	8.398	7.332	-2.093*
Gammaproteobacteria	<i>Pseudomonas</i>	<i>Pseudomonas geniculata</i>	8.884	7.758	-2.182*
Gammaproteobacteria	<i>Saccharospirillum</i>	<i>Saccharospirillum impatiens</i>	7.691	6.592	-2.141*
Gammaproteobacteria	<i>Enterbacteriaceae</i>	<i>Enterobacter cancerogenus</i>	9.442	8.297	-2.211*
Gammaproteobacteria	<i>Enterbacteriaceae</i>	<i>Enterobacter cloacae</i> sub sp. <i>Dissolvens</i>	7.932	6.726	-2.308*
Lactobacillales	<i>Enterococcus</i>	<i>Enterococcus faecalis</i>	9.373	8.191	-2.268*

**Table 3:** Microorganisms decreased by more than two fold at high kimchi intake group, Low: 15 g/day kimchi intake group, High: 150 g/day kimchi intake group. <sup>1</sup>All values are the log<sub>2</sub> transformed values. \*p < 0.05, n = 6.

On the other hand, the percentages of 18 out of the 34 intestinal microorganisms species more than doubled in the high kimchi intake group and *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Lactobacillus mindensis*, *Lactobacillus reuteri*, *Lactobacillus brevis*, *Lactobacillus amylolyticus*, and *Leuconostoc mesenteroides*, which are known to be dominant microorganisms in kimchi, accounted for

38.9% of the 18 species in the high kimchi intake group (Table 4). As the kimchi, fermentation progresses, LAB species, such as *Walsella*, *Leuconostoc*, and *Lactobacillus*, are the dominant microorganisms. As the percentage of dominant microorganisms in kimchi increases in the intestine, the function of kimchi as a probiotic can be expected.

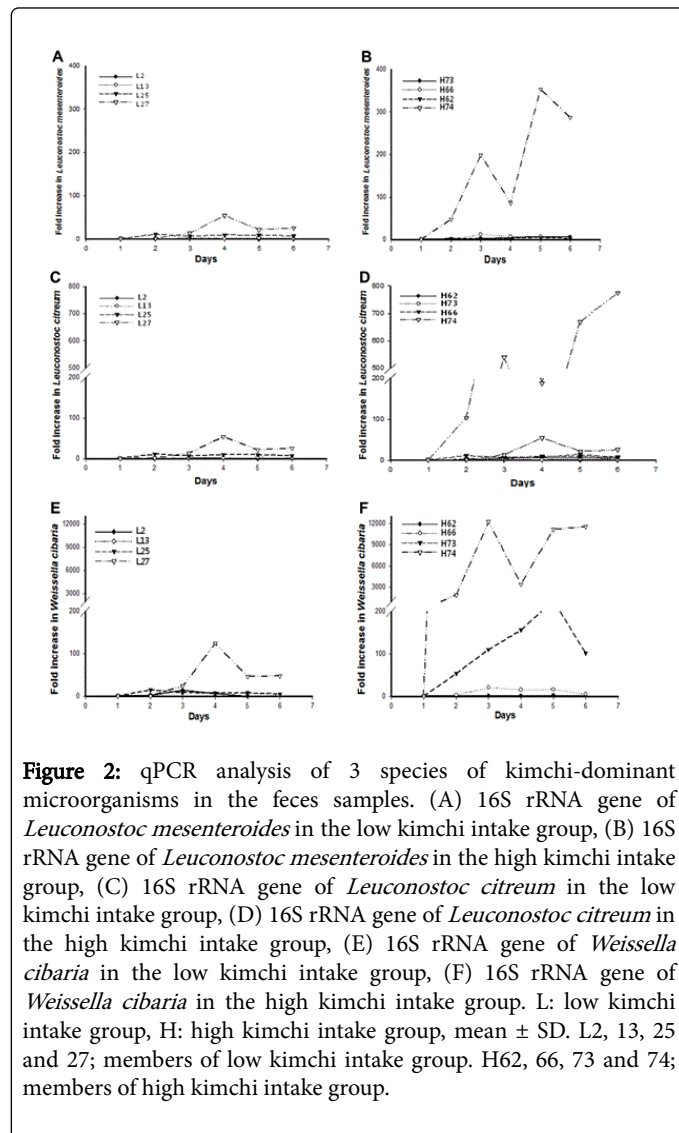
Class	Genus	Species	Low <sup>1</sup>	High <sup>1</sup>	High / Low
Actinobacteria	<i>Bifidobacteria</i>	<i>Bifidobacterium breve</i>	8.471	9.591	2.174*
Alphaproteobacteria	<i>Paracraurococcus</i>	<i>Paracraurococcus ruber</i>	8.723	9.761	2.054*
Alphaproteobacteria	<i>Neorickettsia</i>	<i>Neorickettsia risticii</i>	9.184	10.63	2.717*
Bacilli	<i>Lactobacillus</i>	<i>Lactobacillus acidophilus</i>	7.186	8.281	2.136*
Bacilli	<i>Lactobacillus</i>	<i>Lactobacillus mindensis</i>	7.398	8.793	2.630*
Bacilli	<i>Lactobacillus</i>	<i>Lactobacillus reuteri</i>	8.693	9.855	2.238*
Bacilli	<i>Leuconostoc</i>	<i>Leuconostoc mesenteroides</i>	7.701	8.822	2.174*
Bacilli	<i>Lactobacillus</i>	<i>Lactobacillus brevis</i>	8.852	10.25	2.639*
Bacilli	<i>Lactobacillus</i>	<i>Lactobacillus amylolyticus</i>	6.144	7.301	2.230*
Clostridia	<i>Anaerovibrio</i>	<i>Anaerovibrio lipolyticus</i>	7.918	9.955	4.106*
Clostridia	<i>Lachnospiraceae</i>	<i>Ruminococcus albus</i>	11.7	12.95	2.369*
Clostridia	<i>Lachnospiraceae</i>	<i>Ruminococcus bromii</i>	9.77	10.85	2.113*
Gammaproteobacteria	<i>Halothiobacillus</i>	<i>Halothiobacillus neapolitanus</i>	7.228	8.346	2.171*
Gammaproteobacteria	<i>Thermomonas</i>	<i>Thermomonas brevis</i>	8.748	10.07	2.491*
Lactobacillales	<i>Enterococcus</i>	<i>Enterococcus faecium</i>	11.14	12.61	2.765*
Sphingobacteria	<i>Rapidithrix</i>	<i>Rapidithrix thailandica</i>	76.96	7.987	2.037*
Spirochaetes (class)	<i>Treponema</i>	<i>Treponema bryantii</i>	7.157	8.509	2.552*

Spirochaetes (class)	<i>Treponema</i>	<i>Treponema lecithinolyticum</i>	8.109	9.218	2.157*
----------------------	------------------	-----------------------------------	-------	-------	--------

**Table 4:** Microorganisms increased by more than two fold at high kimchi intake group Low: 15 g/day kimchi intake group, High: 150 g/day kimchi intake group. <sup>1</sup>All values are the log<sub>2</sub> transformed values. \*p < 0.05, n = 6.

### qPCR analysis of LAB in the feces samples

To examine the changes in the intestinal microorganisms over time at different kimchi intake levels, the 16S rRNA genes of the microorganisms were obtained from the feces samples once a day for 7 days, for a total of six times. Then, qPCR was conducted using the 16S rRNA genes, the primers for 12 species of microorganisms including the LAB in the kimchi such as *Leuconostoc mesenteroides*, *Leuconostoc citreum* and *Weissella cibaria*, which are known to be dominant microorganisms in kimchi [1], and universal 16S rRNA primer (Supplementary Table 2). The volunteers who could take their feces samples every day for 7 days were only four of six ones in the low kimchi intake group and the high kimchi intake group, respectively. The qPCR could be conducted only four of them in each group.



The increase in the ratio of *Leuconostoc mesenteroides*, *Leuconostoc citreum*, and *Weissella cibaria* was clearly greater in the high kimchi intake group than in the low kimchi intake group, but no difference was found in the ratios of the other microorganisms between the two intake groups (Figure 2). The ratio of *Leuconostoc mesenteroides* in the feces samples increased by at least five times (and up to 350 times) in the high intake group compared to the low kimchi intake group (Figure 2). However, one of the participants in the low intake group (L27) showed a greater increase in the ratio of *Leuconostoc mesenteroides* than the other participants in that group. The ratios of the increases in *Leuconostoc citreum* and *Weissella cibaria* were also higher in the high intake group compared to the low kimchi intake group (Figure 2). The differences in increases in the rate of the microorganisms among the individuals in the high kimchi intake group were large and the rate of *Leuconostoc mesenteroides* increased significantly in one of the participants in the low intake group (L27). These findings show that differences among individuals are an important factor in the formation of intestinal microbiota [14]. The increases in the rates of the three species of kimchi-dominant microorganisms in the high kimchi intake group, as demonstrated by the qPCR results, are considered to be attributable to the effects of the microorganism transition that resulted from increases in the kimchi intake.

### Discussion

Kimchi is beneficial to health as a synbiotic food. It has the functionality of prebiotics because it contains dietary fibers found in Chinese cabbage, which is the main ingredient, and the functionality of probiotics due to the LAB formed in the process of kimchi fermentation. Studies of the physiological efficacy of kimchi were conducted when researchers first began to investigate kimchi and studies of the changes in the microbiota that naturally exist in kimchi following kimchi fermentation have been conducted recently [1]. The studies of microbiota in kimchi enabled researchers to investigate the numerous microorganisms that live in microorganism ecosystems in certain environments, simultaneously, by using next-generation sequencing technologies that use microarray technology or pyrosequencing to analyze the 16S rRNA genes of microorganisms [25]. However, no study has yet examined changes in human intestinal microorganisms related to kimchi intake. In the present study, we asked healthy Korean adult females to ingest kimchi at different levels for one week while restricting their exercise and other types of food except a supply, and we collected feces samples once every day to examine the effect of kimchi intake on human intestinal microbiota using microarray chips and qPCR.

Food greatly affects the intestinal microbiota and, in particular, non-digestible polysaccharides flow into the large intestine and cause changes in the energy metabolism and the ecosystem of intestinal microorganisms [14-16]. Kimchi contains dietary fibers, as shown in Table 1. Cellulose and hemicellulose are the main dietary fibers found in kimchi. As types of non-digestible polysaccharides, they flow into the large intestine where they act as prebiotic components used by intestinal microorganisms. The non-digestible polysaccharides are

fermented by microorganisms in the large intestines of humans to form short-chain fatty acids, mainly acetate, propionate, and butyrate [26]. The butyrate generated by the microorganisms in the large intestine has anti-inflammatory properties and suppresses colon cancer [25,26]. Butyrate, which is a volatile fatty acid, is a major energy source of large intestinal epithelial cells and, after being absorbed by those cells, it activates growth factor TGF- $\beta$ , which regulates cell growth and differentiation, and Smad3, which is an important mediator in the signaling system. It also suppresses histone deacetylation and cancer cell proliferation by regulating the expression of genes, such as p21. In addition, it induces apoptosis with Ca<sup>2+</sup> accumulation in mitochondria [26-28]. The ecosystem of intestinal microorganisms also shows correlations with obesity [13,17]. As such, human health and the ecosystem of intestinal microorganisms are closely related and intestinal microbiota is affected by a person's eating habits and intake of dietary fiber. Therefore, kimchi which primarily consists of fermented vegetables is considered to be capable of acting as a prebiotic due to its rich non-digestible polysaccharides [29].

A total of 702 species of microorganisms in 17 phyla were identified in the feces samples from the 12 study participants who ingested kimchi at different intake levels (Figure 1 and Supplementary Table 3). While no differences were found in the diversity of the intestinal microorganisms between the low kimchi intake group and the high kimchi intake group (as identified through the feces samples), differences in the percentages of the distribution of the microorganisms were found. It can be seen that the kimchi intake level affects the formation of the intestinal microbiota. The intestinal microorganism microarray analysis method used in the present study identified at least three times more species of microorganisms as compared to the human intestinal microbiota microarray developed by Paliy et al. [30]. Therefore, it can be seen that the customized microarray chips used in the present study are more accurate and sensitive.

When the intestinal microorganisms identified in the feces samples were differentiated into beneficial microorganisms and harmful microorganisms on the basis of genus, the percentages of *Listeria*, *Clostridium*, *Enterobacter*, *Prevotella*, and *Shigella*, which are classified as harmful microorganisms, decreased in the high kimchi intake group compared to the low kimchi intake group. On the other hand, the percentage of *Bifidobacterium*, which is a beneficial microorganism, and *Leuconostoc*, which is a dominant microorganism in kimchi, increased in the high kimchi intake group (Table 2). These experimental results indicate that, in terms of the overall level of the microorganisms in the large intestine, kimchi intake suppresses the formation of harmful microbiota in the intestines and promotes the formation of beneficial microbiota to help support human health. The LABs to be the dominant microorganisms in the process of kimchi fermentation secrete bacteriocins which are natural antibiotics to suppress the growth of the harmful bacteria and other harmful microorganisms. In kimchi, among the LAB that comprise the dominant microorganisms in the process of fermentation, *Lactobacillus* and *Leuconostoc* produce many kinds of bacteriocins to suppress the growth of other microorganisms [11,30]. The bacteriocins produced by *Lactobacillus* and *Leuconostoc* increased in the high kimchi intake group in the present study are considered to suppress pathogenic intestinal bacteria.

*Bifidobacterium breve*, *Lactobacillus acidophilus*, *Lactobacillus mindensis*, *Lactobacillus reuteri*, *Lactobacillus brevis*, *Lactobacillus amylolyticus*, and *Leuconostoc mesenteroides* are the types of

microorganisms that are dominant in kimchi [1]. The percentages of these microorganisms increased in the high kimchi intake group by at least twice the percentage that was found in the low kimchi intake group. The analysis of the feces samples showed that they were transferred from the kimchi to the large intestine (Table 4). *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, and *Bifidobacterium* are widely used commercially. These LABs generate digestive enzymes, such as amylase, cellulase, lipase, and protease, to promote digestion and absorption and they induce the formation of IgA and gamma interferon in blood to enhance the level of immunity [17,31]. In addition, *Bifidobacterium longum* and *Lactobacillus acidophilus* reduce cholesterol levels. The increase in kimchi-dominant microorganisms in the high kimchi intake group demonstrates that kimchi acts as a probiotic.

*Firmicutes* and *Bacteroidetes* are correlated with obesity [32]. The percentages of these microorganisms increase in obese individuals while they decreased in skinny individuals. In the present study, when 17 phyla of intestinal microorganisms were analyzed after kimchi intake, the microorganisms in which the percentages increased were compared with the percentages that decreased. The results showed that the percentage of *Bacteroidetes* decreased in the high kimchi intake group. This finding is contrary to the results of previous studies in terms of kimchi's anti-obesity effects. However, similar results can be seen in the study conducted by Arumugam et al. [33]. The possible reason of the differences in the finding is that the anti-obesity effects of kimchi may arise from the capsaicin in the kimchi rather than the *Firmicutes* and *Bacteroidetes*. The capsaicin in chili that results in the hot taste of kimchi plays an important role in obesity treatment and prevention. Capsaicin acts to promote energy metabolism, the decomposition and burning of body fat. Capsaicin stimulates parasympathetic nerves and increases the secretion of adrenalin [34]. Since these actions promote lipolysis in white adipose tissues and increase heat production in brown fat cells that are an energy consuming organ in the body, they decompose and burn body fat to reduce the amount of fat accumulated in the body [35].

An individual's diet affects the formation of intestinal microbiota, which changes significantly over time; moreover, changes in a person's diet can result in rapid changes in the intestinal microbiota over a short period of time [14]. Feces samples were collected from the 8 study participants for seven days to examine changes in the intestinal microorganisms based on kimchi intake levels using qPCR (Figure 2). To conduct qPCR, a pair of the microorganisms' 16S rRNA gene primers, four pairs of intestinal anaerobic microorganism primers, and eight pairs of kimchi-dominant microorganism primers (Supplementary Table 2) were used. The qPCR of 12 microorganisms was conducted and the results indicated that the ratios of *Leuconostoc mesenteroides*, *Leuconostoc citreum* and *Weissella cibaria* increased in the high kimchi intake group. Changes in the intestinal microorganisms identified through the feces samples were clearly identified from day 2 of the kimchi intake. The microorganisms in the kimchi originated in the raw materials used to make kimchi, which contain many environmental microorganisms including soil microorganisms. Even if the raw materials are washed before the kimchi is produced, some of these environmental microorganisms remain attached to the raw materials act on the kimchi fermentation. Although many species of microorganisms exist in the early stage of kimchi fermentation, as the fermentation progresses the microorganisms' ecosystems are densely populated with LAB [1]. The increases in the rates of these fermenting microorganisms in the large intestine are considered to be directly metastasized, and the increases

in the rates of these kimchi-dominant microorganisms in the large intestine are identical in the microarray chip and qPCR analysis results. The experiment results of some of the study participants indicate that changes in intestinal microbiota are greatly affected by not only kimchi intake but also by individual differences. From these findings, it can be seen that kimchi intake can act as a probiotic because the fermenting microorganisms that form the dominant components of microbiota in the process of kimchi fermentation are metastasized to the large intestine.

In the present study in order to check whether kimchi functions as a synbiotic, changes in the intestinal microorganisms of adult females based on the kimchi intake level were examined through the microarray of 16S rRNA genes and qPCR. Kimchi intake suppressed the proliferation of harmful intestinal microorganisms while promoting the proliferation of beneficial microorganisms. These changes in intestinal microorganisms formed in the kimchi during fermentation. These changes in intestinal microbiota are considered to have been affected by the dietary fiber in the kimchi and the transition of the dominant microorganisms formed in the kimchi during fermentation. These results demonstrate the reasons why kimchi intake causes changes in intestinal microbiota. The metagenomic analysis of the intestinal microbiota is considered to have added data to the limited data on the bioactive effects of kimchi as a synbiotic food.

## Acknowledgement

This research was supported by Priority Research Centers Program through the Ministry of Education, Science, and Technology (2012-0006683), the Ministry of Knowledge Economy, Korea for Advancement of Technology, and Honam Leading Industry Office through the Leading Industry Development for Economic Region (R0001947), and Globalization Research Division, World Institute of Kimchi.

## References

1. Park EJ, Chun J, Cha CJ, Park WS, Jeon CO, et al. (2012) Bacterial community analysis during fermentation of ten representative kinds of kimchi with barcoded pyrosequencing. *Food Microbiol* 30: 197-204.
2. CAC (2001) Codex Standard for Kimchi (CODEX STAN 223-2001). Codex Alimentarius Commission, Rome, Italy.
3. Cheigh HS, Song ES, Jeon YS (1999) Changes of chemical and antioxidative characteristics of chlorophylls in the model system of mustard leaf kimchi during fermentation. *J Korean Soc Food Sci Nutr* 28: 520-525.
4. Choi MJ, Song YS, Song YO (1998) Antioxidative effect of kimchi ingredients on rabbits fed cholesterol diet. *J Korean Soc Food Sci Nutr* 27: 1189-1196.
5. Choi SM, Jeon YS, Rhee SH, Park KY (2002) Red pepper powder and kimchi reduce body weight and blood and tissue lipids in rats fed a high fat diet. *Nutraceut Food* 7: 162-167.
6. Park KY (1995) The nutritional evaluation, and antimutagenic and anticancer effects of kimchi. *J Korean Soc Food Nutr* 24: 169-182.
7. Slavin J (2013) Fiber and prebiotics: mechanisms and health benefits. *Nutrients* 5: 1417-1435.
8. Saarinen MT, Lahtinen SJ, Sørensen JF, Tiihonen K, Ouwehand AC, et al. (2012) Treatment of bran containing bread by baking enzymes; effect on the growth of probiotic bacteria on soluble dietary fiber extract in vitro. *Biosci Biotechnol Biochem* 76: 1135-1139.
9. Van Zanten GC, Krych L, Røytiö H, Forssten S, Lahtinen SJ, et al. (2014) Synbiotic *Lactobacillus acidophilus* NCFM and cellobiose does not affect human gut bacterial diversity but increases abundance of lactobacilli, bifidobacteria and branched-chain fatty acid: a randomized, double-blinded cross over trial. *FMES microbiol Ecol* 90: 225-236.
10. Nam YD, Chang HW, Kim KH, Roh SW, Bae JW (2009) Metatranscriptome analysis of lactic acid bacteria during kimchi fermentation with genome-probing microarrays. *Int J Food Microbiol* 130: 140-146.
11. Sieuwerts S, de Bok FA, Hugenholtz J, van Hylckama Vlieg JE (2008) Unraveling microbial interactions in food fermentations: from classical to genomics approaches. *Appl Environ Microbiol* 74: 4997-5007.
12. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, et al. (2005) Diversity of the human intestinal microbial flora. *Science* 308: 1635-1638.
13. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, et al. (2009) A core gut microbiome in obese and lean twins. *Nature* 457: 480-484.
14. Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, et al. (2011) Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME J* 5: 220-230.
15. Flint HJ, Duncan SH, Scott KP, Louis P (2007) Interactions and competition within the microbial community of the human colon: links between diet and health. *Environ Microbiol* 9: 1101-1111.
16. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, et al. (2008) *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *PNAS* 105: 16731-16736.
17. Duerkop BA, Vaishnav S, Hooper LV (2009) Immune responses to the microbiota at the intestinal mucosal surface. *Immunity* 31: 368-376.
18. Ismail AS, Severson KM, Vaishnav S, Behrendt CL, Yu X et al. (2011) Gammadelta intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface. *PNAS* 108: 8743-8748.
19. Human Microbiome Project Consortium (2012) Structure, function and diversity of the healthy human microbiome. *Nature* 486: 207-214.
20. Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI (2008) Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell Host Microbe* 3: 213-223.
21. Collins MD, Gibson GR (1999) Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *Am J Clin Nutr* 69: 1052S-1057S.
22. Jernberg C, Sullivan A, Edlund C, Jansson JK (2005) Monitoring of antibiotic-induced alterations in the human intestinal microflora and detection of probiotic strains by use of terminal restriction fragment length polymorphism. *Appl Environ Microbiol* 71: 501-506.
23. Mariat D, Firmesse O, Levenez F, Guimarães V, Sokol H, et al. (2009) The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol* 9: 123.
24. Ha JK, Lee SS, Koh JY (2003) Laboratory manual for ruminant nutrition: Manufacturing and quality assessment of fodder. (1stedn), Snupress, Seoul, Korea.
25. Mardis ER (2008) The impact of next-generation sequencing technology on genetics. *Trends Genet* 24: 133-141.
26. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, et al. (2008) Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther* 27: 104-119.
27. Veira EL, Leonel AJ, Sad AP, Beltrão NR, Costa TF, et al. (2012) Oral administration of sodium butyrate attenuates inflammation and mucosal lesion in experimental acute ulcerative colitis. *J Nutr Biochem* 23: 430-436.
28. Kolar SS, Barhoumi R, Lupton JR, Chapkin RS (2007) Docosaheaxaenoic acid and butyrate synergistically induce colonocyte apoptosis by enhancing mitochondrial  $Ca^{2+}$  accumulation. *Cancer Res* 67: 5561-5568.
29. Settanni L, Corsetti A (2008) Application of bacteriocins in vegetable food biopreservation. *Int J Food Microbiol* 121: 123-138.
30. Paliy O, Kenche H, Abernathy F, Michail S (2009) High-throughput quantitative analysis of the human intestinal microbiota with a phylogenetic microarray. *Appl Environ Microbiol* 75: 3572-3579.



- 
31. Kato I, Tanaka K, Yokokura T (1999) Lactic acid bacterium potently induces the production of interleukin-12 and interferon-gamma by mouse splenocytes. *Int J Immunopharmacol* 21: 121-131.
  32. Ley RE, Turnbaugh PJ, Klein S, Gordon JI (2006) Microbial ecology: human gut microbes associated with obesity. *Nature* 444: 1022-1023.
  33. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, et al. (2011) Enterotypes of the human gut microbiome. *Nature* 473: 174-180.
  34. Bloomer R, Canale RE, Shastri S, Suvarnapathki S (2010) Effect of oral intake of capsaicinoid beadlets on catecholamine secretion and blood markers of lipolysis in healthy adults: a randomized, placebo controlled, double-blind, cross-over study. *Lipids Health Dis* 29: 72-78.
  35. Kobayashi A, Osaka T, Namba Y, Inoue S, Lee TH, et al. (1998) Capsaicin activates heat loss and heat production simultaneously and independently in rats. *Am J Physiol* 275: R92-R98.