Thesis for the Degree of Master of Science

Analysis of intestinal microbiome of sea cucumber *Apostichopus japonicus* by next generation sequencing

by

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(차세대 서열 분석을 통한 해삼 *Apostichopus japonicus*의 장내 미생물체 분석 연구)

Advisor: Prof. Kyoung-Ho Kim

by

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Abstract

All animals live in complex interactions with microorganisms and the intestine is the most important organ where such interaction occurs. Understanding the intestinal microbial diversity may be essential to understand the animal. In this study, we investigated the intestinal microbial diversity of the sea cucumber *Apostichopus japonicus*, which is an important economical marine invertebrate in the Asian coasts. We randomly selected nine sea cucumbers at four different sampling times and the intestine was divided into anterior and posterior parts and used for DNA extraction. The 16S rRNA gene amplicon sequencing approach using tagged PCR with the Illumina MiSeq platform, was used to obtain the sequences from each sample. A total of 1,614,748 optimized reads and 350,254 operational taxonomic units (OTUs) were obtained from a total of 72 intestines from 36 sea cucumber samples. The sequences in the anterior intestines and posterior intestines were assigned to 42 and 35 phyla, respectively. The results showed that major phyla are *Proteobacteria*, *Bacteroidetes*, *Verrucomicrobia*, and *Planctomycetes*. The community profiles became similar in the posterior intestine. The taxonomical classification profiles and the Unifrac PCoA chart showed the effect of sampling times on the bacterial communities in the different intestinal regions.

1. Introduction

All plants and animals live together with various microorganisms. Microbiota refers to as the community of all microorganisms which are commensal, mutualistic and pathogenic association with the host organism (Haque 2017). The microbiome is the synonym of the term, microbiota, also means all microorganisms in a specific niche and, in addition, their collective genomes (Bäckhed 2005). Groups of microorganisms such as bacteria, archaea, protists, fungi, and viruses compose the microbiome (Abrahamsson 2012). The microbiome and host can be considered as a synergistic unit in the evolutionary process, sometimes collectively referred to as a holobiont (Gill 2006).

The majority of microbes that make up the microbiome inhabit the intestines. Many experiments have been conducted in various animals to understand the relationship between intestinal microbiome and host. Intestinal microbes synthesize the essential amino acids and vitamins needed for their host and contribute to degrade non-digestible substances like plant polysaccharides (Yang 2017). For example, intestinal microbes of termites and ruminants contribute to the digestion of non-digestible cellulose, and hemicellulose by the host (Yamazaki 2016). And in humans, microbiome in the human intestine have been shown to have mechanisms to synthesize essential substances such as polysaccharides, proteins, and vitamins needed for the host (Rowland 2018). As a result, intestinal microbiomes play a variety of roles in the physiology of the host (Yang 2017). In addition, studies on

the intestinal microbiome in mouse are likely to modulate immune responses, brain development, and behavior (Yamazaki 2016). They have also been shown to have a decisive influence on the regulation of host immune responses, hormonal and metabolic homeostasis (Gill 2006). Although the homeostasis contributed by the intestinal microbiome is crucial to the health of the host, the mechanism that determines the stability of intestinal microbiomes is still difficult to grasp (Sekirov 2010). The bacteria in the digestive tract interact and communicate through complex and diverse networks, delivering energy, materials, and information (Yang 2017). The role of the microbiome in human intestinal microorganisms has been extensively studied, but more researches are still needed in the microbial community of human (Yang 2017).

Marine invertebrates including sea cucumbers, also have symbiotic bacteria which influence the survival of these hosts. The symbiotic microbiota provides a variety of functions such as providing nutrients to the host and/or protecting infection from the invasive pathogens. Marine invertebrates such as starfish and sea cucumbers generally have a soft body. They use chemical substances to protect themselves from predators and other infectious microorganisms (Gao 2010). However, the study of symbiotic microbiota in marine invertebrates has not been fully understood yet and has myriad potential. (Leon-Palmero 2018). To understand that microbiomes maintain ecological stability in hosts, we need to identify the networking method between species-species interactions, the role of

microorganisms and the mechanism of homeostasis (Yang 2017). Genetically, the genes of the microbiome should be included in the genes of the host, rather than viewed separately, and should also be considered to include the network between the host and the microbiome (Gill 2006). Studies on animal intestinal microbial communities have revealed functions such as host growth, development, metabolism, and digestion as well as protection from predators, and we can see that microbes adapt to the environment as the animal evolves (Sommer 2013).

In order to clarify the intestinal microflora composition, amplicon sequencing, one of the culture-independent methods, has been mainly and widely used. The amplicon sequencing usually used PCR to amplifying small subunit rRNA gene and sequenced the amplicons using next generation sequencing (NGS). The amplicon sequencing approach has been widely used in vertebrate studies and also in the studies of invertebrates such as crustaceans, echinoderms, and mollusks to clarify the composition of intestinal microorganisms. The approach has revealed not only the microbial composition in invertebrates, but predicted how groups of microorganisms in the digestive tract affect the host ecologically (Hakim 2015). The development of the high-throughput sequencing technology allows microbes to be completely explored beyond partial information obtained by pure culture technology (Leon-Palmero 2018). An important challenge in natural ecosystems, including microbes, is the identification of species diversity based on gene sequences. Methods to resolve this include operational taxonomic units (OTUs) or

phylogenetic comparisons based on electrophoretic patterns or DNA sequence analysis. OTUs are genetic or phylogenetic species grouped solely by sequence similarity, and generally, are considered as an OTU if they have more than 97% identity (Schloss 2005).

Commercial cultivation of invertebrates has received increasing attention from the middle of the 20th century (Conand 1993). In Asia, sea cucumbers have been ingested from the past and cultivation methods have been developed (Yang 2017). *Apostichopus japonicus* is a sea cucumber in the temperate of the Asian coast, Korea, Japan and China (Sha 2016). *A. japonicus* has a medicinal property (anticoagulant, antifungal, and antitumor agent) and is still used as an important economic resource (Fan 2001). Holothurians, or sea cucumbers, belong to the class *Holothuroidea*, phylum *Echinodermata* (McElroy 1990). There are about 1,700 species of sea cucumbers worldwide, including *A. japonicus*, which is common in Asia (Gao 2014).

Sea cucumber is one of the benthic animals, and it plays an important role in circulating organics by ingesting food through ingestion of marine sediments or filtration of seawater. Sea cucumbers generally have shield-like stretchable tentacles that envelop their mouths and are deposit-feeding animals. Deposit-feeding animals are animals that typically consume sediments containing low nutrients (Kerr 2001). Sea cucumbers are a representative cohort of deposit-feeders. They consume microorganisms, meiofauna, organic debris, minerals and organic

matter of the benthic sediment, and play an important role in benthic nutrients recycling (Yang 2017). Since sediment contain minerals and non-digestible organic matter, an efficient way should be sought to obtain only digestible resources (Lopez 1987). They crawl to the sedimentary layer and ingest substantial amounts of sediments. This behavior has a great influence on the intestinal microflora and the microflora in sediments (Gao 2014). There is a long digestive tract in *A. japonicus*, and a large number of sediments pass through the intestine to selectively absorb available nutrients (Sha 2016).

Bacteria in sediments are considered to be either a direct food source of sea cucumbers or indirectly supplying essential nutrients to the host (Gao 2014). Researchers have demonstrated that microbes in the digestive tract contribute to digestion and absorption of nutrients, growth rates of host, immune responses and antagonism to the other pathogen (Bairagi 2002; Ziaei-Nejad 2006). The intestine microbiota of *A. japonicus* has been shown to produce extracellular enzymes capable of degrading polysaccharides (Zhang 2013). According to another report, intestine bacteria in sea cucumber play a role in supplying certain essential amino acids (Phillips 1984).

Many probiotics have been used to increase the growth rate of sea cucumbers and to increase immunity (Sha 2016). Still unknown species, however, play an important role in their hosts. The total number of microorganisms that are cultivable, are known to be less than 1% of total microbes in the whole microbial

community, therefore, other methods are needed to improve our understanding of microbial diversity (Riesenfeld 2004). NGS or culture-independent can be an important tool for identifying microbial communities (Marguerat 2010). Recently, NGS technology has been used to analyze microbiota diversity in various biome habituated in digestive tracts of human and animal, soils, seawater, sediments, and activated sludge (Roh 2010). NGS technology is capable of analyzing total microbiota up to the genus level in large cohorts (Abrahamsson 2012; De Filippo 2010; Turnbaugh 2009). Using this technology, we can identify the presence and diversity of numerous intestinal microbiota, and furthermore, we can be easily and quickly understanding how microbes play a role in the host (Gao 2014).

2. Materials and methods

2.1 Sample collection

The specimens of sea cucumbers (*Apostichopus japonicus*) were collected in the shallow sea floor (3-5 m under the surface) by scuba diving near Namhae city, South Gyeongsang province, Korea (34°772'N, 127°919'E). The sample collection was conducted at the same location every month (from January to April in 2018) in which they are not dormant. The collected samples were immediately stored at -80 °C before DNA extraction.

2.2 Culture dependent approach

The potential microbiome in the intestine content of sea cucumber was spread onto marine agar (MA, Difco) using the serial dilution method using 0.85% (w/v) saline solution and incubated at 25 ℃ for 5 days. Colonies were picked randomly and transferred to fresh MA plates until verifying their purity. The 16S rRNA gene amplification was carried out using 115 colonies isolated on MA. Four different fingerprinting PCR methods such as ERIC, BOX, REP and GTG PCR were performed to avoid the taxonomical redundancy of colonies as much as possible before sequencing. For ERIC PCR, ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′), ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) primers were used (Gillings 1997). For BOX and GTG, the primers, BOX 5′-CTACGGCAAGGCGACGCTGACG-3′) and GTG (5′-

GTGGTGGTGGTGGTG-3′), respectively, were used (Masco 2003). REP1R (5′-IIIICGICGICATCIGGC-3′) and REP2I (5′-ICGICTTATCIGGCCTAC-3′) primers were used for REP PCR (Masco 2003). For ERIC and REP PCR, 1 μl of each primer (10 pmol) was added and for BOX and GTG PCR, 2 μl of each primer (10 pmol) was added to PCR premix (Maxime™ PCR PreMix (i-Taq), iNtRON). ERIC and BOX PCR conditions are as follows: The pre-denaturation at 95°C for 7 min, 35 cycles of extension (denaturation at 95 °C for 1 min, annealing at 52 °C for 1 min, and extension at 65 °C for 8 min) and final extension at 65 °C for 16 min. REP and GTG PCR conditions used the same conditions except for only annealing temperature of 40 °C. A total of 96 PCR products were sequenced using 16S rRNA gene 8F (5′-AGAGTTTGATCMTGGCTCAG-3′) – 1492R (5′-WACCTTGTTACGACTT-3′) primers (Suzuki 1996).

2.3 DNA extraction

The sea cucumbers were dissected in the laboratory aseptically after their weight and length were measured. The specimen was washed with distilled water to prevent the contamination by the bacteria on the skin before dissected with sterilized knife. The intestine was cut out and divided into anterior region and posterior region at the middle point. Tweezers were used to extract the content of the anterior gut and the posterior gut and transferred to a 5 ml sterilization tube, respectively. Intestinal DNA was extracted using the E.Z.N.A Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA). The extracted DNA was stored at -80 °C.

2.4 Barcode PCR

A specific primer set, 1047F (5'-GGWGBTGCATGGYYG-3') - 1492R (5'-WACCTTGTTACGACTT-3'), was designed to amplify small subunit rDNA of Bacteria, Archaea, and Eukarya. In order to perform tagged PCR, twenty-five different barcode primers as shown in Table 1, were made by attaching 8bp barcode and 2bp linker to specific primers. Intestinal DNA 1ul (10ng) were added to PCR premix which was filled with DNase/RNase free water up to the final volume of 20 μl. PCR condition begins with the pre-denaturation at 95 ℃ for 5 min followed by 38 cycles of denaturation at 95 ℃ for 20 s, annealing at 48 ℃ for 20 s, and extension at 72 ℃ for 20 s, and the final extension at 72 ℃ for 3 min was performed. The PCR product concentration was measured by Nanodrop (Optizen NANO Q, OPTIZEN) and the amplicons from different samples were pooled at the same concentration. Pooled amplicons were sequenced by the Illumina MiSeq sequencing platform at a concentration of minimum standard (26.19 nM)

Table 1. The list of barcoded PCR primer sequences used in this study

Primer set	Barcode primer Set (barcode 8bp+linker 2bp+primer)				Barcode sequences
1	EBA-U1047F1	GTTGGCCGCGGGGWGBTGCATGGYYG	EBA-U1492R1	GTTGGCCGTAWACCTTGTTACGACTT	GTTGGCCG
2	EBA-U1047F2	TATTAACTCGGGWGBTGCATGGYYG	EBA-U1492R2	TATTAACTTAWACCTTGTTACGACTT	TATTAACT
3	EBA-U1047F3	CTAATGGCCGGGWGBTGCATGGYYG	EBA-U1492R3	CTAATGGCTAWACCTTGTTACGACTT	CTAATGGC
4	EBA-U1047F4	AACCAGTCCGGGWGBTGCATGGYYG	EBA-U1492R4	AACCAGTCTAWACCTTGTTACGACTT	AACCAGTC
5	EBA-U1047F5	GAACGGAGCGGGWGBTGCATGGYYG	EBA-U1492R5	GAACGGAGTAWACCTTGTTACGACTT	GAACGGAG
6	EBA-U1047F6	ACTGAAGTCGGGWGBTGCATGGYYG	EBA-U1492R6	ACTGAAGTTAWACCTTGTTACGACTT	ACTGAAGT
7	EBA-U1047F7	TTGGCTATCGGGWGBTGCATGGYYG	EBA-U1492R7	TTGGCTATTAWACCTTGTTACGACTT	TTGGCTAT
8	EBA-U1047F8	TGGCGATTCGGGWGBTGCATGGYYG	EBA-U1492R8	TGGCGATTTAWACCTTGTTACGACTT	TGGCGATT
9	EBA-U1047F9	CCTCTGATCGGGWGBTGCATGGYYG	EBA-U1492R9	CCTCTGATTAWACCTTGTTACGACTT	CCTCTGAT
10	EBA-U1047F10	CTCATGCGCGGGWGBTGCATGGYYG	EBA-U1492R10	CTCATGCGTAWACCTTGTTACGACTT	CTCATGCG
11	EBA-U1047F11	TTCAGCGACGGGWGBTGCATGGYYG	EBA-U1492R11	TTCAGCGATAWACCTTGTTACGACTT	TTCAGCGA
12	EBA-U1047F12	GGATGCCACGGGWGBTGCATGGYYG	EBA-U1492R12	GGATGCCATAWACCTTGTTACGACTT	GGATGCCA
13	EBA-U1047F13	CGGTCGAGCGGGWGBTGCATGGYYG	EBA-U1492R13	CGGTCGAGTAWACCTTGTTACGACTT	CGGTCGAG
14	EBA-U1047F14	AAGACTACCGGGWGBTGCATGGYYG	EBA-U1492R14	AAGACTACTAWACCTTGTTACGACTT	AAGACTAC
15	EBA-U1047F15	AACGCTAACGGGWGBTGCATGGYYG	EBA-U1492R15	AACGCTAATAWACCTTGTTACGACTT	AACGCTAA
16	EBA-U1047F16	GCCTACGCCGGGWGBTGCATGGYYG	EBA-U1492R16	GCCTACGCTAWACCTTGTTACGACTT	GCCTACGC
17	EBA-U1047F17	TGACTGCTCGGGWGBTGCATGGYYG	EBA-U1492R17	TGACTGCTTAWACCTTGTTACGACTT	TGACTGCT
18	EBA-U1047F18	ATTGCCGCCGGGWGBTGCATGGYYG	EBA-U1492R18	ATTGCCGCTAWACCTTGTTACGACTT	ATTGCCGC
19	EBA-U1047F19	CAACCTTACGGGWGBTGCATGGYYG	EBA-U1492R19	CAACCTTATAWACCTTGTTACGACTT	CAACCTTA
20	EBA-U1047F20	GGAGGCTGCGGGWGBTGCATGGYYG	EBA-U1492R20	GGAGGCTGTAWACCTTGTTACGACTT	GGAGGCTG
21	EBA-U1047F21	AATCGATACGGGWGBTGCATGGYYG	EBA-U1492R21	AATCGATATAWACCTTGTTACGACTT	AATCGATA
22	EBA-U1047F22	ACCAATTGCGGGWGBTGCATGGYYG	EBA-U1492R22	ACCAATTGTAWACCTTGTTACGACTT	ACCAATTG
23	EBA-U1047F23	CCTAATAACGGGWGBTGCATGGYYG	EBA-U1492R23	CCTAATAATAWACCTTGTTACGACTT	CCTAATAA
24	EBA-U1047F24	GGATTAGGCGGGWGBTGCATGGYYG	EBA-U1492R24	GGATTAGGTAWACCTTGTTACGACTT	GGATTAGG
25	EBA-U1047F25	GCGTTACCCGGGWGBTGCATGGYYG	EBA-U1492R25	GCGTTACCTAWACCTTGTTACGACTT	GCGTTACC

2.5 Bioinformatic analysis

Paired-end reads from the Illumina MiSeq sequencing were merged to the sequences of 450-550bp in size using QIIME (Kuczynski 2012). The merged reads were assigned to each sample according to the barcodes listed in the mapping file. Reverse complement reads were changed to the forward orientation. The chimera sequence was removed with VSEARCH software (Rognes 2016). OTUs were picked by closed-references method with 97% identity criterion to the SILVA database. The silva132_97.fna and the 97_otus.tre files of the Silva_132_release version of the SILVA database were used as the reference databases. OTUs with fewer than 19 sequence leads were excluded from the analysis (McDonald 2012). After the biom file containing the OTUs information was created, the number of the sequences in each sample were counted. The highest number was 61,382 of AH7 and the lowest was 2,933 of AF7. For alpha and beta diversity analyses of 72 samples, the sequences number of samples was adjusted to the minimum number of 2,933. Sequences assigned to 'unassigned', chloroplasts, and mitochondria were discarded and taxonomy summary plots identifies the types and ratios of bacteria at the various taxonomic levels.

The Unifrac analysis was performed and shown through the EMPEROR program. The OTUs graph was confirmed by drawing the alpha rarefaction curve, and the values of Chao and PD_Whole_tree including the OTUs were numerically confirmed (Kuczynski 2012). All analyses were performed using the version of QIIME 1.9.1.

3. Results and discussion

3.1 Sample data index

Total 72 intestinal samples were analyzed from 36 sea cucumbers (9 sea cucumbers per sampling time). The mean and range of body length were 12.9 ± 1.9 cm and 9.9 cm - 17.2 cm, respectively (Table 2). The mean and range of body weight were 93.3 ± 39.0 g and 34 - 192 g, respectively. The mean and range of intestinal length were 46.4 ± 68.3 g and 31 - 65 cm, respectively. The mean and range of intestinal weight were 9.4 ± 6.1 g and 2.9 - 32.1 g, respectively. Intestines are 3.6 ± 0.6 times longer than body. The intestine weights are 10.3 % ± 4.4% of total body weight. As the length of body increases, the weight also increases (Fig. 1). However, in the case of the intestine, it can be seen that even the same length has a difference in weight, which indicates that the correlation was not clear (Fig. 1). That means that the amount of intestinal content is various according to the feeding status of sea cucumbers.

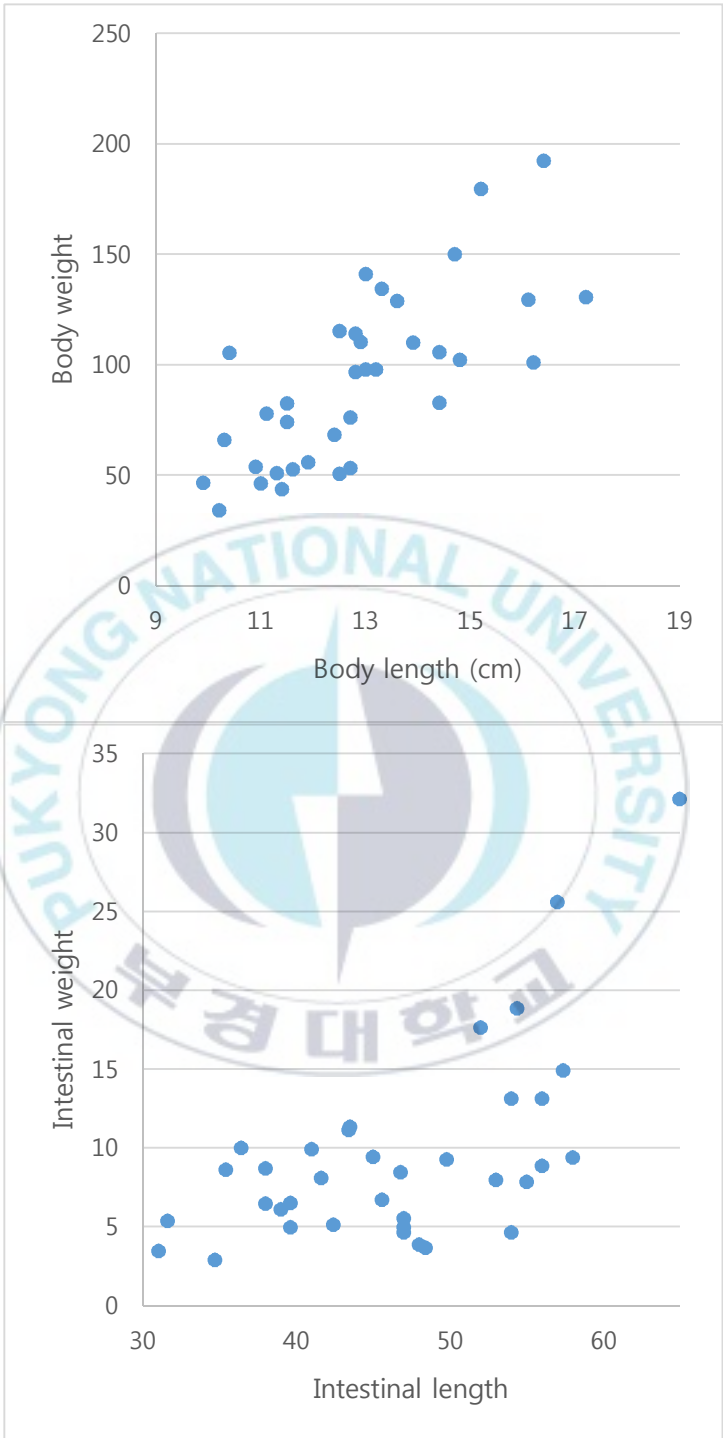


Figure 1. Scatterplots of body length vs weight (upper) and intestinal length vs weight (lower).

Table 2. Weight and length of sea cucumber and its intestine

Jan - A	SC length (cm)	SC weight (g)	Intestine length (cm)	Anterior weight (g)	Posterior weight (g)
A1	17.2	130.67	53	4.06	3.91
A2	16.1	129.48	47	1.59	3.06
A3	14.8	102.34	47	3.03	2.52
A4	14.4	82.76	48.4	2.04	1.64
A5	13.9	110.15	41	5.04	4.9
A6	12.8	96.81	58	5.34	4.05
A7	12.5	50.52	54	2.98	1.66
A8	11.6	52.77	42.4	1.71	3.42
A9	11.4	43.79	48	2.46	1.44
Feb - B	**SC length (cm)**	**SC weight (g)**	**Intestine length (cm)**	**Anterior weight (g)**	**Posterior weight (g)**
B1	16.4	192.4	65	7.04	25.09
B2	15.2	179.75	52	8.25	9.38
B3	14.7	149.95	57	11.14	14.47
B4	12.7	53.38	43.5	6.5	4.86
B5	11.9	55.77	35.4	4.56	4.07
B6	11.5	73.98	36.4	5.41	4.59
B7	11	46.19	31.6	3.11	2.29
B8	10.2	34.08	39	3.21	2.9
B9	9.9	46.7	38	5.45	3.28

Mar - C	SC length (cm)	SC weight (g)	Intestine length (cm)	Anterior weight (g)	Posterior weight (g)
C1	14.4	105.86	54.4	10.16	8.68
C2	13.6	129.01	57.4	6.26	8.67
C3	13.3	134.53	49.8	6.25	3.03
C4	13	141.08	43.4	6.12	5.01
C5	12.5	115.18	41.6	5.49	2.6
C6	12.4	68.28	39.6	4.81	1.7
C7	11.5	82.62	34.7	1.03	1.87
C8	11.1	78.02	47	1.44	3.55
C9	10.4	105.56	55	3.49	4.38
Apr - D	**SC length (cm)**	**SC weight (g)**	**Intestine length (cm)**	**Anterior weight (g)**	**Posterior weight (g)**
D1	16.2	101	54	6.68	6.44
D2	13.2	97.98	45	3.27	6.16
D3	13	97.76	56	3.45	5.41
D4	12.9	110.27	46.8	3.89	4.56
D5	12.8	114.05	56	6.51	6.62
D6	12.7	76.01	45.6	2.39	4.34
D7	11.3	50.81	31	0.97	2.51
D8	10.9	53.71	39.6	1.08	3.91
D9	10.3	65.95	38	2.25	4.24

3.2 Culture dependent taxonomy

A total of 96 sequences from isolated strains were obtained (Table 3). Among them, the most frequently identified bacteria were 13 strains of *Bacillus*, 11 of *Agarivorans*, 11 of *Vibrio*, 7 of *Staphylococcus* and 5 of *Ruegeria*. At the phylum level 50 *Proteobacteria*, 31 *Firmicutes*, 12 *Actinobacteria*, 2 *Bacteroidetes* and 1 *Planctomycetes* (Table 3) were identified.

When intestinal microorganisms of sea cucumber were culturally identified using CMC (carboxymethyl cellulose sodium salt), AL (sodium alginate), XL (xylan), S (soluble starch), LB (Luria-Bertani) medium, but not MA, 53 species were identified. Among them, *Firmicutes* (42 species), *Protebacteria* (9 species), and *Actinobacteria* (2 species) were identified (Zhang 2013). In the MA medium, the ratio of *Proteobacteria* was high, and the presence of vibrio was also confirmed. Culture in a medium with salt concentration similar to that of sea water suggests that *Proteobacteria* and *Vibrio* can be identified in a culture-dependent method. The most frequently found bacteria belonging to *Proteobacteria* were *Agarivorans*, *Vibrio* and *Ruegeria*, and were generally known to exist in the marine environment. *Bacillus* and *Staphylococcus*, which belonged to the *Firmicutes* and occupy a large proportion, were bacteria that have been studied for probiotics. Most of the identified bacteria, including these, showed mainly salt-tolerance and alkaliphilic properties.

Probiotics used as an alternative to antibiotics in aquaculture include *Ameromonas*, *Alteromonas*, *Bacillus*, *Carnobacterium*, *Clostridium*, *Enterococcus*, *Leuconostoc*,

Lactobacillus, *Lactococcus*, *Micrococcus*, *Pseudomona*, *Saccharomyces*, *Shewanella*, *Streptococcus*, *Vibrio* and *Weissella* (Gao 2014; Pandiyan 2013). Among them, *Bacillus*, *Micrococcus*, *Shewanella* and *Vibrio* were identified through culture-dependent method.

3.3 Culture-independent taxonomic assignment results at phylum level

Previous researchers have investigated microbiota in the digestive tract of sea cucumbers, but most of them have been studied through culture-dependent methods. We used next generation sequencing (Illumina Miseq platform) to establish more diverse microbiota.

The total of 72 samples were obtained by separating anterior intestines and posterior intestines from 36 sea cucumbers (Fig. 2). Total 42 phyla were identified at the phylum level. The most abundant phylum was *Proteobacteria* (55.0%). The next abundant phyla (≥ 1%) were *Bacteroidetes* (18.2%), *Verrucomicrobia* (11.3%), *Planctomycetes* (5.0%) and *Tenericutes* (3.8%). *Acidobacteria* (2.6%) and *Actinobacteria* (1.5%). *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, and *Verrucomicrobia* are bacteria commonly known to be intestinal microbiome (gut microbiota) of animals, including people (Mendez-Salazar 2018). Two phyla of Archaea, *Crenarchaeota* and *Euryarchaeota*, were identified with low proportion 0.04% along with the bacteria.

According to a report analyzing the intestinal microbiota of *Holothuria glaberrima*, a type of sea cucumber, *Firmicutes* (39.1%) were the most dominant, followed by

Bacteroidetes (24.4%), *Proteobacteria* (23.8%), *Fusobacteria* (4.2%) and *Actinobacteria* (1.3%) (Pagan-Jimenez 2019). It is interesting that *Fusobacteria* (0.1%), which has a low rate in the results of this report, was relatively high in the other sea cucumber species *Holothuria glaberrima*.

For other type of marine invertebrates, an ascidian, *Ciona intestinalis*, the most abundant phyla were the *Proteobacteria* (80%), *Bacteroidetes* (11.4%), *Fusobacteria* (5.7%), and *Verrucomicrobia* (2.9%) (Dishaw 2014). With the exception of the high proportion of *Fusobacteria*, it was the same to account for a large proportion in the order of *Protebacteria*, *Bacteroides* and *Verrucombia*.

Generally, roughly similar profiles were observed except for several exceptions (Fig. 2). Unlike the other samples, AF6, AF7, and AF8 had very high percentage of *Tenericutes*. AF1, CF9, and DF7 showed very high proportion of *Proteobacteria*. The percentage of *Bacteroidetes* was relatively high in posterior intestines of sea cucumbers sampled in April (DH1 to DH9).

When the samples were separated according to intestinal region, interesting patterns were observed (Fig. 3 and 4). All exceptional samples showing high proportions of *Tenericutes* or *Proteobacteria* were from the anterior region of intestine. The profiles looked more similar in the posterior regions.

Other studies have examined intestinal microorganisms of large and small individuals and identified 8 major phyla. *Proteobacteria* accounted for more than 50%, followed by *Verrucomicrobia*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*,

Cyanobacteria, *Planctomycete*, and *Spirochaetes*. Except for *Actinobacteria*, there was no difference between large and small individuals (Sha 2016). However, the large samples used in the above study were much smaller than the smallest samples used in this report, so it was difficult to make accurate comparisons, but it was confirmed that the dominant species of sea cucumber were *Proteobacteria*, *Verrucomicrobia*, and *Bacteroidetes*.

The results of this report can be verified by previous Gao (Gao 2014) report that confirmed that *γ-Proteobacteria* are the main bacteria in the intestines rather than *α-Proteobacteria*. However, reports by Sha et al. (Sha 2016) showed that *α-Proteobacteria* has stronger ability to adhere to the intestine. Based on the result of this report and other reports, it can be concluded that when the size of sea cucumber is small, the intestinal microorganisms occupy a large proportion of *α-Proteobacteria*, but the proportion of *γ-Proteinobacteria* is relatively increased as the sea cucumber grows.

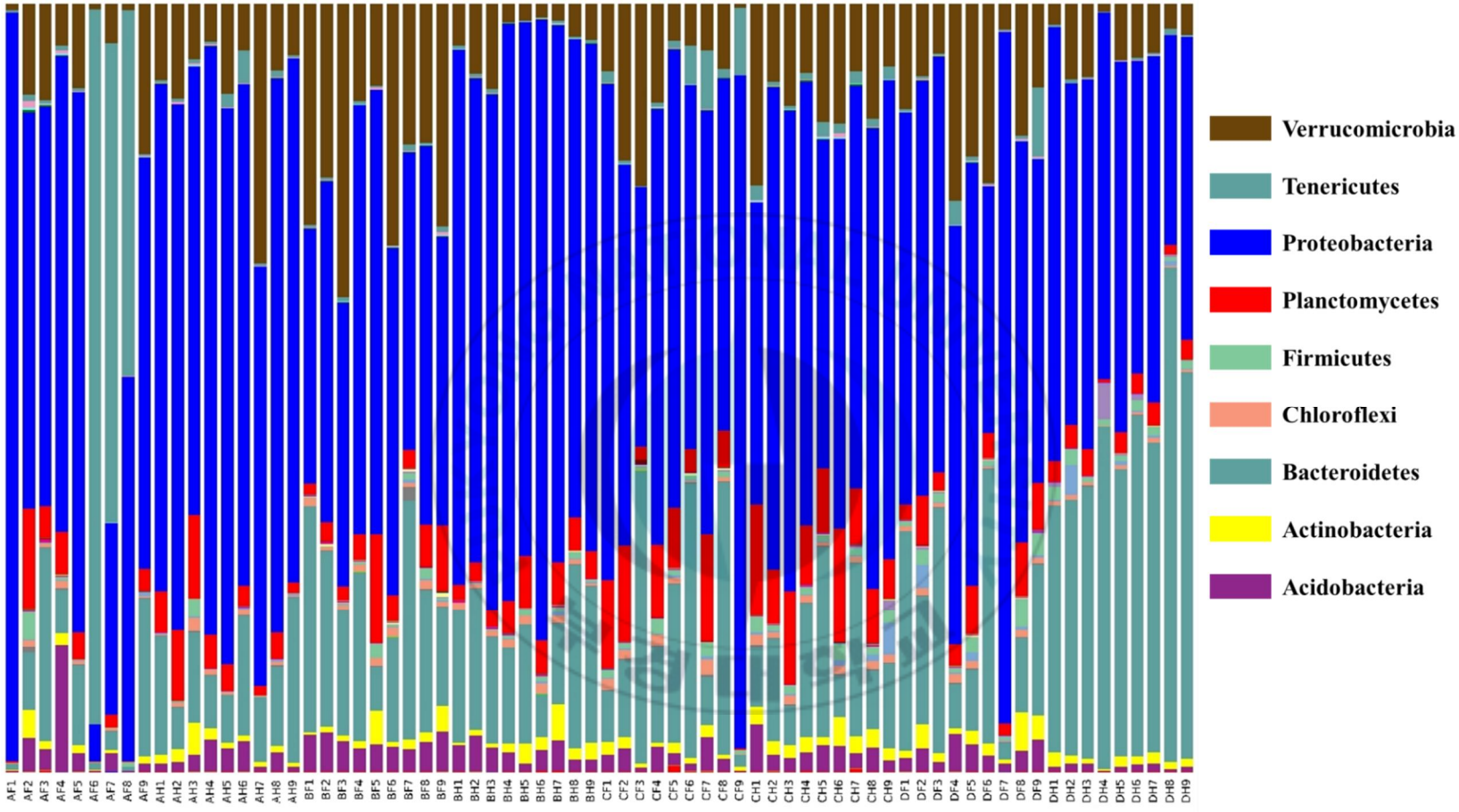


Figure 2. Taxonomic assignment results at the phylum level

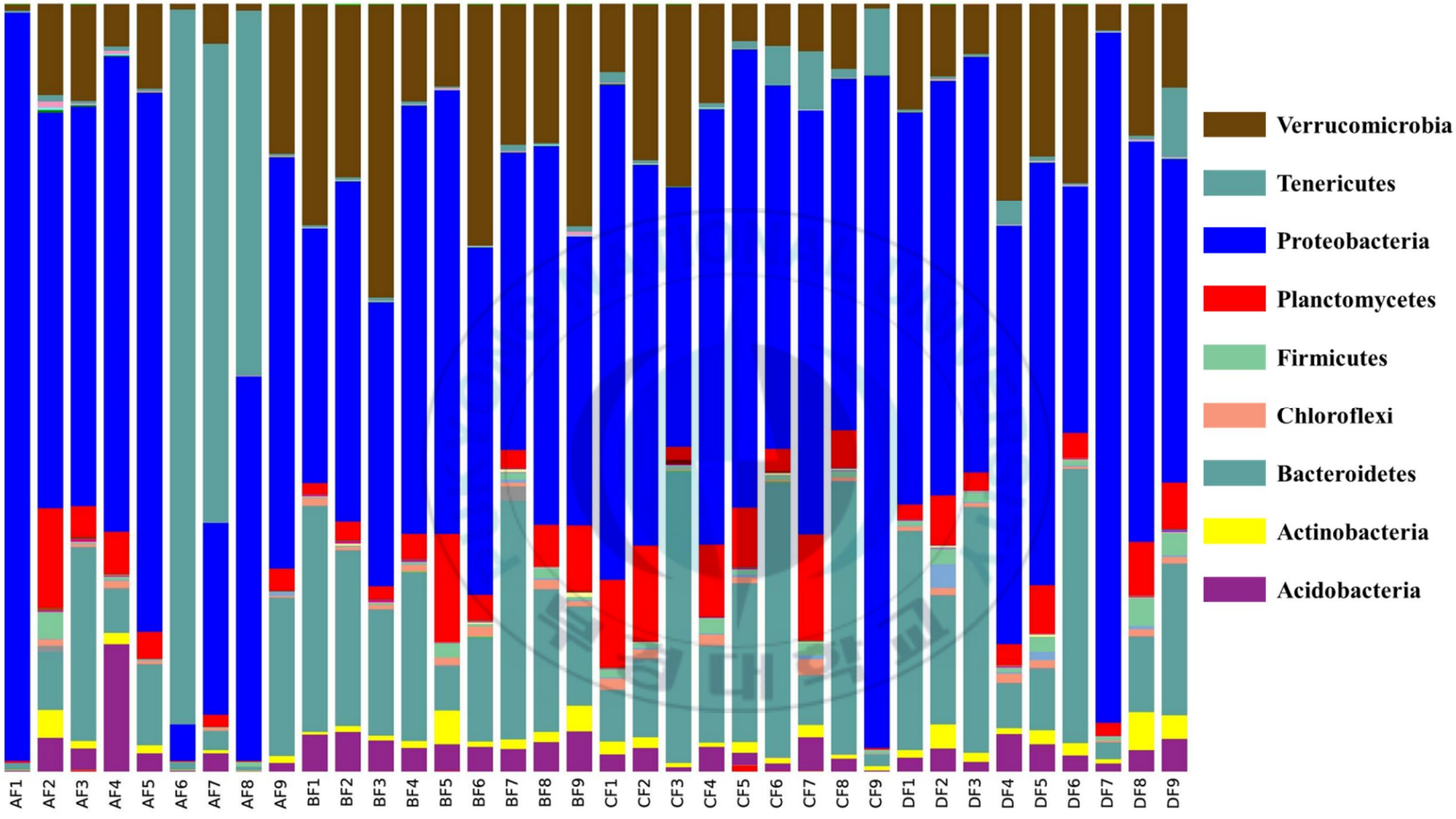


Figure 3. Taxonomic assignment results at the phylum level of the anterior intestines of 36 sea cucumbers

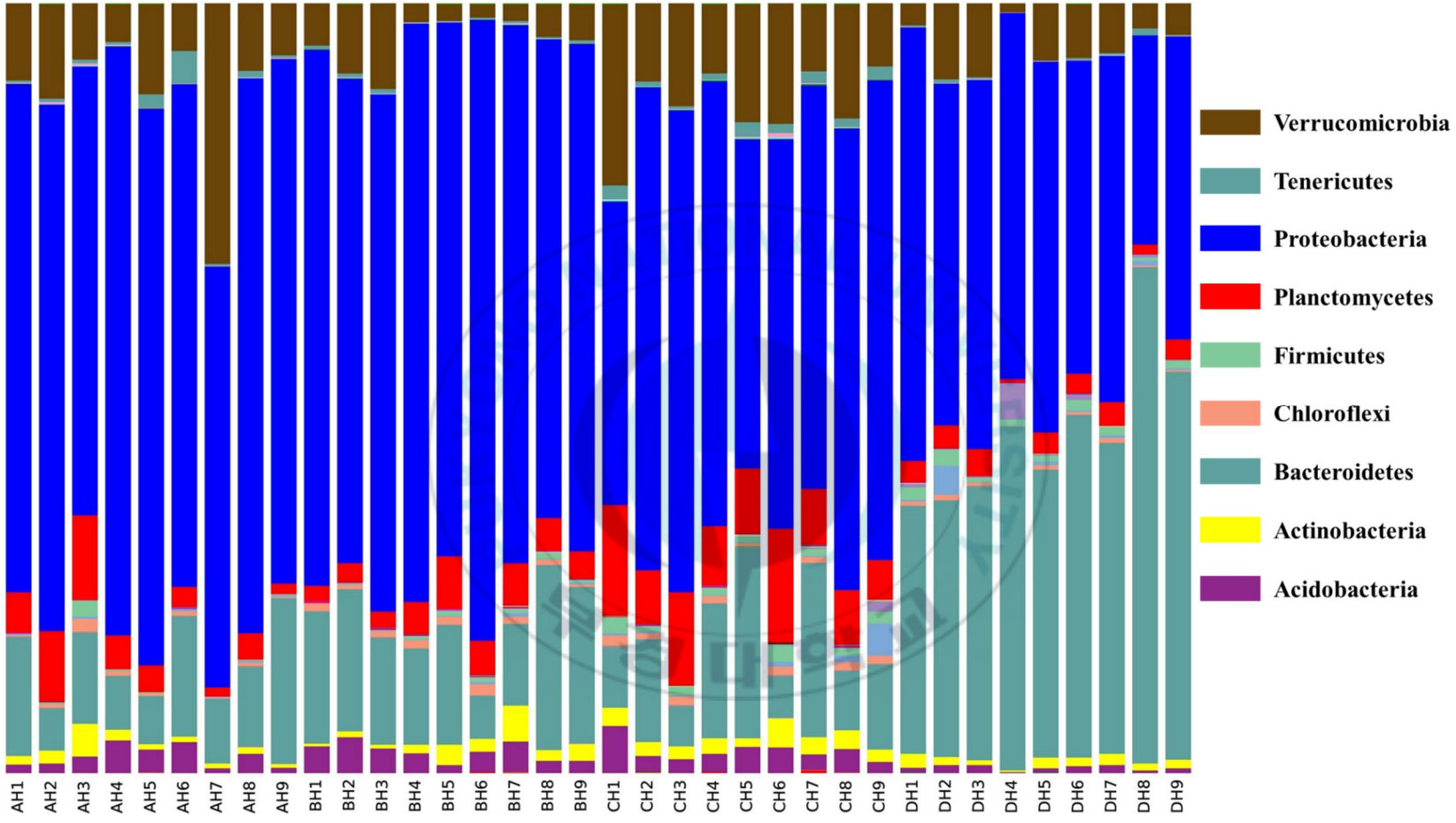


Figure 4. Taxonomic assignment results at the phylum level of the posterior intestines of 36 sea cucumbers

3.4 Taxonomic assignment results at genus level

In the total region of intestine, the bacteria that occupied the largest portion of the results from NGS analysis at the genus level were 17.1% of the genus in the family *OM60*, 14.8% of genus in the family *Flavobacteriaceae*, 5.6% of genus in the family *Desulfobulbaceae*, 5.1% of genus *Rubritalea* in the family *Verrucomicrobiaceae*, 4.2% of the genus in family *Pirellulaceae* and the genus in family *Rhodobacteraceae*. *Bacillus* and *Vibrio*, which were dominant in the culture-based approach accounted for only 0.1% and 0.3%, respectively. *Agarivorans*, *Staphylococcus* and *Ruegeria* accounted for < 0.1%.

The taxonomic assignment results at the genus level were shown in the anterior (Fig. 5) and posterior (Fig. 6) regions separately. The genus level taxonomic summary graphs showed that many anterior and posterior intestinal samples showed similar patterns, but several samples didn't.

The six samples of anterior region which had showed different profiles at the phylum level also showed profiles quite different from other samples at the genus level. A single genus or few genera occupied the majority of the sequences in a sample. For example, the sample AF1 is entirely composed of two groups of unidentified genus and genus *Anaerospora* in the family *Rhodobacteraceae*. In the samples AF6, AF7, and AF8 unidentified genus of class *Mollicutes* accounted for 92.9%, 62.0%, and 47.4%, respectively. Unknown genus of family *Hyphomonadaceae* accounted for 55.4% in the DF7. In the case of CF9, an

unknown genus of family *Caulobacteraceae* and an unknown genus of the class *Alphaproteobacteria* were found to be distinguished from the other anterior samples.

Posterior intestinal samples showed more similar patterns than anterior regions. Unlike to anterior regions, a few samples showed different patterns from other posterior samples, and but the difference was much less than anterior regions. The sample AH7 and DH4 showed different large proportions of few genera which were minor inhabitants in posterior intestines of other sea cucumbers.

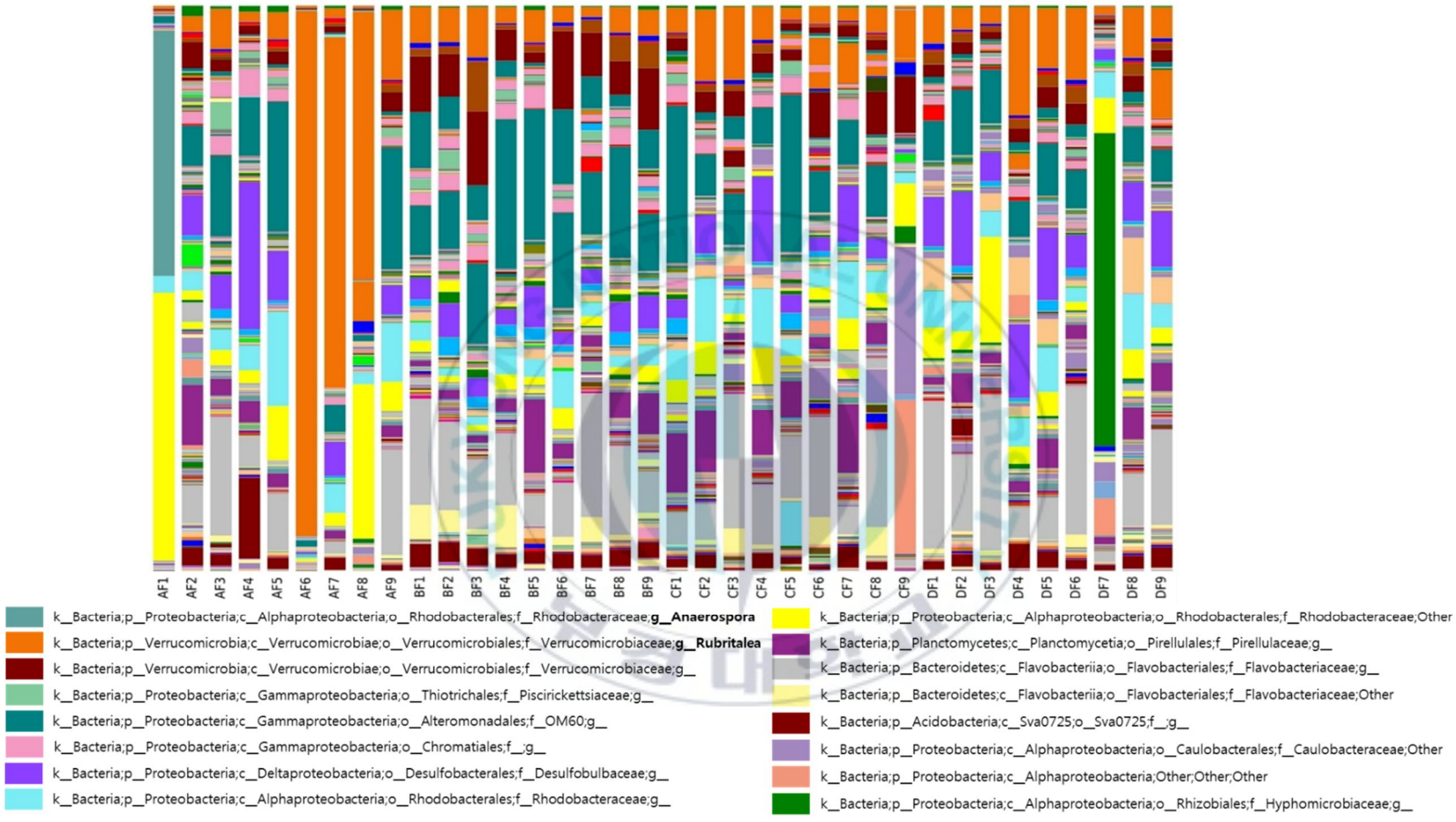


Figure 5. Taxonomic assignment results at the genus level of the anterior intestines of 36 sea cucumbers

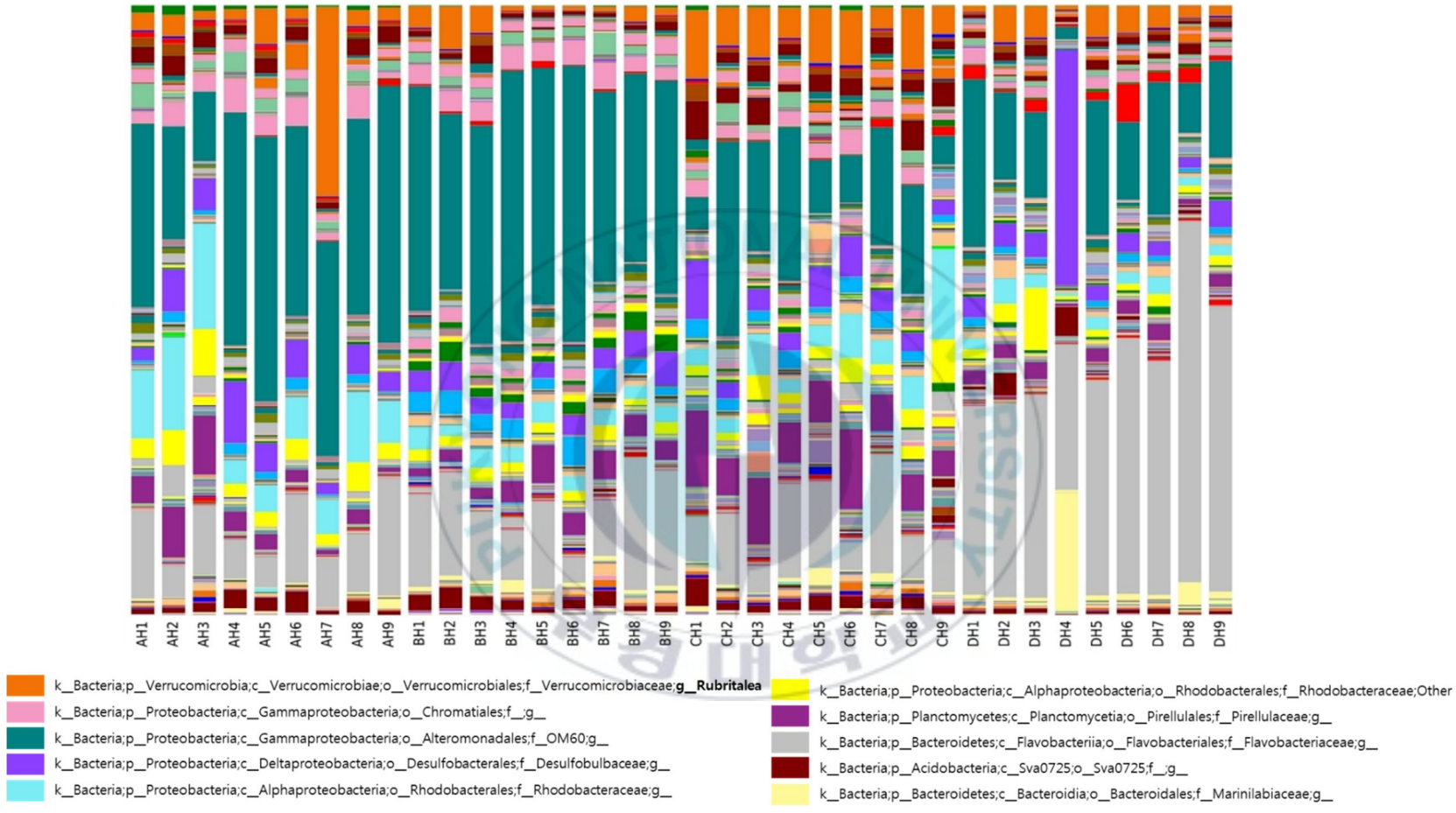


Figure 6. Taxonomic assignment results at the genus level of the posterior intestines of 36 sea cucumbers

Table 3. Results of sequencing analysis of colonies grown in MA

Phylum and genus	Count	Average similarity (%)	Phylum and genus	Count	Average similarity (%)
Actinobacteria	**12**	**99.62**	***Planctomycetes***	**1**	**100**
Janibacter	1	99.51	*Rhodopirellula*	1	100
Kocuria	1	100	***Proteobacteria***	**50**	**99.43**
Microbacterium	3	99.2	*Agarivorans*	11	99.93
Micrococcus	4	99.8	*Marinibacterium*	1	96.52
Nocardioides	1	99.9	*Marinicella*	1	97.31
Rhodococcus	1	99.91	*Marivita*	3	99.94
Serinicoccus	1	99.28	*Microbulbifer*	3	99.87
Bacteroidetes	**2**	**99.95**	*Oceanisphaera*	1	99.52
Gramella	1	100	*Paracoccus*	2	100
Lutimonas	1	99.89	*Phaeobacter*	2	98.59
Firmicutes	**31**	**99.65**	*Psychrobacter*	2	99.8
Bacillus	13	99.7	*Roseibium*	3	98.69
Domibacillus	3	99.68	*Roseovarius*	1	96.3
Halobacillus	1	99.6	*Ruegeria*	5	99.87
Lentibacillus	1	97.31	*Shewanella*	3	99.6
Paenibacillus	1	99.52	*Tateyamaria*	1	99.46
Paralkalibacillus	1	99.81	*Vibrio*	11	99.35
Psychrobacillus	1	99.52			
Sporosarcina	3	99.94			
Staphylococcus	7	99.79			
Total				**96**	

3.5 Rarefaction curve

The rarefaction curves based on the OTUs (Fig. 6) are presented according to intestinal position and sampling time. Anterior and posterior regions showed similar in OTUs numbers. The means of OTUs numbers according to the sample collected also at different seasons also did not show significant differences when the standard deviations were considered. Some samples like those in Jan_A were saturated in the curves by showing less than 800 OTUs at 2,933 read number. However, many other samples were not saturated, by showing up to 1,500 OTUs at the same read number. Because the lowest sample size was selected to compare the diversity indexes of samples, the rarefactions curves were not saturated.

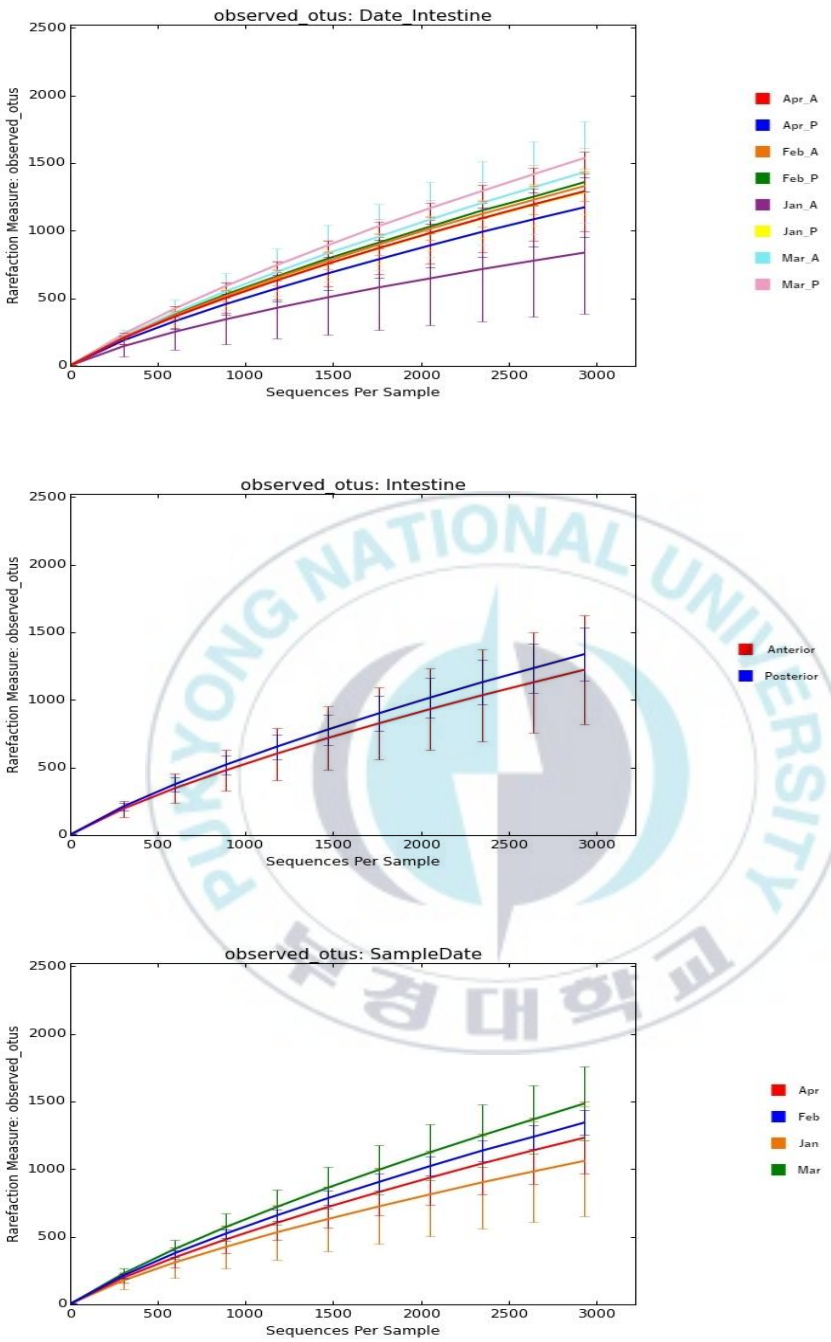


Figure 7. Rarefaction curves according to intestinal position and sampling time

3.6 Comparison of variety of anterior intestine and posterior intestine

The number of taxonomical groups at different taxonomical level in the anterior intestines, whole intestine, and posterior intestines were shown from taxonomic assignment (Fig 8). Totally 42 phyla, 131 classes, 260 orders, 471 families and 832 genera were found. In the anterior intestine, 42 phyla, 128 classes, 252 orders, 447 families and 770 genera were identified while 35 phyla, 106 classes, 222 orders, 408 families and 715 genera were identified in the posterior intestine. At the all taxonomical levels the number of groups is decreased in the posterior region, which means the diversity of posterior regions gets decreased.

This result can be verified by Gao et al. (Gao 2014) comparing phyla numbers of anterior intestine and posterior intestine sea cucumbers *A. japonicus*, and in the ambient surface sediment. According to the study, it can be seen that the diversity of bacteria decreases toward the sediment, anterior intestine, and posterior intestine (Gao 2014).

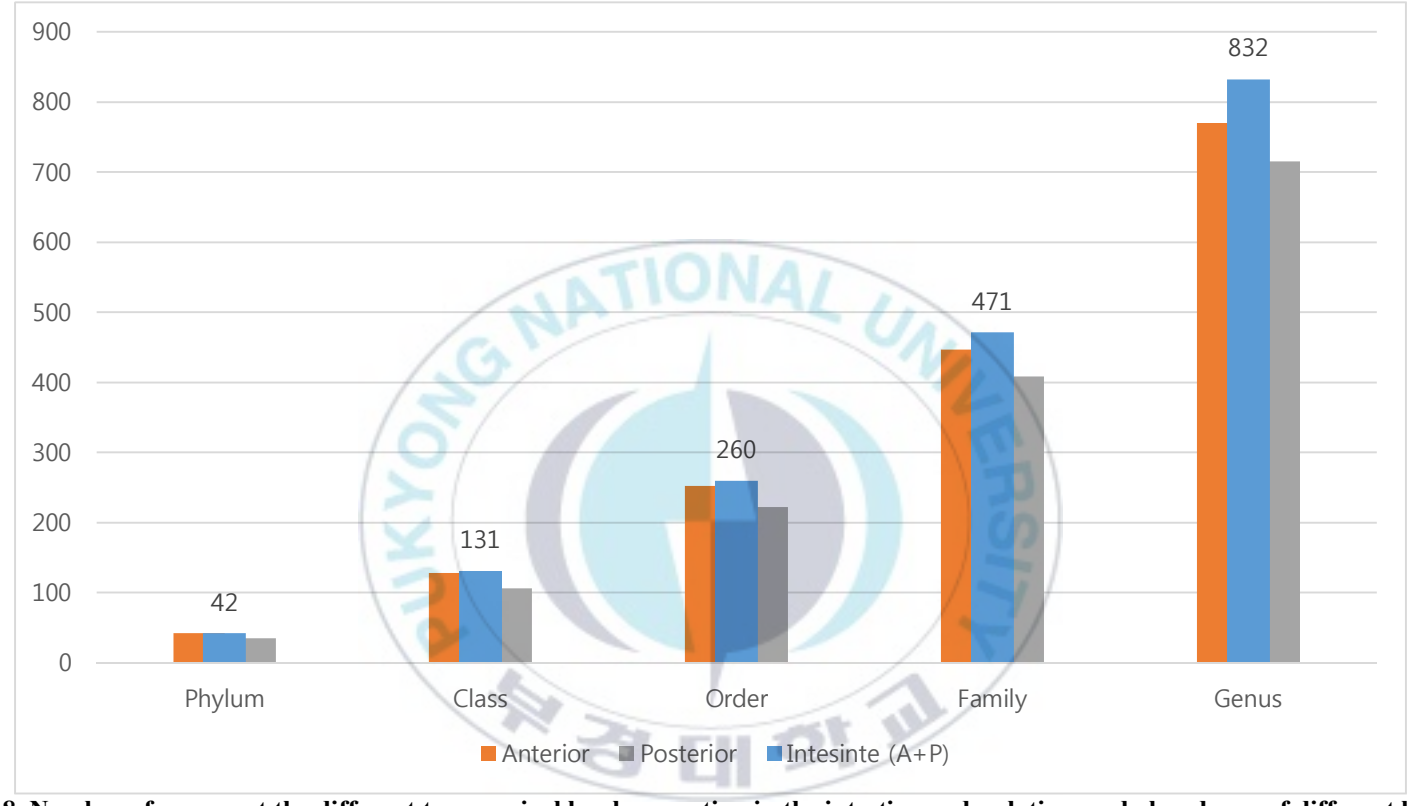
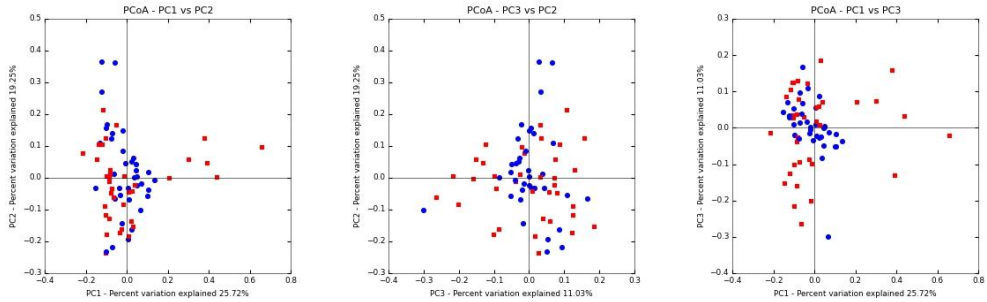


Figure 8. Number of groups at the different taxonomical level presenting in the intestine and, relative read abundance of different bacterial phylum

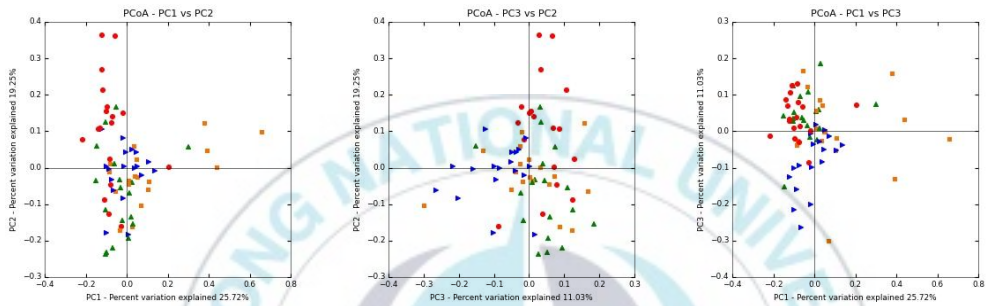
3.7 Unifrac PCoA plot results

The Unifrac analysis uses phylogenetic information and abundance of OTUs to compare sample's similarities. The result can be displayed on the PCoA space to show sample distance (Fig. 9). Some samples from anterior intestines are dispersed widely while posterior region samples can be seen as a loosen cluster. According to the sampling time, the samples in January showed the most scattered shape compared among all samples. After considering the position of intestine and the sampling time at the same time, many groups converge together forming more tightened group. These observations match again the taxonomic assignment results.

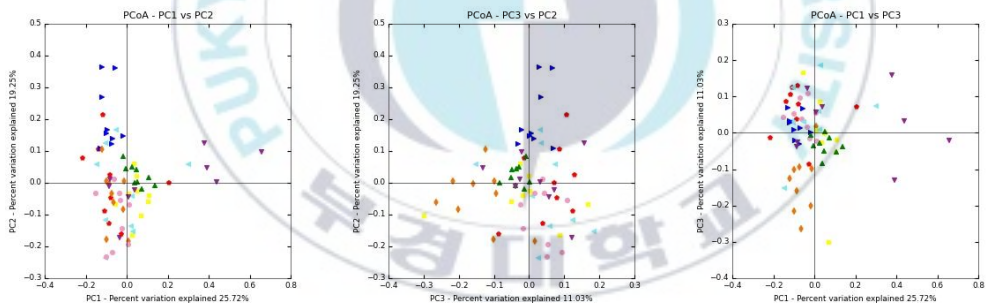
(A)



(B)



(C)



(A) - Anterior intestine - Posterior intestine

(B) - January - February - March - April

(C) - Jan_A - Jan_P - Feb_A - Feb_P

- Mar_A - Mar_P - Apr_A - Apr_P

Figure 9. 2D PCoA plot of samples according to location of intestine and sampling time. The suffix A indicates anterior and P indicates posterior.

3.8 Unifrac distance comparison

To confirm the Unifrac PCoA analysis more clearly, Unifrac distances were compared according to the sampling time and location of intestine (Fig. 10). Each boxplot shows the distribution of the Unifrac distances among the samples in the same group. The spreads of each box distance are generally larger in the anterior than posterior intestines at the all sampling time. In particular, January anterior intestine samples showed a large box range from 0.3 to 0.7 and the median value is about 0.6. The median value was reduced to about 0.2 and the box range also reduced. When the distances of samples in the same position of intestine also showed differences according to the intestinal location. Although the sampling time may cause the effect on the bacterial community change, the positional difference alone can differentiate the samples.

The box plots show the microbial communities in the samples are similar when collected at the same time and also become similar in posterior regions. Even though the microbial community of sea cucumber individual might be different in the anterior region, they get similar in the posterior intestine.

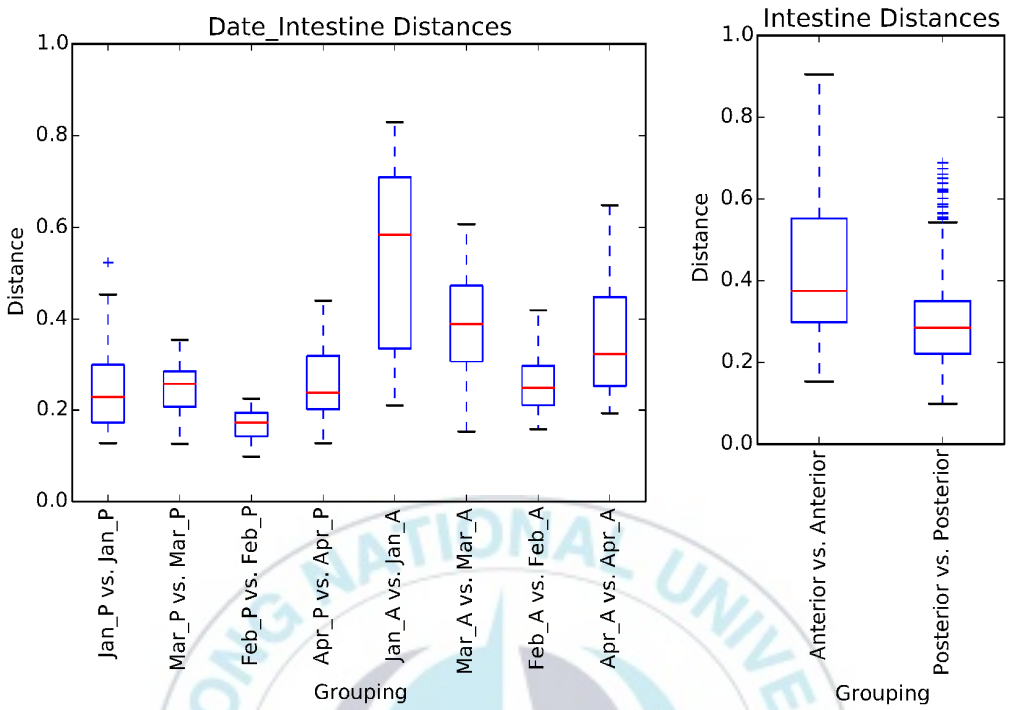


Figure 10. Box plots based on distance on different sampling time and location of intestine

3.9 Different microbiome in the different intestinal regions

Some bacteria were present only in the anterior intestine or in the posterior intestine, respectively. *Clavibacter*, *Methylobacterium*, *Mycoplasma*, *Nitratireductor*, *Phenylobacterium* and *Yersinia* were found only in the anterior intestine. *Clavibacter* was a phytopathogenic actinomycetes (Gartemann 2003). *Methylobacterium* can grow with methanol, methylamine, and promote plant germination and growth (Lidstrom 2002). *Mycoplasma* lack of a rigid cell wall, and infects eukaryotic cells or hybridomas of humans and animals (Drexler 2002). *Nitratireductor* was isolated from an aquarium and showed denitrification ability (Labbe 2004). *Phenylobacterium* was found in a facultative aerobic and salty area (Eberspächer 2006). *Yersinia* is a pathogen of human from livestock and the facultative aerobe (Perry 1997).

Alkaliphilus, *Beggiatoa*, *Desulfobacter*, *Enterovibrio*, *Pleomorphomonas* and *Ruegeria* were found only in the posterior intestine. *Alkaliphilus* was found only in extreme alkaline areas (Takai 2001). *Beggiatoa* was found in a hydrogen sulfide-rich marine environment (Ljungdahl 2007). *Desulfobacter* was found mainly in an anoxic condition and organic matter can be oxidized to CO_2 (Hao 1996). *Enterovibrio* was mainly found in the intestines of fish (Thompson 2002). *Pleomorphomonas* are known as nitrogen-fixing bacteria (Xie 2005). *Ruegeria* was commonly found in the oceans (Uchino 1998). In the anterior intestine, 10 species of archaea were also found, and 9 species of archaea were found in the posterior intestine.

The ratio of bacteria in the anterior intestine and the posterior intestine was compared at the phylum level (Fig. 11). *Proteobacteria* account for 51% and 59% in anterior and posterior region, respectively. The second largest proportion was of *Bacteroidetes*, accounting for 16% and 20%, and the third was of *Verrucomicrobia*, which accounted for 14% and 9%, respectively. The major difference between anterior intestine and posterior intestine was the ratio of *Tenericutes*. *Tenericutes* accounted for 7% of anterior intestine, but only 1% of posterior intestine. The phylum *Tenericutes* contains the class *Mollicutes*, and the most well-known genus is the pathogenic *Mycoplasma* (Huang 2001). Compared to other samples, the abnormally large proportion of *Tenericutes* was observed in AF6, AF7, and AF8, which are believed to have been infected with the pathogen.

At the genus level, the proportion of taxa more than 1 percentage in the anterior and posterior intestine was higher than that in the anterior intestine (Fig. 12). The proportion of *OM60*, *Flavobacteriaceae*, *Desulfobulbaceae*, *Rhodobacteraceae*, *Pirellulaceae* and *Chromatiales* increased in the posterior intestine.

Members of *OM60* have the ability to survive without a carbon source in an anaerobic environment (Spring 2013), and *Flavobacteriaceae* mainly includes symbiotic or opportunistically pathogenic members (Bernardet 2002). *Desulfobulbaceae* is mostly anaerobic and is known to be involved in the nitrogen cycle by acquiring energy using nitrate and nitrite (Kuever 2014). Members in *Rhodobacteraceae* are symbiotic with multicellular organisms including purple

non-sulfur bacteria, and are involved in sulfur and carbon cycle (Pujalte 2014). *Pirellulaceae* contains ammonia-oxidizing bacteria and is involved in the nitrogen cycle (Kellogg 2016). *Chromatiales* is mainly anaerobic, includes purple sulfur bacteria and contributes to the carbon cycle (Hunter 2008). *Piscirickettsiaceae* and *Marinilabiaceae*, which accounted for less than 1% in the anterior intestine, accounted for 1.36 and 1.11% in the posterior intestine, respectively. *Piscirickettsiaceae* contains sulfur oxiding bacteria and causes diseases in fish (Brinkhoff 1999; Wilhelm 2005), and *Marinilabiaceae* contains anaerobic bacteria (Rosenberg 2014).

These results indicate that there are many anaerobic bacteria such as sulfur oxidizing bacteria, ammonia-oxidizing bacteria, and sulfate or nitrate reducing bacteria.

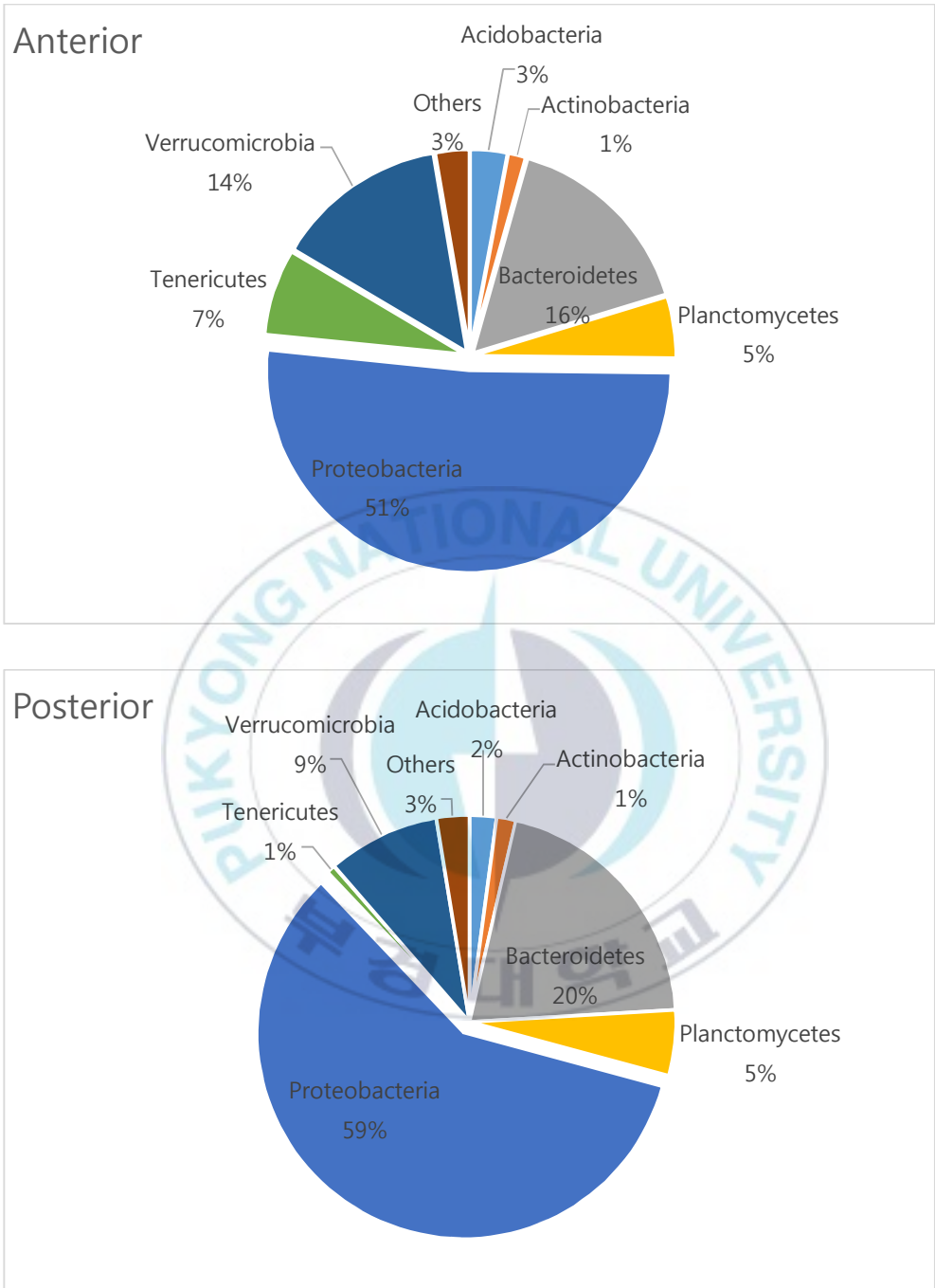
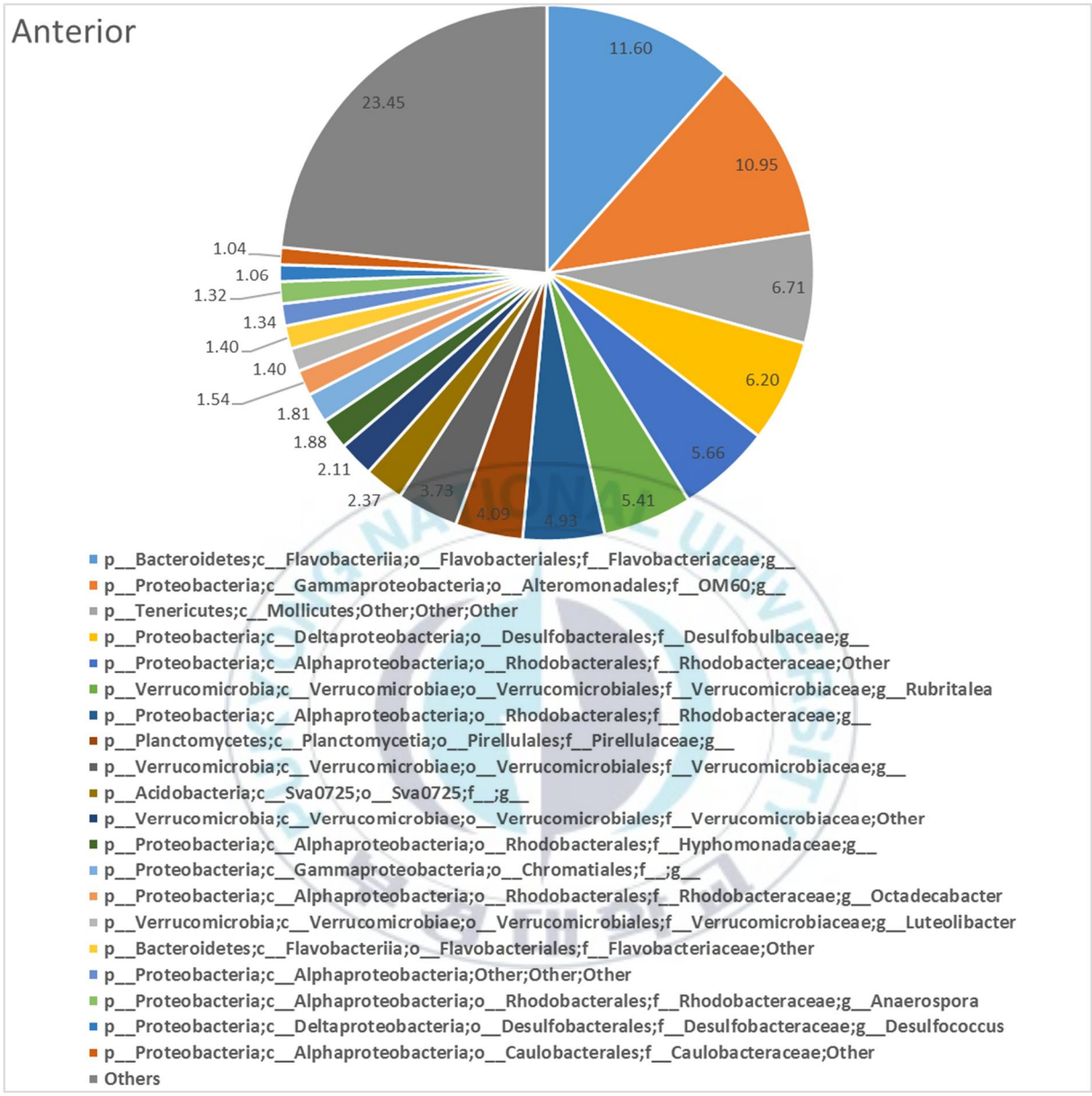


Figure 11. Bacterial phylum ratio of anterior intestine (upper) and posterior intestine (lower)


Anterior
11.60
10.95
6.71
6.20
5.66
5.41
4.93
4.09
3.73
2.37
2.11
1.88
1.81
1.54
1.40
1.40
1.34
1.32
1.06
1.04
23.45
p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__
p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__OM60;g__
p__Tenericutes;c__Mollicutes;Other;Other;Other
p__Proteobacteria;c__Deltaproteobacteria;o__Desulfobacterales;f__Desulfobulbaceae;g__
p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;Other
p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;g__Rubritalea
p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__
p__Planctomycetes;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae;g__
p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;g__
p__Acidobacteria;c__Sva0725;o__Sva0725;f__;g__
p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;Other
p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Hyphomonadaceae;g__
p__Proteobacteria;c__Gammaproteobacteria;o__Chromatiales;f__;g__
p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Octadecabacter
p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;g__Luteolibacter
p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;Other
p__Proteobacteria;c__Alphaproteobacteria;Other;Other;Other
p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Anaerospora
p__Proteobacteria;c__Deltaproteobacteria;o__Desulfobacterales;f__Desulfobacteraceae;g__Desulfococcus
p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;Other
Others

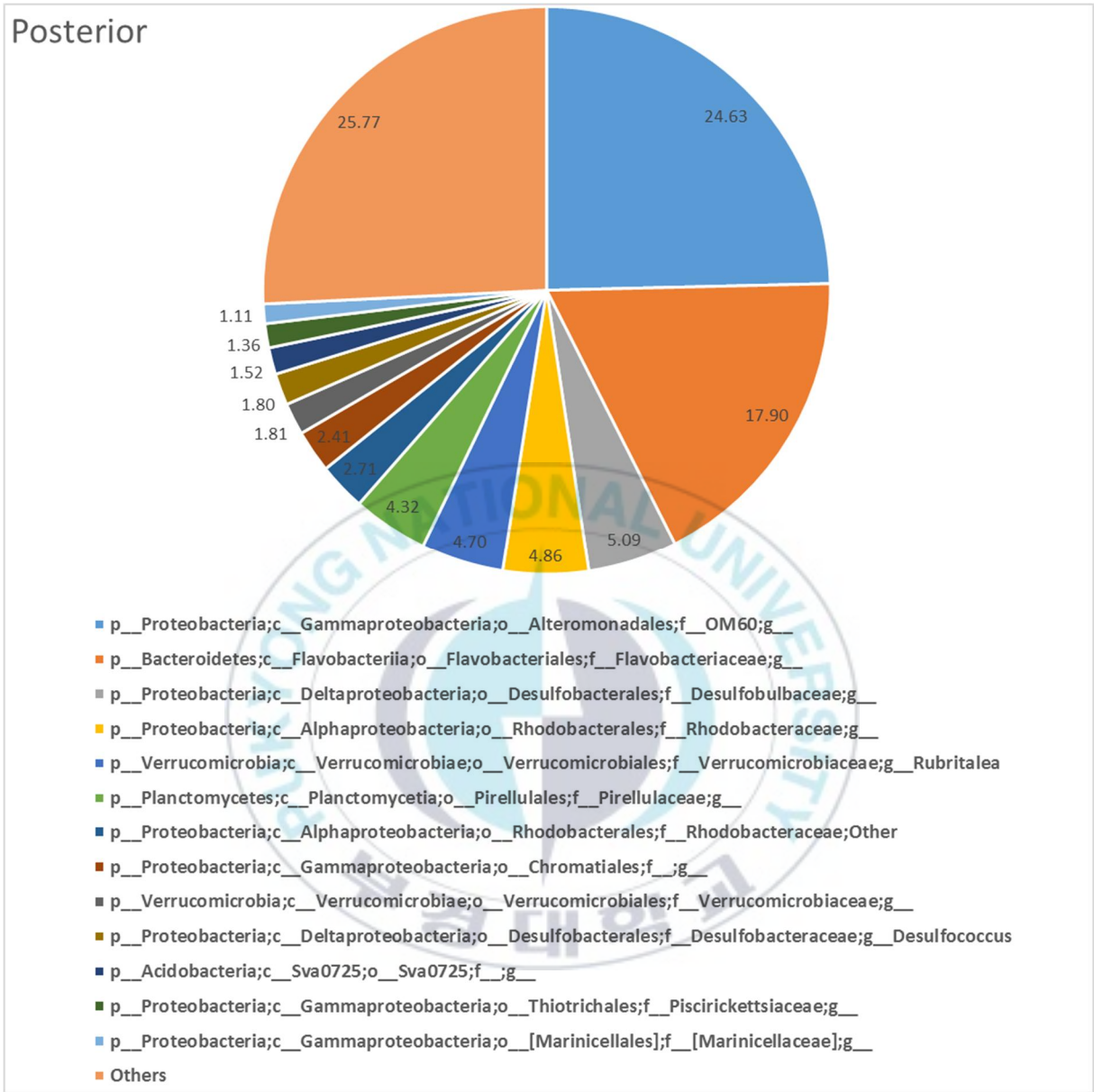


Figure 12. Bacterial genus ratio of anterior intestine (upper) and posterior intestine (lower). The taxa with a proportion less than 1% were merged into 'Others'.

4. Conclusion

As a result of comparing 36 sea cucumbers, there were many different bacteria and archaea in the intestines of sea cucumbers. While there are various microorganisms in the anterior intestines of sea cucumbers, the type of the bacteria in the posterior intestines gets simplified. The bacteria types tended to be similar to other posterior samples. Another factor, the sampling time, had effluence to get the intestinal microbial community similar. Conclusively, regardless of sampling time, it was revealed that the microbes were similar in the posterior intestines of all sea cucumbers. In other words, the anterior intestine of the sea cucumbers is affected by the food intake and the place where the sea cucumbers inhabit, but the posterior intestine of the sea cucumbers shows genuine bacterial community of sea cucumbers without external influence.

5. 국문 초록

모든 동물들은 미생물과 복잡한 상호작용을 하면서 살아가며 장은 그러한 상호작용이 일어나는 가장 중요한 장기이다. 장내 미생물의 다양성을 이해하는 것은 그 동물을 이해하는데 필수적이다. 본 연구에서는 아시아의 연안의 중요한 경제성 해양 무척추 동물인 해삼 *Apostichopus japonicus*의 장의 미생물 다양성을 조사하였다. 4번의 다른 시기에 9개의 해삼 개체를 무작위로 선택하여 장의 앞부분과 뒷부분으로 나누어 시료를 채취하였다. 표지된 PCR과 Illumina MiSeq 기술을 이용한 16S rRNA amplicon sequencing 방법을 이용하여 각각의 시료에서 서열정보를 얻었다. 36개의 해삼 샘플에서 총 72개의 장을 얻었고, 총 1,614,748개의 서열 정보 및 350,254개의 Operational Taxonomic Unit를 확인하였다. 장의 앞부분과 뒷부분에서 각각 42와 35종의 문(phylum)을 확인하였다. 가장 우점하는 문은 *Proteobacteria*, *Bacteroidetes*, *Verrucomicrobia* 및 *Planctomycetes* 임을 보여주었다. 미생물 군집은 장의 뒷부분에서 비슷한 모습을 보였다. 분류학적 분류 프로파일과 Unifrac PCoA 차트에서 장의 위치와 샘플링 시간이 미생물체에 영향을 끼치는 것을 확인하였다.

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