*Journal of Naturopathy Vol. 8(1):1-10*
한국자연치유학회지, 8권 1호, 1-10, 2019년 3월
https://doi.org/10.33562/JN.2019.8.1.1

[Original article]

# Effects of the Proliferation of Beneficial and Harmful Enteric Bacteria after Intake of Soybean Fermentation (Zen) Produced by a Mixture of *Lactobacilli* and *Saccharomyces*

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# *Lactobacilli*와 *Saccharomyces* 혼합균주의 대두발효액(Zen) 섭취 후 장내 유익세균과 유해세균의 증식에 미친 영향

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(Received March 13, 2019 / Revised March 18, 2019 / Accepted March 18, 2019)

**Abstract** **Purpose:** The purpose of this study was to investigate the increase or decrease of important intestinal beneficial bacteria and inhibitory bacteria in 30 stools of clinical subjects after ingesting Zen fermentation broth as a mixed microbial fermentation solution for eight weeks. **Methods:** Intestinal bacteria were identified by PCR amplification using specific primers. **Results:** *Bifidobacterium* genus gi% of test group ingested Zen-fermented broth was 55.15% before and 70.1% after ingestion, so it was a significant difference ($p$<.009). *Lactobacillus* genus of the test group was 46.87% before and 60.91% after ingestion, it was a significant difference ($p$<.01). *Clostridium* genus of the test group was 85.64% before and 65.99% after ingestion. There was a significant difference ($p$<.017) as the pre-post-difference decreased to -19.65%. *Bacteroides* genus of the test group was 17.11% before and 20.22% after ingestion. There was a significant difference ($p$<.048) as the pre-post-difference increased to 3.11%. *Prevotella* genus of the test group was 14.01% before and 16.79% after ingestion, so it was not a significant difference. **Conclusions:** Intestinal bacteria increased the proliferation of beneficial bacteria and suppressed harmful bacteria in the intestines after ingesting the Zen-fermented broth of the mixed microorganism. The Zen fermentation broth evaluated as a beneficial drink for intestinal health.

**Key words** *Lactobacillus Bifidobacterium*, *Clostridium*, Intestinal bacteria, Fermented solution

**초록** **목적:** 본 연구는 임상대상자 30명에게 미생물발효용액인 Zen발효용액을 8주간 섭취시킨 후에 임상대상자들의 변에서 중요한 장내 유익세균 및 저해세균의 증식이 촉진되는지 또는 감소하는지를 연구하는 것이 목적이었다. **방법:** 장내세균은 특정 Primer를 이용하여 PCR 증폭기로 동정 검색하였다. **결과:** Zen발효액을 섭취한 임상군의 *Bifidobacterium* genus 유전자복제지수(gi%)는 섭취 전 수치는 55.15%, 섭취 후에는 70.1%로 나타났으며, 섭취 후에 14.95% 차이로 유의성이 있게 증가하였다. 아래 모든 대조군은 유의성이 없었다. 임상군의 *Lactobacillus* genus지수는 섭취 전이 46.87%, 섭취 후가 60.91%로 나타났으며, 섭취 후에 14.04% 차이로 유의성은 있게 증가하였다($p$<.01). 임상군의 *Clostridium* genus지수는 사전이 85.64%, 사후가 65.99%로 나타났으며, 섭취 후에 -19.65% 차이로 유의성이 있게 감소를 하였다($p$<.017). 임상군의 *Bacteroides* genus지수는 사전이 17.11%, 사후가 20.22%로 나타났으며, 섭취 후에 3.11% 차이로 유의성이 있게 증가하였다. 임상군의 *Prevotella* genus지수는 사전이 14.01%, 사후가 16.79%로 나타났으며, 섭취 후에 2.78%차이로 증가하였으나 유의성은 없었다. **결론:** 장내세균은 혼합미생물의 발효액Zen을 섭취 후에 장내에서 유익균은 증식이 증가하고, 유해균은 억제되는 현상을 발견하였다. Zen발효액은 장 건강에 유익한 음료라 평가한다.

**주제어** *Lactobacillus Bifidobacterium*, *Clostridium*, 장내세균, 발효용액

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## Introduction

Intestinal microorganisms can be classified into *Bifidobacterium, Lactobacillus, Streptococcus, and Clostridium, Eubacterium* and *Veilonella. Bifidobacterium*, a representative bacterium, produces beneficial substances in the intestine, whereas *Clostridium*, a common pest, is a pathogen that is involved in various enterotoxins such as enteritis (Smith, 1979; Bartlett, 2002). Dominguez-Bello *et al*., (2010) reported that intestinal microbes in the neonate were *Lactobacillus* and *Prevotella*, and were similar to maternal microorganisms. Schwartz *et al*., (2012) found that infant intestinal bacteria in breastfeeding are more abundant and heterogeneous in biodiversity than infant formula infants. Koenig *et al*., (2011) suggested that when intestinal microorganisms settled, they would prevent invading pathogens including other viruses and bacteria.

Intestinal microorganisms use interactions that reinforce the immune system, using biocomponents separated from food and digestive tracts while maintaining symbiotic or antagonistic relationships in the intestinal tract of the body (Ley *et al*., 2006; Clemente *et al*., 2012). These intestinal microorganisms highly influenced by human age and diet, and the composition of these microorganisms are known to be deeply related to aging, constipation, and intestinal diseases (Mitsuoka, 1990; Lee *et al*., 2001).

The bacterial populations in the intestine are diverse and need to be classified. Weinstock (2012) developed a method for analyzing the 16S ribosomal RNA gene (rRNA) and analyzing many bacteria in the intestines. 16S rRNA has a conserved region that is common to all species and a hypervariable region that can classify specific species so that microorganism species can be isolated through sequencing. Depending on the phylotype, species correspond to more than 97% of the 16S rRNA, more than 75% of the phylum, more than 80% of the class, more than 85% of the order (90%), and the genus (94%).

A joint expert committee of the World Health Organization (WHO) and the International Food Agency (FAO) has defined probiotics as a good germ as a living microorganism (FAO/WHO, 2001). *Lactobacillus* genus, *Saccharomyces* genus, *Bifidobacterium* genus, and *Bacillus* genus, which produce organic acids and antibiotics, inhibit the growth of pathogens and the overgrowth of harmful bacteria (Oh, 2008).

Prebiotics refers to the nutrients required for the growth of probiotics, including lactinol, lactulose and oligosaccharide (FAO/WHO, 2001). An oligosaccharide is a small molecule of 300~20,000 combined with about 2~10 monosaccharides such as glucose or galactose. It reaches the large intestine without digesting into digestive enzymes in vivo, and *Bifidobacterium,* which is a useful bacterium, can be selectively used (Ku *et al*., 1997). Recently, many forms of lactic acid bacteria and prebiotics have mixed in health foods containing many types of lactic acid bacteria, and these combined products are called synbiotic (Schrezenmeir & Vrese, 2001; Choi *et al*., 2004).

The intake of the several lactic acid bacteria diet inhibits the early development of colon adenomas, and the inhibition of microadenomas results in a reduction of subsequent polyp and tumor yield in the mouse colon.

Harrison & Peat (1975) found that serum cholesterol decreased as the number of *Lactobacillus acidophilus* strains increased in newborns fed artificial feeding, Grunewald (1982) reported that serum cholesterol was lowered when rats fed L. acidophilus fermented milk. Takahashi *et al*., (2005) found that oral administration of Lactobacilli to mice selectively activate CD1d-independent NK1.1+ T cells in the large intestine to produce IFN-gamma and therefore modulate Th1 immune responses.

Odashiro *et al*., (2014) reported that the randomized double-blind clinical trial of LDS ingestion in patients with colonic polyps has confirmed that a 6-months ingestion of 10 ml of LDS per day induced elimination of existing colonic polyps or significant reduction of polyps size in approximatey 60% of the subjects, which is expressed to have significant effects on colonic polyps regression without any side effects.

*B. subtilis* AK fermentation was found to be effective in NK (natural killer) cell activity in rats and human subjects (Takeda *et al*., 2016). Ryu & Lee (2018b) also found that the intake of *B. subtilis* AK fermentation was effective in NK cell activity and bone growth. Thus, the microbial fermentation broth was reported to be beneficial to the health of the human body, thus motivating the study.

The purpose of this study was to investigate whether the beneficial bacteria in the intestinal tract promoted the proliferation or the number of the harmful bacteria decreased in the subjects who consumed the product Zen fermentation broth of twelve kinds of *Lactobacillus* and four kinds of Yeast mixed fermentation solution for Koreans.

## Materials amd Methods

### Subjects, Experimental periods and place

Test subjects consisted of 15 and control subjects are

**Table 1.** General characteristics of Subjects

| Control group | | | | Experimental group | | | |
|---|---|---|---|---|---|---|---|
| No. | Sex | Age | Weight (kg) | No. | Sex | Age | Weight (kg) |
| 1 | M | 59 | 65 | 1 | M | 65 | 60 |
| 2 | M | 58 | 73 | 2 | M | 67 | 62 |
| 3 | M | 56 | 75 | 3 | M | 67 | 65 |
| 4 | M | 52 | 55 | 4 | M | 62 | 62 |
| 5 | M | 67 | 75 | 5 | M | 68 | 65 |
| 6 | M | 45 | 80 | 6 | M | 56 | 67 |
| 7 | M | 70 | 72 | 7 | M | 56 | 67 |
| 8 | M | 50 | 65 | 8 | M | 54 | 58 |
| 9 | M | 52 | 65 | 9 | F | 52 | 53 |
| 10 | F | 50 | 50 | 10 | F | 53 | 59 |
| 11 | F | 53 | 65 | 11 | F | 57 | 49 |
| 12 | F | 64 | 60 | 12 | F | 61 | 52 |
| 13 | F | 57 | 52 | 13 | F | 62 | 58 |
| 14 | F | 59 | 50 | 14 | F | 63 | 52 |
| 15 | F | 70 | 50 | 15 | F | 70 | 53 |
| Mean | 15 | 56.8 | 63.5 | Mean | | 60.9 | 58.8 |

15. The control group consisted of eight males and seven females. There were eight male and seven female subjects in the test group (Table 1). The subjects participated in this study fully understood the purpose of the research and arbitrarily decided to join and participated after all agreed and signed. The study conducted according to the code of ethics.

The research performed from August 26, 2018 to October 26, 2018, for eight weeks. The research plan and venue jointly planned by RSW Dongeu Research Institute (16-1, Uirim Daero 50-gil, Jecheon, Chungbuk, Korea) and Department of Biological Sciences, Konkuk University (120 Neungdongro, Gwangjin-gu, Seoul, Korea). The microorganism assay was commissioned and analyzed by "Kim Seok-jin Probioticslab R&D Institute," (Bio-Eleven Co., Ltd. Teheranro 34 Gil 6 Gangnam-gu, Seoul, Korea).

### Outline of lactic acid bactera-fermented soymilk extract process

The soybeans are poured into water and ground and then heated. Then, the soy milk is squeezed to isolate, and heat sterilize. Then, the sterilized soymilk solution treated with enzymes, and 12 kinds of lactic acid bacteria and four kinds of yeast (Table 2) added, and after the symbiotic fermentation conducted at 30°C for four days, the pH was adjusted, sterilized, and completed. The final product was named Zen fermentation broth (Japan Patent 2009-190661, September 2, 2013).

**Table 2.** Names of symbiotic fermenting 12 lactic acid bacteria and 4 yeasts

| *Bacillus* strains & sources | |
|---|---|
| *Enterococcus feacalis* | *Lactobacillus plantarium* |
| *Lactobacillus acidophilus* | *Lactobacillus salivarius* |
| *Lactobacillus brevis* | *Bifidobacterium breve* |
| *Lactobacillus case* | *Bifidobacterium bifidum* |
| *Lactobacillus heliveticus* | *Bifidobacterium longum* |
| Coccus strains | |
| *Streptococcus lactis* | *Streptococcus thermophilus* |
| Yeast strains | |
| *Saccharomyces cerevisiae* | *Saccharomyces malirisler* |
| *Saccharomyces intermedius* | *Saccharomyces rosei* |

### Ingestion of Zen-fermented broth and preparation of stools

Ten ml of tap water, a similar drink in the morning, was administered to the control group, which was a clinical subject, and 10 ml of Zen fermentation broth (Zen Lot. K2171, Nihon Bio Co., Tokyo, Japan). It was taken before breakfast. After eight weeks of ingestion, the stools were received in sterile tubes and stored in a -20°C freezer for home use and then stored at -80°C in the assay room, and then this sample used for PCR analysis.

### Primers used for PCR

To determine five target intestinal bacteria, PCR primers prepared by amplifying 16S rRNA gene of a specific bacterial group shown in Table 3 (Kook *et al*., 2018).

### Extraction of bacterial DNA from stool samples of subjects

The stools of subjects were collected and frozen after eight weeks of ingestion of Zen fermentation broth and then performed DNA extraction using the frozen seats (Kook *et al*., 2018). Subjects randomly divided into 3 ml of physiological saline (200 mg) taken from the side of the plastic tube kit and homogenized with glass beads (0.1 mm diameter) (Biospec, Orlando, FL). Bacterial DNA was extracted using QIAamp stool DNA extraction kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. After the extracted DNA concentrated, the purity determined by spectrophotometer (NanoDrop Technology, Washington, DE, USA). All extracted DNA stored at -80°C in a cold freezer (Ryu & Lee, 2018).

**Table 3.** Primer sequences and sizes used in PCR

| Bacterial genus | Primer directions | Primer Sequences (5'→3') | PP size (bp) |
|---|---|---|---|
| *Lactobacillus* | forward | AGCAGTAGGGAATCTTCCA | 341 |
| | reverse | CACCGCTACACATGGAG | |
| *Bifidobacterium* | forward | GGGTGGTAATGCCGGATG | 442 |
| | reverse | TAAGCGATGGACTTTCACACC | |
| *Bacteroides* | forward | ATAGCCTTCCGAAAGRAAAGAT | 495 |
| | reverse | CCAGTATCAACTGCAATTTTA | |
| *Clostridium* | forward | CGGTACCTGACTAAGAAGC | 429 |
| | reverse | AGTTTYATTCTTGCGAACG | |
| Universal | forward | TCCTACGGGAGGCAGCAGT | 467 |
| | reverse | GGACTACCAGGGTATCTAATCCTGTT | |

pp size: predicted PCR product size (bp), bp: base pair.
Quoted from Kook *et al*. (2018).

### Identification of intestinal bacteria using a PCR

The target bacteria are shown in Table 4. Bacteria were extracted from the stool samples of the subjects using qPCR methods such as Rinttila *et al*., (2004), Schmittgen & Livak (2008), and Kook *et al*., (2018) respectively. Probe DNAs amplified from 16s rRNA gene of the specific bacterium of Table 4 were mixed with ABI SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) to perform qPCR. After the DNA extracted from the samples concentrated, the purity was measured by spectrophotometer at 230 nm, 260 nm and 280 nm (Nano-Drop Technology, Washington, DE, USA). After PCR, DNAs concentrated by the genus of each bacterium quantitated by the threshold cycle value (ΔCT) of bacteria (Schmittgen & Livak, 2008).

ΔCT values = (CT gene of specific bacterial species – CT of 16s RNA)

In this study, the Bacteria gene index (= ΔCT values) value derived by measuring the expression level of the target gene and the reference gene by the relative quantification method of the gene. The number of beneficial bacteria can be judged to be more or less than the average when bacteria are indexed (%) by comparison with the intestinal bacterial composition data of Koreans accumulated in the 'Kim Seokjin Institute' (Kook *et al*., 2018).

In this study, ΔCT values were expressed as% Bacteria gene copy index (%), expressed as gi%. The number of analyzed bacteria was compared with that of the Korean intestinal bacterial composition database (Kook *et al*., 2018).

Gene copy index (gi) was derived by measuring the expression level of the target gene and the reference gene by relative quantification of the gene. The number of *Bifidobacterium* gene clones can be judged to be higher or lower than the average when the number of *Bifidobacterium* gene clones is indexed (Kook *et al*., 2018).

### Statistic analysis

Statistical analysis was performed using the SPSS WIN statistical program 22.0. First, the independent sample *t*-test was conducted to investigate the difference of intestinal bacteria according to the group, and the corresponding sample *t*-test was performed to examine intestine bacterial differences before and after the test.

**Table 4.** Target bacterial strains for determination of qPCR

| Bacterial genus | Standard strains | qPCR effc. (%) | Sources |
|---|---|---|---|
| *Lactobacillus* | *Lactobacillus casei* ATCC393 | 103.5 | |
| *Bifidobacterium* | *Bifidobacterium animalis* subsp. *lactis* JCM10602 | 90.7 | |
| *Bacteroides* | *Bacteroides fragilis* ATCC25285 | 96.9 | Kook *et al*. (2018) |
| *Clostridium* | *Clostridium sphenoides* ATCC19403 | 96.8 | |
| Universal | *Lactobacillus casei* ATCC393 | 101.6 | |

effic: efficiency. Quoted from Kook *et al*. (2018).

## Results and Discussions

### Characteristics of subjects

The study subjects were 15 individuals in the test group and the control group, respectively. The control group consisted of eight males and seven females, and aged 45-70 years. The test group was 52-70 years old. As a whole, the elderly are the main groups. The mean weight of the control group was 63.5 kg, and the mean weights of the test group were 58.8 kg (Table 1).

### Gene index *of Bifidobacterium* genus

Fifteen individuals were taken 10 ml of Zen fermentation broth for eight weeks, and then stool samples were taken from the subjects. The gene of *Bifidobacterium* genomic DNA identified by the qPCR method. Control groups drinking water were similarly analyzed. The gene copy index (gi%) of *Bifidobacterium* was calculated as a percentage (Table 5 and Fig. 1). Gene index is a numerical value converted to a percentage (%) by comparison with the intestinal bacterial composition database of Koreans (Korean intestinal bacterial composition data accumulated in the 'Kim Seokjin').

In the control group that drank water, the pre-test was 59.65%, and the post-test was 57.75%. *Bifidobacterium* index levels were lower by about 1.9% after ingestion than the before values.

In the case of the test group, the value before and after treatment was 55.15% and 70.1%, respectively, and the index of *Bifidobacterium* was significantly increased by 14.95% after ingestion of Zen fermentation broth. The increase after intake is thought to be due to the nutrients ingested by the Zen fermentation broth.

In the before examination of the test group, the overall ratio was in the range of 11.5~99.7%, and the average value was 46.9%. After the intake of Zen fermentation broth of the test group, the index range was 28.5~99.7%, and the mean value was 60.9%. The test group showed higher post-value than pre-value (Fig. 2).

The subject No. 6 of the test group was 47.7% in the pre-test and 99.7% in the post-test, which was 52% significantly higher than the pre-test. The test group No.12 was 5.2% in the pre-test and 50% in the post-test, and the difference was 44.8%. Overall, the gene index was varied in the ratio of 15 individuals (Fig. 2). This result implies that the *Bifidobacterium* genus increased after ingesting Zen fermentation broth.

Kook *et al*., (2018) confirmed the presence of 28.69% of the *Bifidobacterium* genus in intestinal bacteria using qPCR. This study was consistent with the results of establishing the presence of the *Bifidobacterium* genus using rRNA primer.

In the case of Ryu & Lee (2018a), *Bifidobacterium* was increased by about 6.01% in the test group treated with *Bacillus subtilis* fermentation, 63.51% and 69.53%, respectively. However, there was no significant difference. The Zen fermentation broth was also significantly increased in the test group.

### Gene index of *Lactobacillus* genus

Fifteen individuals of the test group were taken 10 ml

**Table 5.** Differences in gene index of intestinal *Bifidobacterium* genus between before and after ingestion of the Zen broth

| Group | Time tested | *N* | M (gi%) | SD | *t* | *p** |
|---|---|---|---|---|---|---|
| Con | Before | 15 | 59.65 | 35.75 | 1.251 | .106 |
| | After | 15 | 57.75 | 31.28 | | |
| Test | Before | 15 | 55.15 | 31.13 | -3.019** | .009 |
| | After | 15 | 70.10 | 26.74 | | |

*$p$<.05, **$p$<.01, ***$p$<.001.

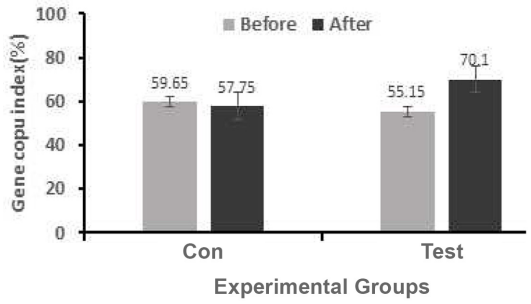


**Fig. 1.** Difference graphs in gene index of *Bifidobacterium* between before and after ingestion of the Zen broth.

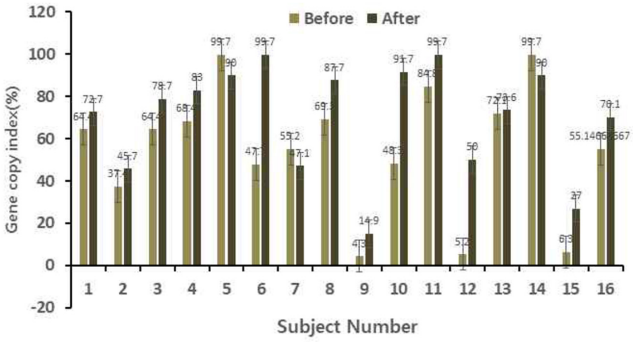


**Fig. 2.** Comparison of *Bifidobacterium* gene index between before and after ingestion of Zen broth of Test group. The subjects are from 1 to 15, and 16 is the average.

of Zen fermentation broth for eight weeks, and then stool samples were taken from the subjects. The gene of *Lactobacillus* gene identified by the qPCR method. The control group (15 individuals) receiving water were similarly analyzed. The gene copy index (gi%) of *Lactobacillus* calculated as a percentage in Table 6 and Fig. 3.

In the control group, water intake was 51.91% in the pre-treatment and 45.15% in the post-treatment, and 6.76% lower in the post-treatment. It was lower after ingestion than before water intake but was not significant.

In the test group, Zen broth intake was 46.87% in the pre-treatment and 60.91% in the post-treatment, and 14.04% in the post-treatment. It was higher after ingestion than before Zen broth intake, so was significant.

In the test group, the overall ratio before ingestion of Zen broth was in the range of 11.5~99.7% and the average value was 46.9%. After the intake of Zen broth, the index range was 28.5~99.7%, and the mean value was 60.9%. Gene index of test group showed higher post-value than pre-value (Fig. 4).

The subject No. 9 was 17% in the pre-test and 66.7% in the post-test, which was 49.7% significantly higher than the pre-test. Overall, the gene index was varied in the ratio of 15 individuals (Fig. 4). This result means that the *Lactobacillus* genus increased after ingesting Zen fermentation broth.

Ryu & Lee (2018a) reported a significant increase in *Lactobacillus* levels by 53.9% and 66.43%, respectively, before and after ingestion of *Bacillus subtilis* fermented broth. The results are similar to those of the present study.

### Gene index of *Clostridium* genus

The control group drank 10 ml of water in the morning, and test group ingested 10 ml of Zen fermentation broth for eight weeks. Stool samples of study subjects collected and the genes of intestinal bacteria *Clostridium* genus identified by the qPCR method. The prevalence of *Clostridium* gene index before and after treatment calculated in Table 7 & Fig. 5.

The *Clostridium* gene index of the control group was 80.68% before water intake and 82.86% after treatment. The *Clostridium* gene index increased 2.18% after ingestion but was not significant.

*Clostridium* gene index in the test group was 85.64% before the intake of Zen broth and 65.99% after ingestion of Zen broth. *Clostridium* gene index was significantly decreased by 19.65% after ingestion ($p$<.017).

The gene index range before feeding Zen fermentation

**Table 6.** Differences in gene index of intestinal *Lactobacillus* genus between before and after ingestion of the Zen broth

| Group | Time tested | *N* | M | SD | *t* | *p** |
|---|---|---|---|---|---|---|
| Con | Before | 15 | 51.91 | 31.04 | 2.503 | .052 |
| | After | 15 | 45.15 | 24.97 | | |
| Test | Before | 15 | 46.87 | 27.04 | -2.979 | .010* |
| | After | 15 | 60.91 | 21.64 | | |

*$p$<.05, **$p$<.01, ***$p$<.001.

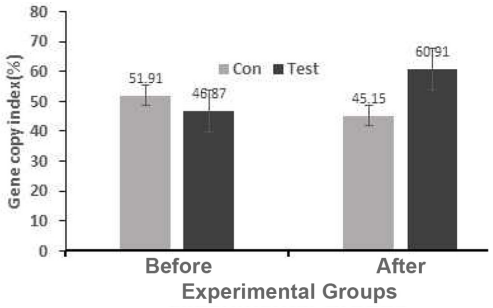


**Fig. 3.** Difference graphs in gene index of *Lactobacillus* between before and after ingestion of the Zen broth.

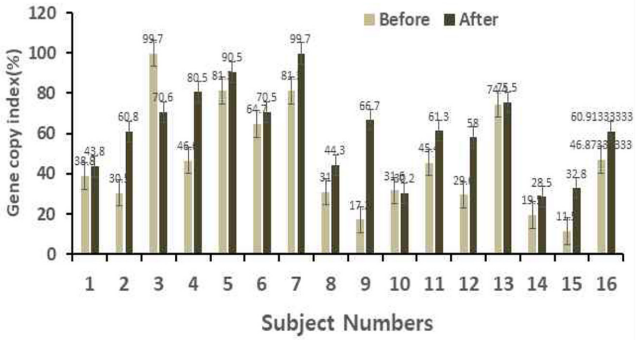


**Fig. 4.** Comparison of *Lactobacillus* gene index between before and after ingestion of Zen broth of Test group. The subjects are from 1 to 15, and 16 is the average.

to 15 subjects in the test group was 27.3~99.7%, and the mean value was 85.64%. The gene index range after ingestion was 37.4~99.7% and the mean value was 65.99%. The difference of after ingestion of Zen broth was 19.65% lower than before drinking water. Therefore, it decreased significantly (Fig. 6).

The *Clostridium* index of the subject No. 1 in the test group was 99.7% in the pre-test and 27.3% in the post-test, which was 72.4% significantly lower than the pre-test. He was the highest one. That of the test group No.8 was 99.7% in the pre-test and 37.4% in the post-test, and the mean difference was 62.3% lower after ingestion. Overall, the gene index was varied in the ratio of 15 individuals

**Table 7.** Differences in gene index of intestinal *Clostridium* genus between before and after ingestion of the Zen broth

| Group | Time tested | *N* | M (%) | SD | *t* | *p** |
|---|---|---|---|---|---|---|
| Con | before | 15 | 80.68 | 23.67 | -.748 | .467 |
| | after | 15 | 82.86 | 18.58 | | |
| Test | before | 15 | 85.64 | 19.48 | 2.703 | .017* |
| | after | 15 | 65.99 | 30.56 | | |

*$p$<.05, **$p$<.01, ***$p$<.001.

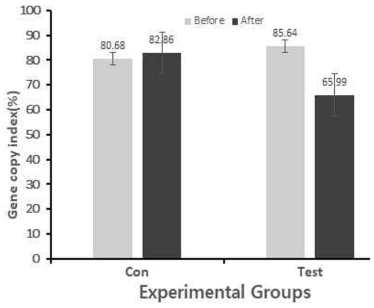


**Fig. 5.** Difference graphs in gene index of *Clostridium* between before and after ingestion of the Zen broth.

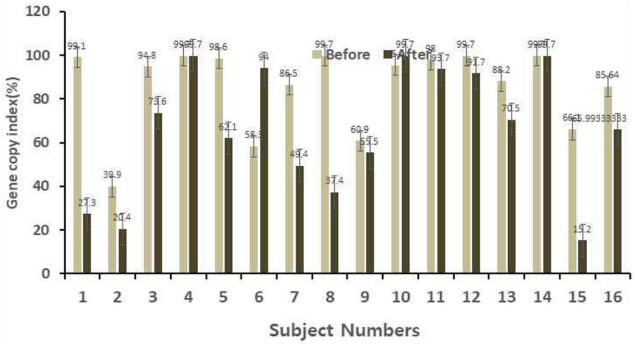


**Fig. 6**. Comparison of *Clostridium* gene index between before and after ingestion of Zen broth of Test group. The subjects are from 1 to 15, and 16 is the average.

(Fig. 6). This result indicated that the *Clostridium* genus decreased after ingesting Zen fermentation broth.

In this study, the results of confirming the presence of the *Clostridium* genus using rRNA primers were consistent with the results of Kook *et al*., (2018). Ryu & Lee (2018a) also found that the proliferation of *Clostridium* genus, a harmful bacterium, was also reduced in the ingestion of ENM solution, a fermentation product of *Bacillus subtilis*. Zen fermentation broth is estimated to reduce the number of inhibitory bacteria in the intestines. The reason is that many nutrients needed for beneficial bacteria increased the number of beneficial bacteria so that the inhibitory bacteria relatively eliminated from the survival competition and the number was decreased.

### Gene index of *Bacteroides* genus

Test group ingested 10 ml of Zen fermentation broth in the morning, and the control group drank 10 ml of water in the morning for eight weeks. Stool samples of study subjects collected and the genes of intestinal bacteria *Bacteroides* genus identified by the qPCR method. The prevalence of *Bacteroides* gene index before and after treatment calculated in Table 8 & Fig. 7.

The difference in *Bacteroides* gi% of before and after ingestion of Zen broth or water between the control and the test groups showed in Table 8 & Fig. 7. In the control group, water intake was 15.85% in the pre-treatment and 14.17% in the post-treatment, and 1.8% lower in the post-treatment. It was lower after ingestion than before water intake but was not significant.

In the test group, Zen broth intake was 17.11% in the pre-treatment and 20.22% in the post-treatment, and 3.11% were higher, and there was a significant difference.

The gene index range before feeding Zen fermentation to 15 subjects in the test group was 1.5~66.5%, and the mean value was 17.11% (Fig. 8). The gene index range after ingestion was 2.7~56.4% and the mean value was 20.22%. The index difference after ingestion of Zen broth was 3.11% higher than before drinking water. Therefore, they increased significantly (Fig. 8).

The subject No. 1 in the test group was 18.9% in the intake-water and 33.1% in the Zen broth ingestion, which was 14.2% significantly higher than the water-intake. He was the highest one. The test group No.9 was 27% in the water-intake and 45% in the Zen broth, and the difference was 18% higher after ingestion of Zen broth (Fig. 8).

Overall, the gene index was varied in the ratio of 15 individuals (Fig. 8). This result indicated that the *Bacteroides* genus significantly increased after ingesting Zen fermentation broth.

Ryu & Lee (2018a) reported a significant increase in *Bacteroides* genus index by 4.74% after ingestion of *Bacillus subtilis* fermented broth. The results are similar to those of the present study.

### Gene index of *Prevotella* genus

Experimental group ingested 10 ml of Zen fermentation broth in the morning, and the control group drank 10 ml of water in the morning for eight weeks. Stool samples of the study subjects collected and the genes of intestinal bacteria *Prevotella* genus identified by the qPCR method.

**Table 8.** Differences in gene index of intestinal *Bacteroides* genus between before and after ingestion of the Zen broth

| Group | Time tested | *N* | M | SD | *t* | *p** |
|---|---|---|---|---|---|---|
| Con | Before | 15 | 15.85 | 14.85 | 1.695 | .077 |
| | After | 15 | 14.17 | 13.66 | | |
| Test | Before | 15 | 17.11 | 17.46 | -1.962 | .048* |
| | After | 15 | 20.22 | 17.04 | | |

*$p$<.05, **$p$<.01, ***$p$<.001.

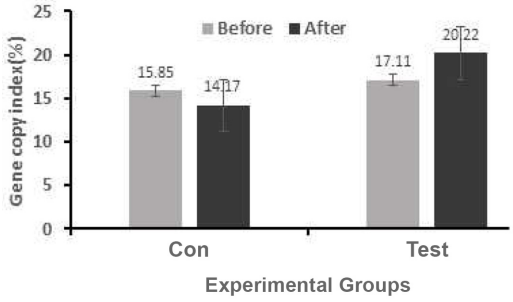


**Fig. 7.** Difference graphs in gene index of *Bacteroides* between before and after ingestion of the Zen broth.

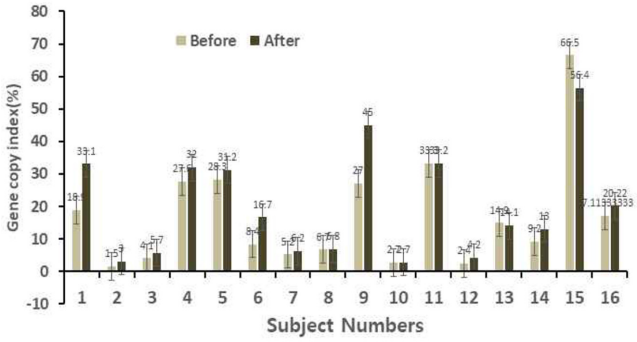


**Fig. 8.** Comparison of *Bacteroides* gene index between before and after ingestion of Zen broth of Test group. The subjects are from 1 to 15, and 16 is the average.

**Table 9.** Differences in gene index of intestinal *Prevotella* genus between before and after ingestion of the Zen broth

| Group | Time tested | *N* | M (%) | SD | *t* | *p** |
|---|---|---|---|---|---|---|
| Con | Before | 15 | 11.64 | 14.13 | 1.735 | .105 |
| | After | 15 | 8.19 | 9.60 | | |
| Test | Before | 15 | 14.01 | 14.47 | -1.658 | .120 |
| | After | 15 | 16.79 | 16.27 | | |

*$p$<.05, **$p$<.01, ***$p$<.001.

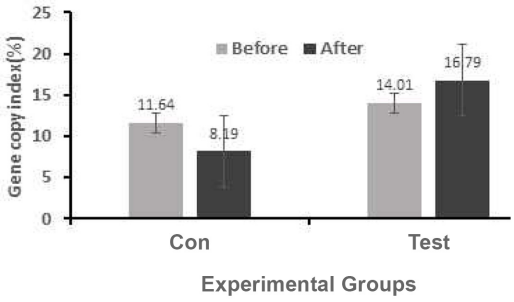


**Fig. 9.** Difference graphs in gene index of *Prevotella* between before and after ingestion of the Zen broth.

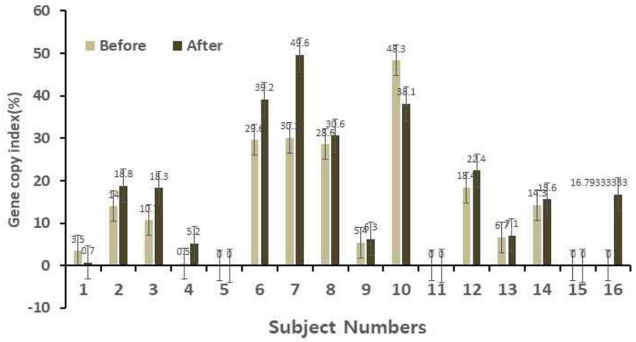


**Fig. 10.** Comparison of *Prevotella* gene index between before and after ingestion of Zen broth of Test group. The subjects are from 1 to 15, and 16 is the average.

*Bacteroides* gene index before and after treatment presented in Table 9 & Fig. 9.

In the control group, *Prevotella index* was 11.64% in the pre-water treatment and 8.19% in the post-Zen broth treatment, and 3.45% lower in the post-treatment. It was lower after ingestion than before water intake but was not significant.

In the test group, *Prevotella index* was 14.01% in the pre-treatment and 16.79% in the post-treatment, and 2.78% were higher, but it was not significant.

Fifteen subjects in the test group had *Prevotella* indexes ranging from 0.5% to 48.3% before the intake of Zen fermented broth, and the mean value was 14.01%. *Prevotella* index after ingestion was 0.7~49.64% and the mean value was 16.79%. The index increased by 2.78% after than before (Fig. 10).

*Prevotella* index of the subject No. 7 in the test group was ranged from 30.1% before ingestion to 49.6% after the ingestion. The Index after ingestion increased by 19.5% (Fig. 10). Three of the subjects were unclear in Fig. 10. Overall, the indexes of 12 subjects varied (Fig. 10). This result was not statistically significant although the *Prevotella* index increased after ingesting the Zen fermentation broth.

The reason is that among Zen fermentation broth components, beneficial components for beneficial bacteria,

oligosaccharide or amino acid components influence on the proliferation of *Prevotella* species. Kook *et al*., (2018) also confirmed the presence of intestinal bacteria as qPCR specific primers as our data. Ryu & Lee (2018a) showed that *Prevotella* index was significantly 5.78% higher when *Bacillus* fermentation broth ingested ($p$<.01). In this study, the intake of Zen fermentation broth increased by 2.78%, but there was no statistical significance. We think that the effect of Zen fermentation broth was not significant in this bacterium.

### The growth ratios of five intestine bacteria

The order of increase of intestinal bacteria after ingestion of Zen-fermented broth was *Bifidobacterium* genus (14.95%) > *Lactobacillus* (14.04%) > *Bacteroides* (3.11%) > *Prevotella* (2.78%) > *Clostridium* (-19.65%)(Fig. 11). The intake of Zen fermentation broth promoted the proliferation of the beneficial bacteria *Bifidobacterium* genus, *Lactobacillus* genus, *Bacteroides* genus, and *Prevotella* genus, and the harmful *Clostridium* genus showed the effect of reducing proliferation.

## Conclusions

The purpose of this study was to investigate the increase or decrease of important intestinal beneficial bacteria and inhibitory bacteria in 30 stools of clinical subjects after ingesting Zen fermentation broth as a mixed microbial fermentation solution for eight weeks. Intestinal bacteria were identified by PCR amplification using specific primers.

1) *Bifidobacterium* genus gi% of the test group ingested Zen-fermented broth was 55.15% before and 70.1% after ingestion, so it was a significant difference ($p$<.009).

2) *Lactobacillus* genus gi% of the test group was 46.87% before and 60.91% after ingestion, so it was a significant difference ($p$<.01).

3) *Clostridium genus* gi% of the test group was 85.64% before and 65.99% after ingestion, so it was a significant decrease ($p$<.017).

4) *Bacteroides* genus gi% of the test group was 17.11% before and 20.22% after ingestion, so it was a significant difference ($p$<.048).

5) *Prevotella* genus gi% of the test group was 14.01% before and 16.79% after ingestion, so it was not a significant difference.

In conclusions, intestinal bacteria increased the proliferation of beneficial bacteria and suppressed harmful bacteria in the intestines after ingesting the Zen-fermented broth produced by the mixed microorganism. The Zen fermentation broth evaluated as a beneficial drink for intestinal health.

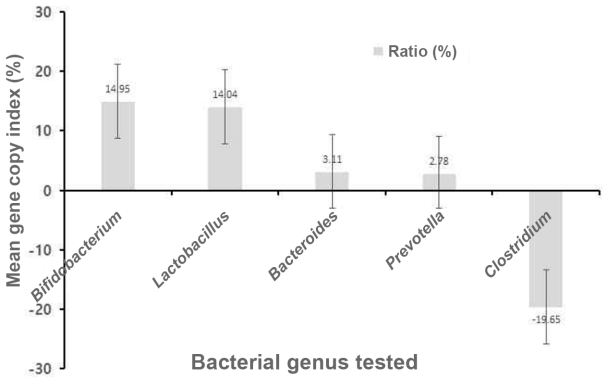


**Fig. 11.** Comparison of growth rate of intestinal bacteria according to difference of pre and post after ingestion of Zen fermentation broth.

## Acknowledgements

We are grateful to Mr. S.J. Kim and Ms. E.H. Yang. Probioticslab R&D Institute. Bioeleven Co., Seoul, Korea for their technical assistance.

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