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High-throughput 16S rRNA gene sequencing reveals alterations of mouse intestinal microbiota after radiotherapy

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ABSTRACT

The mammalian gastrointestinal tract harbors a highly complex microbial community that comprises hundreds of different types of bacterial cells. The gastrointestinal microbiota plays an important role in the function of the host intestine. Most cancer patients undergoing pelvic irradiation experience side effects such as diarrhea; however, little is currently known about the effects of irradiation on the microorganisms colonizing the mucosal surfaces of the gastrointestinal tract. The aim of this study was to investigate the effects of gamma irradiation on the compositions of the large and small intestinal microbiotas. The gut microbiotas in control mice and mice receiving irradiation treatment were characterized by high-throughput sequencing of the bacterial 16S rRNA gene. Irradiation treatment induced significant alterations in the bacterial compositions of the large and small intestines at the genus level. Unexpectedly, irradiation treatment increased the number of operational taxonomic units in the small intestine but not the large intestine. In particular, irradiation treatment increased the level of the genera *Alistipes* in the large intestine and increased the level of the genus *Corynebacterium* in the small intestine. By contrast, compared with that in the corresponding control group, the level of the genera *Prevotella* was lower in the irradiated large intestine, and the level of the genera *Alistipes* was lower in the irradiated small intestine. Overall, the data presented here reveal the potential microbiological effects of pelvic irradiation on the gastrointestinal tracts of cancer patients.

1. Introduction

Recent studies have shown that the human body is inhabited by at least ten times more microorganisms than the number of somatic and germ-line cells it contains [1]. The host gut and its microbiota have co-evolved intricate relationships, and the mammalian gastrointestinal tract is colonized by 10–100 trillion microorganisms that are essential to host cell maintenance in health and disease; therefore, the human gut microbiota has attracted increasing interest from medical researchers. Comprehensive 16S rRNA gene-based analyses of fecal microbial communities have demonstrated that the gut microbiota is highly diverse between individuals; however, *Bacteroidetes* and *Firmicutes* are the predominant phyla in most individuals [2,3]. The majority of studies examining the beneficial and/or pathogenic influences of microbes on intestinal diseases have focused on irritable bowel or inflammatory bowel diseases [4,5]; however, recent evidence has linked the composition of the gut microbiota to gastrointestinal responses to irradiation [6–9].

Radiotherapy is a common treatment for cancers, especially gynecological and colorectal cancers. Approximately 70% of all cancer patients receive radiotherapy treatment [6,10,11] and these patients typically present clinical symptoms of gastrointestinal irradiation injury [8,12,13]. Pelvic radiotherapy of gynecological cancer patients causes changes in the microbial composition of the gut; Nam et al. [14] demonstrated that radiation therapy changes the intestinal levels of the *Firmicutes* and *Fusobacterium* phyla significantly. Furthermore, Crawford and Gordon [7] used germ-free mice models to demonstrate microbial regulation of intestinal radio-sensitivity. These two studies focused on the large intestine microbiota and cell function, respectively; however, several

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studies [6,8,15,16] have suggested that injury to the small intestine in cancer patients receiving pelvic irradiation leads to adverse side effects such as diarrhea. Nevertheless, analyses of the small intestine microbiota are lacking because sampling of this region is challenging. Therefore, the objective of this study was to investigate and compare the microbial compositions of fecal samples from irradiated mouse large and small intestines using Illumina MiSeq high-throughput sequencing.

2. Materials and methods

2.1. Animal preparation

Male 8–10-week-old C57BL/6 mice were purchased from Orient Bio (Seongnam, Republic of Korea) and housed with access to irradiated food and water. All mouse experiments were performed in accordance with animal protocols approved by the Institutional Animal Care and Use Committee of Jeju National University. The mice were irradiated with a single 8 Gy dose using a Cobalt 60 source irradiator [17]. The small and large intestinal contents were collected three days after irradiation. During the study, the mice were fed a commercial AIN-76A Purified Rodent Diet (Dyets Co. Ltd, Bethlehem, PA) *ad libitum*; however, the food consumption was limited to 2–3 g/day.

2.2. Sample collection, DNA extraction, and sequencing

Immediately after the mice were euthanized, the contents of the small and large intestines, excluding the cecum, were obtained by manual extrusion. All samples were placed immediately into sterile plastic tubes using alcohol-sterilized spatulas. In total, five mice were sampled (two from the control group and three from the irradiation group), resulting in a total of ten samples from the large and small intestines. All of the samples were snap-frozen in liquid nitrogen and stored at −80 °C until analysis. DNA was extracted from the samples using the UltraClean® Fecal DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA), according to the manufacturer's instructions. Total DNA was quantified using a NanoDrop® ND-1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE). For paired-end sequencing, the bacterial 16S rRNA genes were amplified from the fecal samples using a barcode-tagged primer set designed for use with the MiSeq platform (Illumina, San Diego, CA). The sequences of the primers targeting the V4 hyper-variable region of the bacterial 16S rRNA genes [18,19] were as follows: 515F, 5′- GTGCCAGCMGCCGCGGTAA-3′; and 806R, 5′-GGACTACHVGGGTWTCTAAT-3′. Polymerase chain reaction (PCR) was performed using a published protocol [20,21] with slight modifications. Briefly, the 20 μl of PCR mixtures contained 10 μl of 2 × PCR Master Mix Solution (Solgent, Daejeon, Republic of Korea), 1 μM primers (final concentration), and approximately 10 ng of template DNA. The following thermal cycling conditions were used: initial denaturation at 95 °C for 5 min; followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and then a final extension at 72 °C for 5 min. The amplified products were purified using the LaboPass Gel and PCR Clean-up Kit (Cosmogenetech, Seoul, Republic of Korea). DNA was quantified using a spectrophotometer and mixed in equivalent proportions. Sequencing was performed by Macrogen (Seoul, (Republic of Korea)) using the Illumina MiSeq system (Illumina), according to the manufacturer's instructions.

2.3. Bioinformatics data analysis

The modified pipeline described on the mothur website (http://www.mothur.org/wiki/MiSeq_SOP) was used for the bioinformatics analysis. The bacterial sequence reads were compared to a reference database of known 16S rRNA genes obtained from the Ribosomal Database Project (RDP). The bacterial sequences were assigned taxonomically based on the RDP classifiers [22]. Rarefaction curves, Shannon indices, Good's coverages, and Chao1 nonparametric richness estimators were determined using the mothur package [23]. To increase the analysis quality, reads that included chimeric sequences that were unassigned and/or related to non-bacterial species, such as chloroplasts and mitochondria, were disregarded. Before performing diversity calculations, the library sizes were normalized to that of the smallest library. The calculations were repeated 100 times using random sub-samples of sequences. A 3% dissimilarity level between sequences was used to calculate the diversity estimators. The microbial community structures in different samples were compared using the Fast-UniFrac tool [24], based on the phylogenetic relationships between representative reads (operational taxonomic units or OTUs) from different samples. Trees were constructed using the FastTree program [25]. The Fast-UniFrac sample clustering results were used to compare the hierarchical relationships of the samples [24]. A Mann–Whitney U-test [26] was performed to compare the diversity indices between two groups. Unless otherwise stated, the proportion of total reads representing each taxonomic group was calculated.

3. Results

3.1. Clustering of the mouse gut microbiotas

Table 1 summarizes the sequencing reads, diversity indices, and sample coverages of the mouse intestinal samples included in the study. The control group comprised mice 1 and 2, and the irradiation group comprised mice 7, 8, and 9. After quality control processing and removal of chimeric reads, a total of 51,505 sequences (from 60,394 raw reads) were used for abundance and diversity analyses, as well as taxonomic comparisons. Based on unweighted pair group method with arithmetic mean clustering (using the mothur program) and a principal coordinates analysis using the UniFrac tool, the bacterial communities of the small intestinal samples were divided into two clear groups (Fig. 1); the first group comprised control samples 1 and 2, and the second group comprised irradiated samples 7, 8, and 9. By contrast, a clear division of these groups was not observed for the large intestinal microbiota.

Next, the diversity indices were compared using the qualified sequence reads. The detailed diversity estimates of the large and small intestinal microbiota are shown in Table 1. In the control group, the large intestinal microbiota was significantly different to that of the small intestine (ANOVA, $P = 0.004$). Although the irradiated large and small intestinal samples clustered separately, the difference between the microbiotas in these organs was not significant (ANOVA, $P = 0.292$). The small intestinal microbiota differed significantly between the control and irradiated groups (ANOVA, $P = 0.003$), whereas the large intestinal microbiota did not (ANOVA, $P = 0.586$). Based on the observed number of OTUs, the number of OTUs estimated using the Chao method, and the Shannon indices, the diversity of the small intestinal microbiota in the irradiated group was higher than that in the control group (Table 1 and Fig. 2); in particular, the OTUs were significantly different ($P < 0.05$) between these two groups (Fig. 2b). By contrast, the diversity indices of the large intestinal microbiota were comparable for the control and irradiation groups (Fig. 3).

Rarefaction curves of the OTUs in the large and small intestinal microbiotas did not appear to approach a horizontal asymptote, indicating that the sequencing effort did not saturate diversity

Table 1
An overview of the mouse large and small intestine mucosa samples and estimates of sequence diversity and phylotype coverage of the MiSeq data. The diversity indices and richness estimators were calculated using mothur software. Diversity was estimated using operational taxonomic units (OTUs) and was defined as groups with ≥97% sequence similarity.

Sample[a]	Group	Total reads	Analyzed reads	Observed OTUs[b]	Shannon	Simpson	Chao	Good's coverage
1L	Control	5424	4470	915	4.75	24.57	4034.64	0.84
1S	Control	2058	1972	193	2.27	3.87	950.65	0.92
2L	Control	8323	6933	1419	5.05	29.23	7084.60	0.84
2S	Control	4037	3313	465	3.59	10.00	2020.74	0.89
7L	Radiation	6145	5091	1221	5.01	27.48	5083.20	0.81
7S	Radiation	15,377	13,089	1795	4.55	24.03	8497.97	0.89
8L	Radiation	7998	6761	1290	5.08	37.88	7226.69	0.85
8S	Radiation	2462	2213	313	3.33	10.22	1589.17	0.89
9L	Radiation	4161	3655	678	4.38	14.94	3456.88	0.86
9S	Radiation	4409	4008	560	3.27	6.77	2356.18	0.89

[a] L and S denote the large and small intestine, respectively.
[b] The OTUs were determined based on 97% of 16S rRNA gene similarity.

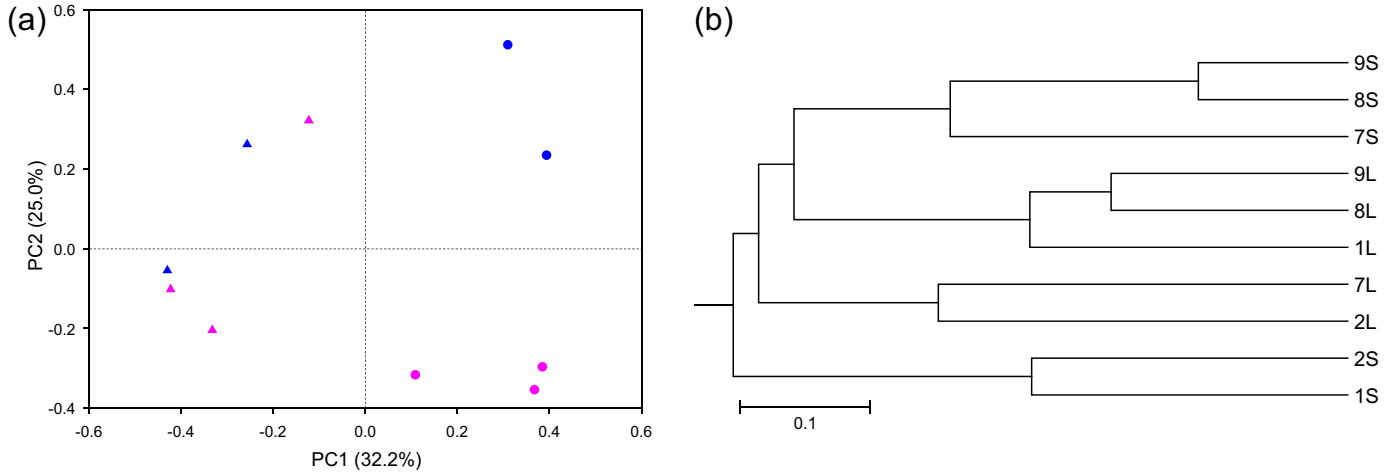

Fig. 1. The relationships between the bacterial profiles of the samples, represented by a principal coordinates analysis plot from a weighted UniFrac analysis (a) and an unweighted pair group method with arithmetic mean (UPGMA) clustering tree (b). Operational taxonomic units (OTUs) were determined based on 97% similarity of reads. The large and small intestine samples are denoted by L (triangle) and S (circle), respectively. (a) Blue and pink coloring denote the control and irradiation groups, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(data not shown). Nevertheless, the unweighted pair group method with arithmetic mean tree, diversity indices (Good's coverage), and principal coordinates analysis showed that the microbiota of the irradiation group was clearly distinguishable from that of the control group in terms of bacterial composition at the level of the OTU (Table 1, and Figs. 1–3).

3.2. Classification of assigned sequencing reads from the large and small intestines

3.2.1. Phylum resolution

The dominant microbial groups of the mouse large and small intestines were the phyla *Bacteriodetes* and *Firmicutes* (Supplementary Fig. S1). *Actinobacteria*, *Proteobacteria*, *Spirochaetes*, *Deferribacteres*, *Tenericutes*, *Verrucomicrobia*, and unclassified bacteria were also identified as minor bacterial groups using the RDP database. The phylum *Deferribacteres* was identified in the large intestine only. The small intestine contained a larger amount of the phyla *Actinobacteria* and *Proteobacteria* than the large intestine. Notably, *Verrucomicrobia* was identified in the irradiated large and small intestine samples, but not the control samples (Supplementary Fig. S2). In addition, reads assigned to the phyla *Bacteroidetes* and *Proteobacteria* were slightly more abundant in the irradiated large intestine group than the control large intestine group (Supplementary Fig. S2a). By contrast, the abundances of the phyla *Firmicutes* and *Actinobacteria* in the large intestine tended to be lower in the irradiated group than the control group. Similar tendencies of phyla abundances were also observed for the small intestine groups (Supplementary Fig. S2b). Notably, the magnitudes of the irradiation-induced alterations in the levels of *Bacteroidetes* and *Firmicutes* differed between the large and small intestinal samples; for example, irradiation treatment increased the amount of *Bacteroidetes* in the large intestine by approximately 4 percentage points, and increased the amount of *Firmicutes* in the small intestine by approximately 18 percentage points.

3.2.2. Genus resolution

More than 1% of the total taxon-assigned reads were analyzed at the genus level of taxonomic resolution (Supplementary Fig. S3). As expected, several specific genera were identified in the large and small intestinal microbiota. *Turicibacter* (74.0%) and *Corynebacterium* (17.2%) were the most abundant genera in the small intestine, and *Alistipes* (1.8%) was detected as a minor taxon in this organ. By contrast, the *Turicibacter* and *Corynebacterium* genera were not identified in the large intestinal microbiota. Other genera, including *Lactobacillus*, *Akkermansia*, and *Mucispirillum*, accounted for less than 1% of the small intestinal microbiota. Overall, at the genus level, the small intestinal microbiota was much less diverse than

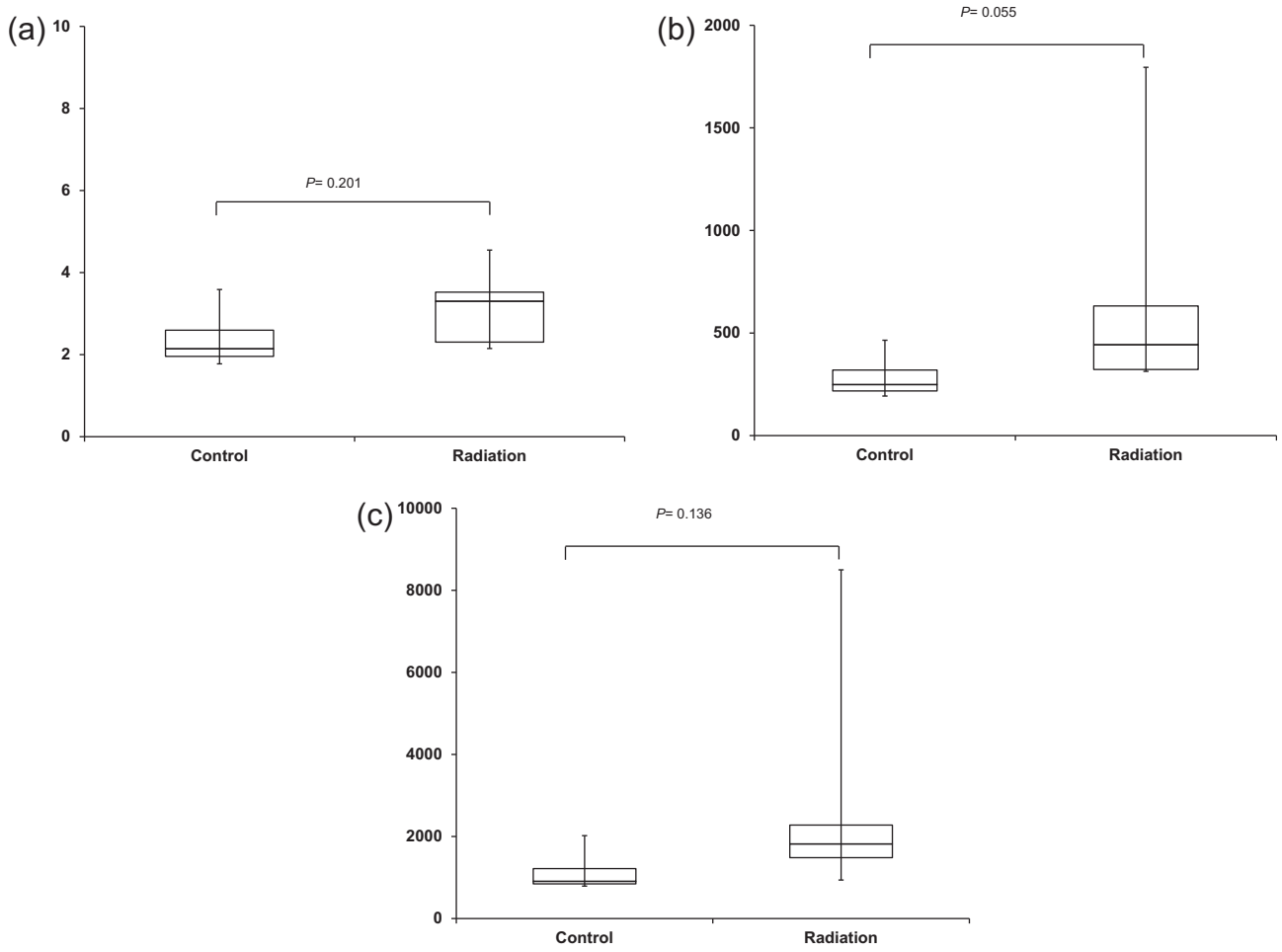

Fig. 2. Box plots of the Shannon indices (a), operational taxonomic units (OTUs) (b), and OTUs estimated using the Chao method (c) of the small intestinal microbiota groups. The plots are based on the data shown in Table 1. The *P*-values were estimated using a Mann–Whitney *U*-test.

that of the large intestine, which included *Alistipes*, *Barnesiella*, *Lactobacillus*, *Prevotella*, *Bacteroides*, *Oscillibacter*, *Akkermansia*, *Pseudoflavonifractor*, and *Mucispirillum*.

3.3. Core microorganisms affected by gamma irradiation in the large and small intestines

The results described above identified several genera in the large and small intestines that are affected by gamma irradiation. In the large intestine, irradiation increased the proportions of the genera *Alistipes*, *Lactobacillus*, and *Akkermansia*, but reduced the proportions of the genera *Barnesiella*, *Prevotella*, *Bacteroides*, *Oscillibacter*, *Pseudoflavonifractor*, and *Mucispirillum* (Supplementary Fig. S3). In particular, the abundances of the genera *Alistipes*, *Lactobacillus*, *Prevotella*, and *Akkermansia* were altered dramatically (>5%) by irradiation treatment. Although there were two major genera in the small intestine (*Turicibacter* and *Corynebacterium*), five genera (*Turicibacter*, *Corynebacterium*, *Alistipes*, *Lactobacillus*, and *Mucisprillum*) could be considered as irradiation-susceptible microorganisms based on the differences in their abundances in the control and irradiation groups; in particular, irradiation treatment caused a marked increase and decrease in the abundances of *Corynebacterium* and *Alistipes*, respectively (Supplementary Fig. S3).

4. Discussion

It was reported recently that the human body possesses at least ten times more intestinal microbial cells than human cells [27]. The composition of the gut microbiota can have a profound impact on human health and disease. To date, most studies have focused on pathogenic or beneficial microorganisms that influence gastrointestinal disorders, such as irritable bowel and inflammatory bowel diseases [4,5]; however, the relationships between the human and microbial cell communities remain unclear.

Gamma radiation treatment (radiotherapy) is typically prescribed to more than 50% of patients with colorectal, ovarian, or cervical cancers [11]. Although the small intestine is rarely the target of irradiation treatment, due to its size and location, it is often exposed to irradiation as collateral damage, and radiosensitive mucosal cells of the small intestine might be proliferated rapidly and/or injured by this exposure [6]. Hence, during and/or after irradiation treatment, most patients suffer side effects, such as diarrhea, malabsorption, bloating, nausea, anorexia, or fecal incontinence. These symptoms of small intestinal injury affect a patient's quality of life, resulting in extended hospitalization. Damage to the small intestine is one of the major causes of dose limitation or discontinuation of radiotherapy.

Next-generation sequencing has been used to examine the functional roles of the gut microbiota in a number of mammals [28–37]. Here, we used the Illumina MiSeq high-throughput

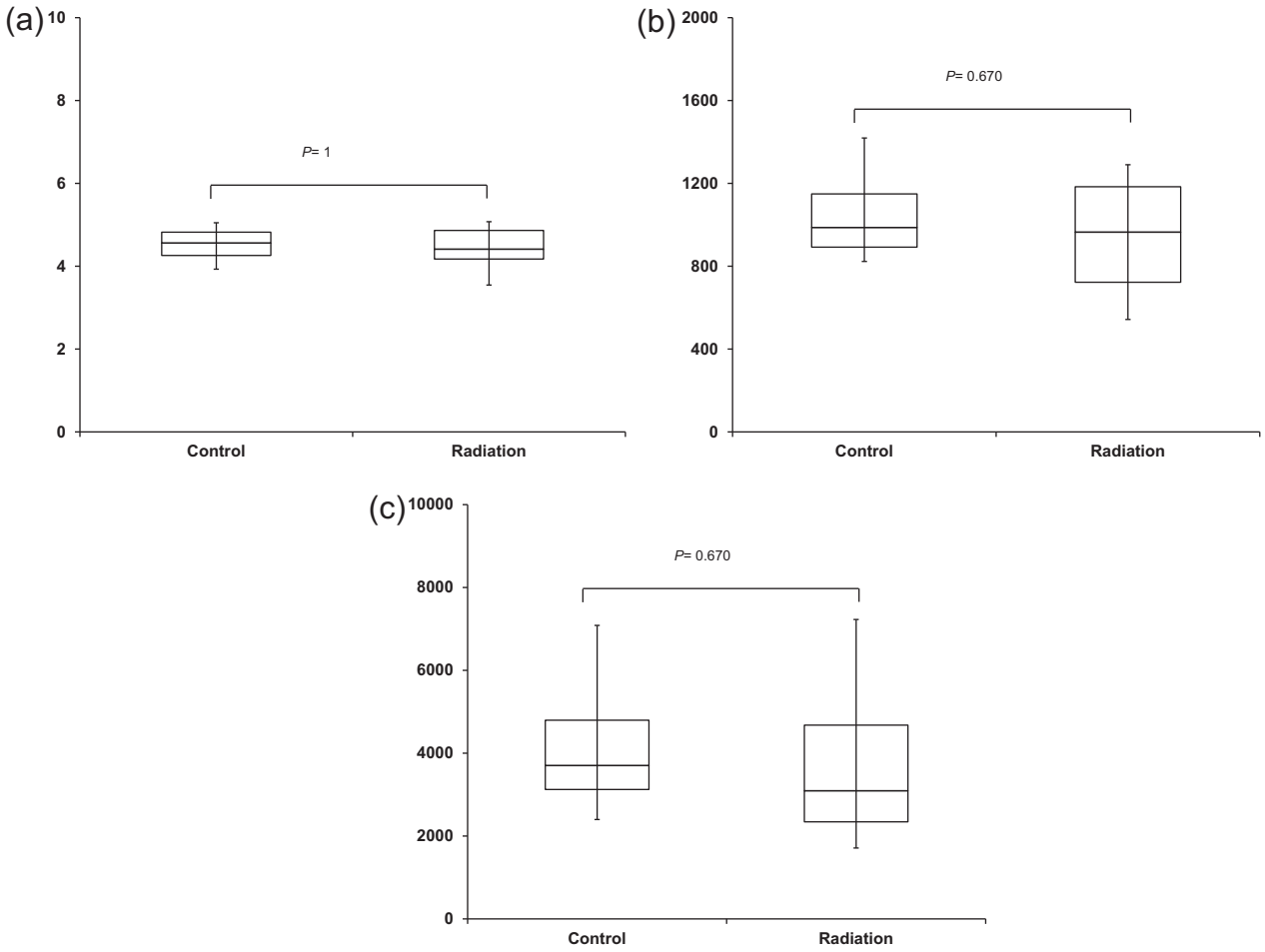

Fig. 3. Box plots of the Shannon indices (a), operational taxonomic units (OTUs) (b), and OTUs estimated using the Chao method (c) of the large intestinal microbiota groups. The plots are based on the data listed in Table 1. The *P*-values in the plot were estimated using a Mann–Whitney *U*-test.

sequencing platform and analysis of the bacterial 16S rRNA gene to study the effects of gamma irradiation on the community structure, microbial replacement, and core microorganisms of the mouse large and small intestines. We identified the relative abundances of the predominant phyla and genus level taxa in large and small intestinal samples, and identified significant differences between the control and irradiation groups. For the small intestine, an analysis at the OTU level revealed a clear separation between the control and the irradiation groups. Other studies have reported that the harmful effects of irradiation, such as injuries induced by X-ray irradiation, are caused by proliferation of the small intestinal bacteria [reviewed in 16]. Therefore, we focused on the small intestinal microbiota with the aim of identifying microorganisms that are affected by irradiation treatment.

As reported in several previous studies of the mammalian gut, including those of human and swine [32,37,38], *Bacteriodetes* and *Firmicutes* were identified as the two major phyla of the mouse small and large intestines. Although the abundances of several phyla differed between the control and the irradiation groups, we did not observe community replacements and/or changes at the phylum level. Notably, *Verrucomicrobia* was identified in the irradiated large and small intestines, but not the control samples, suggesting that the proliferation of microbial cells belonging to this phylum might be increased after irradiation. Although the relationship between the host and *Verrucomicrobia* has not been characterized, Dubourg et al. [39] reported dysbiosis (microbial imbalance) and an increase in the proportion of sequences related to *Verrucomicrobia* in the gut after broad-spectrum antibiotic treatment. The observed detection of *Verrucomicrobia* in the irradiated mouse gut suggests that this treatment may have induced an abnormal condition/state of health [40–42].

At the genus level, the large intestine was more diverse than the small intestine, and the microbial community structures also differed significantly between these two organs. The results presented here support the notion that the nature and functions of the small intestine contribute to the microbial community structure and affect the relationship between the human gut and its microbiota [43,44]. The abundances of intestinal microorganism genera were also altered by irradiation. In the large intestine, irradiation treatment reduced the abundances of some genera, such as *Prevotella* and *Mucispirillum*. These genera have been identified as normal gut microorganisms that may play important roles in regulating host gene expression and cellular functions as mucosa-associated microbiota [35,45]. Moreover, several studies have shown that *Prevotella* spp. may be associated with rheumatoid arthritis [46], autism [47], and gut health, including ulcerative colitis [48]. Unexpectedly, irradiation treatment increased the abundance of the genus *Alistipes* in the large intestine from 7.7% to 20.6% of the total assigned taxa. *Alistipes* spp. strains (such as *Alistipes finegoldii*) have been isolated from tissue samples of brain abscesses and appendicitis patients [49,50]. In addition, the proportion of *Alistipes* in the gut microbiota is reportedly increased in patients

with myalgic encephalomyelitis/chronic fatigue syndrome [51]; therefore, an elevated level of *Alistipes* spp. might be considered a biomarker of pathologic gastrointestinal conditions. In the large intestine, irradiation treatment increased the proportion of sequencing reads of the genus *Akkermansia* from an undetectable level to 5.8%. *Akkermansia muciniphila* was originally isolated from the human intestine as a mucin-degrading bacterium [52]. Everard et al. [53] suggested that *A. muciniphilla* might positively contribute to obesity and type 2 diabetes; in addition, the results presented here suggest that *Akkermansia* spp. may also be related to irradiation injuries of the gut. It is possible that radiation therapy induces symptoms of enteritis symptoms, including mucus discharge; hence, the abundance of *Akkermansia* spp. may be increased to promote mucus degradation. Irradiation-induced mucus secretion is recovered over time or by treatment of patients with drugs [54–57]. It is possible that the ionizing effects of gamma irradiation damage the chromosomal DNA in rapidly proliferating tumor cells but not normal cells [58]. Human chromosomes do not normally untangle and divide without specific cellular signals. If *Akkermanisa* spp. have functional activities that contribute to normal gut health, their activities might be decreased after irradiation. Although the mechanisms involved in irradiation injuries to the gut are currently unknown, it is clear that some gut microorganisms contribute to host health.

The irradiated small intestine contained two dominant genera, namely *Corynebacterium* and *Turicibacter*. The genus *Turicibacter* was originally isolated from a febrile patient with acute appendicitis [59]. Notably, irradiation treatment increased the abundance of *Corynebacterium* in the small intestine from 5.4% to 21.5% of the total assigned taxa. Although some *Corynebacterium* spp. have useful industrial applications [60], other strains of *Corynebacterium* cause human diseases such as diphtheria [61]. Therefore, it is possible that *Corynebacterium* and *Turicibacter* contribute to diarrhea in irradiated patients.

There are several limitations to this study. First, analysis of the mouse gut microbiota was performed three days after irradiation; analyzing the time-course of the effects of irradiation on the mouse gut would provide more detailed information regarding intestinal dysbiosis [62]. Second, the sample size of the irradiated group was relatively small ($n = 3$ mice). The results presented here differ from those of previous studies that analyzed the intestinal microbiota after irradiation exposure; because bacterial composition is influenced by host genetics, diet, or development, this discrepancy could be due to the use of different animal models in each study [28,63]. Nevertheless, our analyses revealed identified several microorganisms that are related to irradiation injuries. In addition, we demonstrate that quantitative alternations in the proportions of several genera, such as *Alistipes*, *Corynebacterium*, and *Mucisprillum*, may be useful biomarkers of irradiation exposure in the gut [62]. Future studies should focus on the functional roles of irradiation-related microorganisms such as *Alistipes* and *Mucisprillum* in the gastrointestinal tract.

In summary, this study demonstrates that the levels of some microorganisms in the gut, such as *Alistipes* and *Mucisprillum*, are associated with gamma irradiation exposure and suggests that the composition of the small intestinal microbiota may be associated with host gut health. The bacterial composition traits described here expand current understanding of the relationship between the microbial community structure and irradiation injuries to the human gut. In addition, this study provides a baseline for understanding the complex gut microbiome, which will aid the development of effective clinical diagnosis methods or treatments that improve the quality of life of cancer patients.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.anaerobe.2015.01.004.

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