Fecal microbiota changes with fermented kimchi intake regulated either formation or advancement of colon adenoma

Jong Min Park,¹ Won Hee Lee,² Hочan Seo,² Ji Young Oh,³ Dong Yoon Lee,³ Seong Jin Kim,⁴ and Ki Baik Hahm⁴,5,*

¹College of Oriental Medicine, Daejeon University School of Oriental Medicine, Daehak-ro 62, Dong-gu, Daejeon 34520, Korea
²MD Healthcare, Inc., Seoul 03986, Korea
³CJ Food Research Center, Gwanggyo-ro, Yeongtong-gu, Suwon 16495, Korea
⁴CHA Cancer Preventive Research Center, CHA Bio Complex, 330 Pangyo-dong, Bundang-gu, Seongnam, 13497, Korea
⁵Medpacto Research Institute, Medpacto Inc., 92, Myeongdal-ro, Seocho-gu, Seoul 06668, Korea

(Received 29 July, 2020; Accepted 16 September, 2020; Published online 26 December, 2020)

Gut bacteria might contribute in early stage of colorectal cancer through the development and advancement of colon adenoma, by which exploring either beneficial bacteria, which are decreased in formation or advancement of colon adenoma and harmful bacteria, which are increased in advancement of colon adenoma may result in implementation of dietary interventions or probiotic therapies to functional means for prevention. Korean fermented kimchi is one of representative probiotic food providing beneficiary microbiota and exerting significant inhibitory outcomes in both APC−/− polyposis model and colitis-associated cancer. Based on these backgrounds, we performed clinical trial to document the changes of fecal microbiota in 32 volunteers with normal colon, simple adenoma, and advanced colon adenoma with 10 weeks of fermented kimchi intake. Each amplicon is sequenced on MiSeq of Illumina and the sequence reads were clustered into Operational Taxonomic Units using VSEARCH and the Chao Indices, an estimator of richness of taxa per individual, were estimated to measure the potential of colon adenoma relates to the size, type, and degree of carcinomas at the time of removal, whereas those measuring less than 1 cm have less than 1–2% risk.⁴ However, exceptional cases that even smaller size progress to adenocarcinoma exist and no way of definite preventive strategy threatened general population who was diagnosed as simple colon adenoma and made them to perform frequent colonoscopy or led to intake of unnecessary medications/general health medicine.

Meanwhile, the multiple reports that higher numbers of Fusobacterium nucleatum, Enterococcus fecalis, Streptococcus bovis, Enterotoxigenic Bacteroides fragilis, and Porphyromonas spp. were detected in patients with colon adenoma, from simple adenoma to advanced adenoma, in contrast to samples from the normal or hyperplastic polyp and the contrary results that lower number of Lactobacillus spp., Roseburia spp., and Bifidobacterium spp. were detected in colon adenoma compared to the normal signified the contribution of altered microbiota in formation and progression of colon adenoma rendered us to adopt fecal microbiota analysis as predictive functional analyses based on the candidate bacterial quantity of the adenoma and non-adenoma groups and tool to assess the function of fermented foods relevant to colon adenoma.⁵-¹³ Therefore, the alteration of the microbiota may be a useful to preventing and altering the trajectory of colorectal cancer as well as advancement of colon adenoma,¹⁴,¹⁵ usually through intake of fermented foods or probiotics.

Supported with facts that gut microbiota represents a natural defense against various kinds of gastrointestinal diseases and exerts anticarcinogenic effects, maintaining the gut homeostasis and the modulation of intestinal microbiota with probiotics foods, fermented kimchi in the current study, can be basis for either preventing recurrence of colon adenoma or blocking advancement of colon adenoma.¹⁶,¹⁷ we have performed clinical trials to explore fecal microbiota changes relevant to probiotic foods and found significant contribution with probiotic kimchi was noted against colon adenoma.

Material and Methods

Bacteria DNA extraction from human stool samples.

The human stool sample is filtered through a 40 µm-pore sized cell strainer after being diluted and incubated in 10 ml of PBS for 24 h. To separate the bacteria from human stool, bacteria in stool samples is isolated using centrifugation at 10,000 x g for...
10 min at 4°C. After centrifugation, the pellet is comprised of bacteria. To extract the DNA out of the bacteria, bacteria is boiled for 40 min under 100°C. DNA is extracted by using a DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany). The standard protocol is followed as the kit guide. The DNA from bacteria in each sample is quantified by using QIAxpert system (QIAGEN). The PowerSoil Kit (QIAGEN, Hilden, Germany); The standard for 40 min under 100°C. To extract the DNA out of the bacteria, bacteria is boiled for 10 min at 4°C. DNA is extracted by using a DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany). The standard protocol is followed as the kit guide. The DNA from bacteria in each sample is quantified by using QIAxpert system (QIAGEN).

**Bacterial metagenomic analysis using DNA from human stool samples.** Bacterial genomic DNA was amplified with 16S V3 F (5'-TTC GCG AGC GGG TAT CTA ATC C-3') and 16S V4 R (5'-GTC TCG TGG GCT CAG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3') primers, which are specific for V3–V4 hypervariable regions of 16S rDNA gene. The libraries were prepared using PCR products according to MiSeq System guide (Illumina, San Diego, CA) and quantified using a QIAxpert (QIAGEN). Each amplicon is then quantified, set equimolar ratio, pooled, and sequenced on MiSeq (Illumina) according to the manufacturer’s recommendations.

**Analysis of bacterial composition in the microbiota.**

Paired-end reads that matched the adapter sequences were trimmed by cutadapt ver. 1.1.6. The resulting FASTQ files containing paired-end reads were merged with CASPER and then quality filtered with Phred (Q) score based criteria described by Bokulich. Any reads were shorter than 350 bp and longer than 550 bp after merging, were also discarded. To identify the chimeric sequences, a reference-based chimera detection step was conducted with VSEARCH against the SILVA gold database. Next, the sequence reads were clustered into Operational Taxonomic Units (OTUs) using VSEARCH with de novo clustering algorithm under a threshold of 97% sequence similarity. The representative sequences of the OTUs were finally classified using SILVA 132 database with UCLUST (parallel_assign_taxonomy_uclust.py script on QIIME ver. 1.9.1) under default parameters. The Chao Indices, an estimator of richness of taxa per individual, were estimated to measure the diversity of each sample.

**Volunteers recruitment and clinical trials.**

Normal control (healthy control) was defined as subject who underwent screening colonoscopy for health promotion purpose without any symptom and in whom the result of colonoscopy was normal up to terminal ileum. Volunteer subjects with simple colon adenoma and advanced colon adenoma were recruited at clinical trial center located at Digestive Endoscopy Center, Digestive Disease Center, CHA University Bundang Hospital (Seongnam, Korea). The informed consents were obtained from the Digestive Disease Center of Bundang Cha Medical Center (Seongnam, Korea) after explaining the aim and the object of the current study that fermented kimchi showed significant protection from either development or advancement of colon adenoma in mice model [APC<sup>-Min</sup> model developing multiple intestinal neoplasms (MIN) via truncated <i>apc</i> mutation and azoxymethane-initiated, dextran sulfate sodium-promoted colitis-associated cancer model]. As shown in Fig. 1, they were administered with fermented kimchi for 10 weeks after informed consent and stools were donated for microbiota analysis (MD Healthcare, Inc., Seoul, Korea) and blood samplings were saved for biochemical analysis including CBC, biochemistry, and levels of IL-1β, malondialdehyde for lipid peroxidation, and prostaglandin E<sub>2</sub>. Before starting the kimchi intake, the volunteers were advised not to take any medications like probiotics, proton pump inhibitor, antibiotics, and medications influencing gut microbiota as 4 weeks of wash-out period before taking feces for microbiota analysis. Compliance for taking kimchi was checked above intake more than 90% and kimchi was prepared every 5 days to keep optimal fermentation state. All of these studies were approved with IRB (IRB #18-0201) of CHA University Bundang Hospital. There was no significant difference in mean ages, gender difference, and history of smoking and alcohol among groups on demographic analysis of simple and advanced adenoma. simple colon adenoma in 12 patients [male:female = 4:8, mean ages, 51 ± 3, mean colon adenoma size = 0.8 cm, smoking:non-smoking = 4:8, alcohol:non-alcohol = 6:6, family history of colon cancer:non-family history of colon cancer = 1:1, rectum, descending colon, transverse colon; ascending colon of colon adenoma location=5, 2, 0, 5, positive stool occult blood (OB) in 1 case], advanced colon adenoma in 9 patients (male:female = 4:5, mean ages, 54 ± 4, mean colon adenoma size = 1.3 cm, smoking:non-smoking = 3:6, alcohol:non-alcohol = 3:6, family history of colon cancer:non-family history of colon cancer = 2:7, rectum, descending colon, transverse colon, ascending colon of colon adenoma location = 4, 1, 1, 3, positive

![Fig. 1. Clinical trial with fermented kimchi (A) Schematic protocol for fecal microbiota measurement All volunteers were administered with fermented kimchi (CJ Food, Seoul, Korea), 100 g/day for 10 weeks prepared according to SOP recipe, delivered every 3 days to all volunteer home exactly. All the volunteers were included after informed consent and their compliance for kimchi intake were more than 95%.](image-url)
stool OB in 1 case). All volunteers were administered with fermented kimchi (CJ Food, Blossom Park, Suwon, Korea), 100 g/day for 10 weeks, delivered to the home refrigerator every 3 days after generating SOP-recipe kimchi in same condition. All the volunteers were included after informed consent and their compliance for kimchi intake were more than 95%.

Kimchi preparation and extracts for in vitro experiment. Kimchi preparation was based on the standardized kimchi recipe of the Kimchi (sKimchi) in CJ Food Research Center, Suwon, Korea. First of all, sKimchi is made of birned baechu cabbage (a kind of Chinese cabbage), red pepper powders, garlic, ginger, anchovy juice, sliced redish, green onion, some sugar, then fermented for some periods yielding lactobacillus like L. plantarum. In addition to these ingredients necessary for sKimchi preparation, additional supplements such as mustard leaf, Chinese pepper, pear, mushroom, and sea tangle juice instead of anchovy juice were included in cancer preventive kimchi (epkimchi). Kimchi was prepared every 5 days to keep fermentation and was delivered to house of volunteers on exact day of delivery and keep at refrigerator. 100 g of kimchi was packed and supplied.

Statistical analysis. Comparison of relative abundances (RA) of OTUs and α diversity between groups was performed with the Mann-Whitney U test. Statistical significance was considered if the p value was <0.05. The α diversity of microbial composition was measured using the Observed, Chao1, Shannon, Simpson index and rarefied to compare species richness. Statistical analyses were performed with R software (ver. 3.6.0).

Results

α and β Diversity analysis according to colon status, normal colon, simple colon adenoma, and advanced colon adenoma. α Diversity is the analysis of species diversity in samples, for which Chao1, Shannon, and Simpson indexes were explored in order to describe the diversity features of colorectal community according to colon status, normal healthy colon, colon with simple adenoma, and colon with advanced adenoma, respectively, all were calculated based on OTU species and abundance, respectively in Fig. 2A. In detail, Shannon, diversity indexes used to describe the diversity features of our colorectal community, Chao indexes used to reflect the species richness in the sample, that is, the number of OTU, and Simpson indexes used to reflect community diversity including species richness and species evenness were calculated in Fig. 2 according to patient group.24,25 Usually, the larger the Shannon index and the smaller the Simpson index, the higher the species diversity in the sample were noted. Regarding α diversity according to colon pathology as shown in Fig. 2A, there was no significant difference in these indexes between normal control, colon with simple adenoma, and colon with advanced adenoma. In order to further display differences in species diversity among samples, principal coordinate analysis (PCoA) was used to display differences among samples. If the two samples are close together, the species composition of the two samples is similar. No significant separation in bacterial community composition between normal colon, colon with simple adenoma, and colon with advanced adenoma were seen (Supplemental Fig. 1A*, phylum level and Supplemental Fig. 1B*, genus level). Relative abundance of colon adenoma associated microbiota including microbial composition of normal colon, colon with simple adenoma, and colon with advanced adenoma at the phylum level was shown in Fig. 2B. The most abundant phylum in total samples was Firmicutes (88.9%), followed by followed by Bacteroidetes (62.3%), Proteobacteria (45.7%), and Actinobacteria (23.4%) (Fig. 2B). Conclusively, relative abundance of the phylum level in feces by pyrosequencing showed that there was no significant difference in relative abundance of most abundant phyla among the three groups (p>0.05). In addition, relative abundance of colon adenoma associated microbiota including microbial composition of normal colon, colon with simple adenoma, and colon with advanced adenoma at the genus level was shown in Fig. 2C and Fig. 3. Relative abundance of microbiota among the three groups, normal colon, colon with simple adenoma, and colon with advanced adenoma, in the genus level was shown in Fig. 4 depicting Streptococcus, Citrobacter, and Pseudomonas were significantly increased in advanced adenoma, whereas Fecalibacterium and Akkermansia were significantly decreased in advanced colon adenoma. Individual microbiom was compared according to group, which was presented in Fig. 4 that Actinobacteria, Cyanobacteria, Clostridium sensu stricto, Turicibacter, Gastronaeophillales, H. pittma HK B5 were proven to be increased of patients with advanced colon adenoma, whereas Enterococcaceae Roseburia, Coryobacteriaceae, Bifidobacterium spp., and Akkermansia were proven to significantly decreased in feces from patients with advanced colon adenoma (Fig. 4). Individual separate Heatmap results compared between group before kimchi and group after kimch at genus level were shown on Supplemental Fig. 4*.

Evaluation of α and β diversity in the 3 groups of normal control, simple colon adenoma, and advanced colon adenoma after 10 weeks of kimchi intake. The Chao1 Richness index of the fecal microbiota was significant different between normal control and normal control with kimchi intake (p<0.052), while other index of observed, Shannon, and Simpson was changed, but not significance. Similar results were observed between simple colon adenoma control and simple colon adenoma with kimchi intake (p<0.014 observed index and 0.00037 Chao1 richness index, Fig. 5B), and between advanced colon adenoma control and advanced colon adenoma with kimchi intake (p<0.05 Chao1 richness index, Fig. 5C). Overall differences in these diversity analyses were shown in Fig. 5D, significant difference in Shanon index with kimchi intake. In order to further display differences in species diversity among samples, PCoA based on the unweighted UniFrac distance metrics is used to display differences among samples. (Supplemental Fig. 2*).

Relative abundance of colon adenoma associated microbiota; microbial composition of normal colon, colon with simple adenoma, and colon with advanced adenoma after 10 weeks of kimchi intake at the phylum level. The most abundant phylum in total samples was Firmicutes (90.1%), followed by followed by Bacteroidetes (71.4%), Proteobacteria (43.7%), Actinobacteria (35.7%), Verrucomicrobia (16.6%) (Fig. 6A). Figure 6B showed relative abundance of the phylum level in feces by pyrosequencing and there was no significant difference in relative abundance of most abundant phyla among the three groups (p>0.05). Individual separate Heatmap results compared between group before kimchi and group after kimchi at phylum levels were shown on Supplemental Fig. 3*.

Relative abundance of colon adenoma associated microbiota; microbial composition of normal colon, colon with simple adenoma, and colon with advanced adenoma at the genus level. Relative abundance of microbiota among the three groups, normal colon, colon with simple adenoma, and colon with advanced adenoma, in the genus level was shown in Fig. 6C depicting Streptococcus, Citrobacter, and Pseudomonas were significantly increased in advanced adenoma, whereas Fecalibacterium and Akkermansia were significantly decreased in advanced adenoma. Individual microbiom was compared according to group, which was presented in Fig. 6 that Actinobacteria, Cyanobacteria, Clostridium sensu stricto, Turicibacter, Gastronaeophillales, H. pittma HK B5 were proven to be increased of patients with advanced colon adenoma, whereas Enterococcaceae Roseburia, Coryobacteriaceae, Bifidobacterium spp., and Akkermansia were proven to significantly decreased in feces from patients with advanced colon adenoma (Fig. 6). Individual separate Heatmap results compared between group

*See online. https://doi.org/10.3164/jcbn.20-121

Fig. 2. Relative abundance of the phylum level (% similarity) in collected feces samples by pyrosequencing. (A) $\alpha$ Diversity in normal colon, simple colon adenoma, and advanced colon adenoma. (B) Microbiota at phylum levels, heatmap and bar display. (C) Microbiota at genus levels. See color figure in the on-line version.
before kimchi and group after kimchi at genus level were shown on Supplemental Fig. 4*.

Changes of IL-1β after kimchi intake. Kimchi intake significantly decreased sera levels of IL-1β. We have compared CBC and blood chemistry including cholesterol level, triglyceride level before and after 10 weeks of kimchi intake in all participants and there were no significance differences. Also, we have measured three kinds of parameters, IL-1β, MDA, and PGE₂ blood levels and there were significant decreases in IL-1β in patients with advanced colon adenoma after kimchi intake (*p<0.05, Supplemental Fig. 5A*), while there were no significant changes in MDA and PGE₂ between levels before kimchi intake and after kimchi intake.

Discussion

Well-fermented kimchi showed significant inhibitory actions against intestinal adenoma formation as well as colitic cancer.

*See online.  https://doi.org/10.3164/jcbn.20-121

J.M. Park et al.
development (26) during which we could identify significant changes in fecal microbiota from the current study after clinical trial of fermented kimchi in patients with colon adenoma. Kimchi effectively changed fecal microbiota implicated in colon either adenoma formation or adenoma advancement. Detailed microbiota changes were displayed in Fig. 7, explaining the contributive role of fermented kimchi intake in either preventing colon adenoma or inhibiting the progression of simple colon adenoma. The most abundant phylum in total samples was Proteobacteria (55.6%), followed by Firmicutes (27.4%) and Bacteroidetes (11.6%). Though there was no significant difference in relative abundance of the phylum level among the four groups, Fusobacterium nucleatum, known to be frequently detected during colorectal carcinogenesis, was found in patient with advanced adenoma. The diversity of mucosal communities of patients with adenoma was higher in patients with adenoma than that of healthy control and its diversity varies significantly with kimchi intake. Though we should wait for long-term effect of kimchi intake on inhibitory action of colon adenoma, together with previous publication that fermented kimchi intake significantly inhibited colon adenoma as well as colitic cancer and current investigation to explore microbiota changes with kimchi intake in patients with colon adenoma, we could conclude fermented kimchi can be of potential nutritional intervention to inhibit colon adenoma.

Here in this investigation, we could include one patient, 17 years old female diagnosed as attenuated type of familial adenomatous polyposis (FAP) presenting with multiple polyps in stomach and colon, who participated in 10 weeks of kimchi trial of fermented kimchi in patients with colon adenoma. Kimchi effectively changed fecal microbiota implicated in colon either adenoma formation or adenoma advancement. Detailed microbiota changes were displayed in Fig. 7, explaining the contributive role of fermented kimchi intake in either preventing colon adenoma or inhibiting the progression of simple colon adenoma. The most abundant phylum in total samples was Proteobacteria (55.6%), followed by Firmicutes (27.4%) and Bacteroidetes (11.6%). Though there was no significant difference in relative abundance of the phylum level among the four groups, Fusobacterium nucleatum, known to be frequently detected during colorectal carcinogenesis, was found in patient with advanced adenoma. The diversity of mucosal communities of patients with adenoma was higher in patients with adenoma than that of healthy control and its diversity varies significantly with kimchi intake. Though we should wait for long-term effect of kimchi intake on inhibitory action of colon adenoma, together with previous publication that fermented kimchi intake significantly inhibited colon adenoma as well as colitic cancer and current investigation to explore microbiota changes with kimchi intake in patients with colon adenoma, we could conclude fermented kimchi can be of potential nutritional intervention to inhibit colon adenoma.

Fig. 4. Significant microbiota changes at genus level. (A) Between simple adenoma cases before and after kimchi intake. (B) Between advanced adenoma cases before and after kimchi intake.
significantly tells fundamental role of intestinal microbiota in adenoma-carcinoma sequence. As like in the current investigation denoted in advanced colon adenoma, *S. bovis*, *Helicobacter pylori*, *B. fragilis*, *E. faecalis*, *C. septicum*, *Fusobacterium* spp., and *Escherichia coli* were reported to be pathogen responsible for colon tumorigenesis including adenocarcinoma. As results, modulation of intestinal microbiota can alter colitis-associated cancer susceptibility as evidenced that the severity of chronic colitis directly correlates to colitic cancer, during which bacterial-induced inflammation drives progression from adenoma to invasive carcinoma.

In this study, after informed consent, 5 ml bloods were obtained in all volunteers before and after kimchi intake, by which we had additional measurements of serum levels of IL-1β, MDA, and PGE2 in all cases and significant differences in IL-1β were noted (Supplemental Fig. 5a). We have conducted RNAseq analysis in animal model of colitis-associated cancer with pellet diet containing fermented kimchi and found significant changes in COX-2, 15-PGDH, oxidative stress related genes, and inflammases including IL-1β. Based on these in vivo finding, we decided to measure the changes of serum IL-1β, MDA, and PGE2 and found the significant decreases in IL-1β after kimchi intake. Since IL-1β promotes cell growth, stemness and invasiveness of colon cancer cells as well as colitic cancer, serum levels as well as genetic polymorphisms, IL-1RN +2018T>C, can influence colon carcinogenesis.

Though we have omitted results regarding the results from cross intake (10 weeks of fermented kimchi followed with non-fermented kimchi or reverse) of fermented kimchi, we could realize fermented kimchi yielded positive changes in patients with colon adenoma, signifying the importance of fermentation in preventing colon adenoma. Small in size, short-follow up interval,
and analysis in phylum and genus level, but combined with our in vitro and in vivo model study, we dare to conclude long-term intake of well-fermented kimchi can be anticipating strategy either to prevent adenoma formation or to block advancement of colon adenoma. Though fermented kimchi intake was dealt in this study, food we consume is essential for energy, but also provides a diverse community of microbiota within our GI tract. Therefore, more understanding of the nutrient–microbiota interplay and large-scale studies to evaluate the efficacy of dietary modification and gut microbiota modulation in reversing dysbiosis and restoring health could offer novel preventative and/or therapeutic strategies. However, considering different ethnicity, different dietary style, and different cultural habits, our study should be extended more to reach to conclusion.

Fig. 6. Significant changes in microbiota among normal colon, simple adenoma, and advanced adenoma with 10 weeks kimchi intake. (A) Phylum level analysis Heatmap analysis and bar display. (B) Genus levels analysis and bar display. See color figure in the on-line version.
Author Contributions

Study concept and design: JMP and KBH; acquisition of data: JMP, WHL, HS, DYL, SHC; analysis and statistical analysis: JMP, WHL, and KBH; interpretation of data: HS, JYO and KBH; drafting of manuscript: KBH.

Acknowledgments

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through High Value-added Food Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (116015-03-1-CG000).

Abbreviations

CAC  colitis-associated cancer
CRC  colorectal cancer
FAP  familial adenomatous polyposis
FFPE  formalin-fixed and paraffin-embedded
H. pylori  Helicobacter pylori
MDA  malondialdehyde
MIN  multiple intestinal neoplasia
PCoA  principal coordinate analysis
OUTs  operational taxonomic units
PGE$_2$  prostaglandin E$_2$
STRING  Search Tool for the Retrieval of Interacting Genes/Proteins
RA  relative abundancy
Conflict of Interest

No potential conflicts of interest were disclosed.

References

2. Appelman HD. Con: high-grade dysplasia and villous features should not be part of the routine diagnosis of colorectal adenomas. Am J Gastroenterol 2008; 103: 1329–1331.