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Analysis of the bacterial microbiome in the small octopus, *Octopus variabilis*, from South Korea to detect the potential risk of foodborne illness and to improve product management

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ABSTRACT

The small octopus (*Octopus variabilis*) is a popular seafood in many countries including South Korea. Because it is often consumed uncooked, the microorganisms in it often cause food poisoning. Therefore, analyzing the microbiome of the small octopus can help to understand the risk of food poisoning and manage octopus products better. A total of 40 small octopuses were collected from four sites in November and August. The microbiota was analyzed using Illumina Miseq sequencing, and the amount of bacteria was quantified by real-time PCR. In addition, we analyzed the influence of *Vibrio vulnificus* infection on the microbiome of the small octopus through artificial infection experiments. *Bacteroidetes* was the predominant phylum in August, and *Proteobacteria* was predominant in November. The composition of the microbiota in octopus depended on sampling region and season. The potential risk of foodborne illness from small octopus consumption might be higher in August than in November due to the abundance of potential pathogens. In the infection experiment, the proportion of *V. vulnificus* increased only at 27 °C. The composition and functional gene profiles of the microbiota varied in a similar manner between non-infected and infected samples over time at the same temperature. These results indicated that the indigenous microbiota in small octopus could inhibit colonization by *V. vulnificus* during storage. Although further studies are necessary to clarify these results, our results could help us better understand food poisoning through octopus ingestion and manage products.

1. Introduction

The small octopus (*Octopus variabilis*) is one of the most popular seafood as healthy food in Asian countries. Octopus mainly comprises water and protein and has low fat content and high proportions of polyunsaturated fatty acids (Vaz-Pires & Barbosa, 2004). Fishing rates have increased in recent times due to the increased consumption of octopus (Boyle & Rodhouse, 2005). Because of their habits of filter-feeding and inhabiting tidal flat sediments, small octopus accumulate rich and diverse microbiota (Prieur, Mével, Nicolas, Plusquellec, & Vigneulle, 1990). The small octopus was reported to be in the top five of the 50 most potentially hazardous foods consumed in South Korea (Park et al., 2013). Raw small octopus is generally eaten in Korean restaurants, and this can cause food poisoning through foodborne pathogens. The microbiota of small octopus includes indigenous bacteria as well as transient or non-indigenous bacteria, including potentially pathogenic microorganisms, can be causal agents of food poisoning (Berg, 1996; Moriarty, 1997). The composition of the microbiota is affected by various environmental conditions of the habitat; it can also vary during storage and delivery to consumers. However, there is very little data on microbiota in small octopus. When the raw octopus is consumed, the whole body is digested; therefore, investigating the entire microbiota of the small octopus is important for understanding the risk of food poisoning and managing the products.

Vibrio spp. have been reported to be the major bacterial agents of foodborne illnesses in Korea and Japan (Lee, Lee, Kim, & Park, 2001). They inhabit from the deep sea to shallow aquatic environments, and are predicted to be important etiological agents of diseases in the coming years, due to ocean warming (Paillard, Le Roux, & Borrego, 2004; Reen, Almagro-Moreno, Ussery, & Boyd, 2006). Pathogenic bacteria of the *Vibrio* spp. have been isolated from seafood worldwide and include several important pathogens of aquatic organisms (DaSilva et al., 2012). Among them, *V. vulnificus* has been the focus of research in the last decade because of its pathogenicity to both humans and fish; therefore, improved methods for its detection in small octopuses are available (Arias, Macian, Aznar, Garay, & Pujalte, 1999; Lee,

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Eun, & Choi, 1997). *Vibrio* can directly colonize octopus or contaminate them from other seafood during processing or storage in the market. *Vibrio* can influence the microbiota of the small octopus by interacting with the indigenous microbes. However, there is very little data on these issues, including uncultured bacteria. Therefore, it is necessary to analyze the effects of *Vibrio* infection on indigenous microbiota in small octopus before consumption.

In this study, we investigated the microbiota of small octopuses, collected from different sampling sites in August and November, using the MiSeq system with the 16S rRNA genes. We then compared the differences of microbiota, based on the regions and the sampling times. In addition, we assessed the influence of pathogens on the composition and potential functions of the indigenous microbiota through artificial infection experiments. Results in this study can extend our understanding of food poisoning by small octopus, and help to manage products according to information of microbiome.

2. Materials and methods

2.1. Sample collection

A total of 40 small octopus samples were collected from four different sites in November 2015 (relatively low temperature of seawater; 16.7 ± 0.7 °C) and August 2016 (relatively high temperature of seawater; 25.7 ± 2.2 °C) (Fig. 1). The sampling sites were chosen based on the areas with maximum production in the western and southern coasts of South Korea, and the sampling months were chosen based on the season with maximum consumption (KOSIS, 2017). Five small octopuses were randomly selected at each site, and transported in an icebox to the laboratory. Bacterial cells were detached from whole octopus using a spindle (microorganism homogenizer, Korea patent registration: 10-2010-0034930). Rotation and vibration were conducted by a direct drive motor in a stomach bag in the spindle. The bacterial cells obtained from each sample were diluted in 255 mL of buffered peptone water (10 g peptone, 5 g sodium chloride, 3.5 g disodium phosphate, 1.5 g potassium dihydrogen phosphate with pH 7.2), and stored at − 80 °C before metagenomic DNA extraction.

2.2. Artificial infection of Vibrio vulnificus on small octopus

The influence of *V. vulnificus* infection on the indigenous microbiota of small octopuses during storage was analyzed by artificially infecting octopuses with *V. vulnificus* and stored in sterile containers at 27 °C (room temperature) and 4 °C. A total of 32 small octopus samples obtained from site A in November were used to artificial infection experiments based on consumption amount (KOSIS, 2017). *V. vulnificus* FORC_037 was cultivated at 30 °C in a Luria-Bertani medium (LB; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2% NaCl for 4 h until an optical density at 600 nm (OD_{600}) of 2.5 was achieved. The number of infected cells was 1.0×10^6 cells/g, which was the reported infective dose of *Vibrio* spp. (Huss, Ababouch, & Gram, 2003; Jeong & Satchell, 2012). *V. vulnificus* was evenly sprayed on the small octopuses. The temporal shifts of the microbiota were investigated after 1 h, 4 h, and 12 h. Since small octopuses tended to decompose after 12 h of storage at 27 °C, we analyzed the microbiota until 12 h of storage to compare between different temperatures. The shifts in the microbiota of uninfected samples during the same storage period were also investigated as the control. These infection experiments were performed in duplicate. Bacterial cells on the small octopuses were detached as described earlier, and the obtained cells were stored at − 80 °C before DNA extraction.

2.3. Metagenomic DNA extraction and quantitative real-time polymerase chain reaction

Metagenomic DNA was extracted from each sample using the phenol DNA extraction method as described in previous studies (Lee, Lee, Chung, Choi, & Kim, 2016; Naravaneni & Jamil, 2005). Briefly,

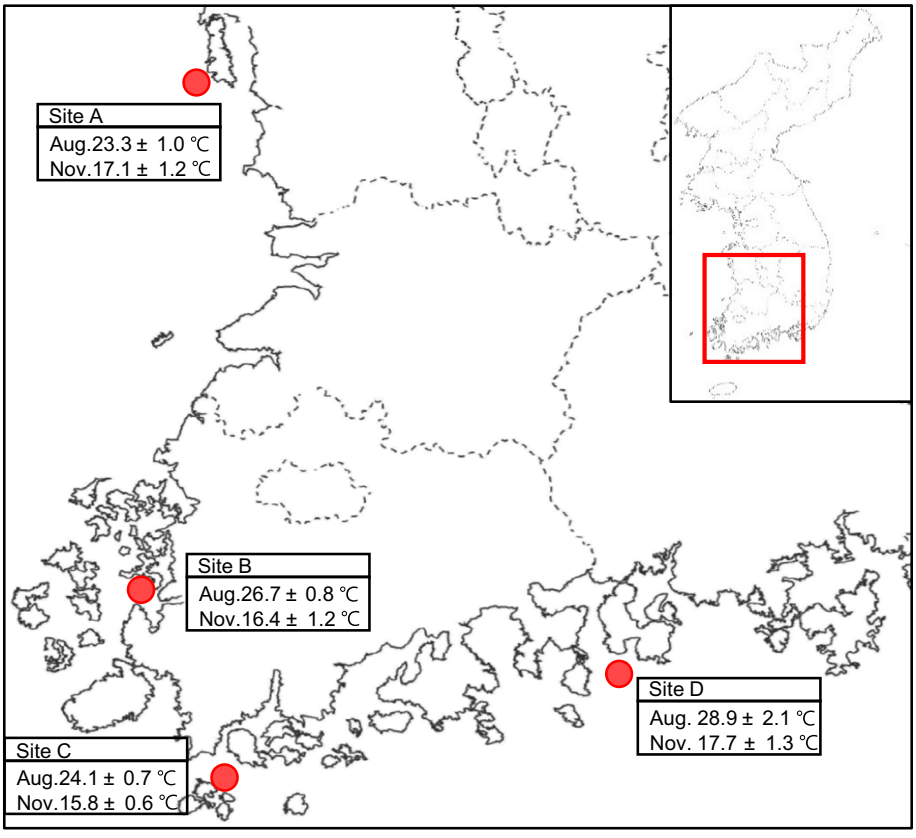

Fig. 1. Sampling sites of small octopuses. Samples were obtained from four different sites in November 2015 and August 2016. Each site was chosen based on the production of small octopuses in South Korea. The water temperature at the sampling time is presented below each sampling site.

bacterial cells in 255 mL of buffer peptone water were filtered through a sterilized gauze filter and centrifuged at 9000 rpm for 15 min. The pellets were dissolved in 10 mL of TES buffer (10 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid, 0.1 M NaCl), and centrifuged at 6000 rpm for 10 min. The pellets were suspended in 400 μL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Then, 50 μL lysozyme solution (100 mg/mL) and 200 μL Proteinase K mixture (140 μL of 0.5 M EDTA, 20 μL of 20 mg/mL Proteinase K, 40 μL of 10% sodium dodecyl sulfate) were treated at 37 °C for 1 h. After treatment, 5 M NaCl and 80 μL of CTAB/NaCl solution were added, and the mixture was incubated for 10 min at 65 °C. One milliliter of phenol/chloroform/isoamly alcohol (25:24:1 v/v/v) was added and mixed well, and then centrifuged at 5000 rpm for 5 min. The upper phase was transferred to 10 μL of RNase A (30 mg/mL) and the mixture was incubated at 37 °C for 1 h. Then, 80 μL of 3 M sodium acetate solution was added and the tubes were inverted several times. One milliliter of 100% ethanol was added to the mixture, then cooled at − 20 °C for 1 h, and centrifuged at maximum speed for 20 min. The pellet was washed with 75% ethanol and dried. The DNA pellet was resuspended in 100 μL of TE buffer and incubated at 55 °C for 1 h. The extracted metagenomic DNA was purified using the PowerClean Pro DNA Clean-up kit (Mo Bio Laboratories, Carlsbad, CA, USA), and confirmed by 1% agarose gel electrophoresis. Two samples (small octopus from site C and D in November) did not extracted DNA, and these were excluded from further experiments.

The total amount of bacteria in the sample was determined by quantitative real-time PCR with 16S rRNA genes. The 16S rRNA gene was amplified using the primers 340F (5′-TCCTACGGGAGGCAGCAG-3′) and 518R (5′-ATTACCGCGGCTGCTGG-3′) with the TaKaRa PCR Thermal Cycler Dice Real Time System III (TaKaRa, Shiga, Japan). Triplicate reactions were performed for each sample with a final volume of 25 μL, comprising 12.5 μL of 2× SYBR Premix Ex Taq (TaKaRa), 2 μM of each primer, and 1 μL of DNA template (ten-fold diluted metagenomic DNA) or distilled water (negative control). The conditions for the reaction were as follows: initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 15 s; and final extension at 72 °C for 10 min. Standard curves were generated from parallel PCRs with serial log-concentrations (1×10^2–1×10^8) of the copy number of 16S rRNA gene from *Escherichia coli* K12 w3110. Regression coefficients (r^2) for all standard curves were found to be ≥ 0.99. The differences between samples were analyzed using the Student's *t*-test in the R software (ver. 3.2.0). Results with *P* value < 0.05 were considered statistically significant.

2.4. MiSeq sequencing

The extracted metagenomic DNA was amplified using primers with adapters (targeting V1-V3 region of the 16S rRNA gene). PCR amplification was followed by the protocol for preparing a 16S metagenomic sequencing library for the MiSeq system (Illumina, Inc., San Diego, CA, USA). Briefly, the first amplification was performed with a final volume of 25 μL comprising 12.5 μL of 2× KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Inc., Wilmington, MA, USA), 1 μM of each primer, and 2.5 μL of template (sample DNA). The reaction was performed under the following conditions: initial denaturation at 95 °C for 3 min; 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; and final extension at 72 °C for 5 min. The amplicons were verified by 2% agarose gel electrophoresis, and purification and size selection were performed using the Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA). The index PCR was performed using 5 μL of the initial PCR product in a final volume of 50 μL, using the Nextera XT Index Kit (Illumina, Inc.). The index PCR was performed under the following conditions: initial denaturation at 95 °C for 3 min; 8 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; and final extension at 72 °C for 5 min. The amplicons of each sample were purified again using Agencourt AMPure XP beads (Beckman Coulter). The library was quantified using real-time PCR. Equimolar concentrations of each library from the different samples were pooled and sequenced using an Illumina MiSeq system (300 bp-paired ends), according to the manufacturer's instructions.

2.5. Sequence data analysis

Sequences obtained from the Illumina MiSeq sequencer were analyzed using the CLC genomic workbench (ver. 8.5.1) with the Microbial Genomics Module (Qiagen, Redwood city, CA, USA). Briefly, raw sequences of short read lengths (< 200 bp in each pair) and low quality scores (Q < 25) were removed, and paired reads were merged. Adapter sequences and primer sequences were removed from these merged sequences. The sequences were then clustered to OTUs (Operational Taxonomic Units) using the 97% sequence similarity based on reference database of SILVA (Quast et al., 2013). Taxonomic position of representative sequence in each OTU cluster assigned by the SILVA database. To compare the diversity indices between samples, various read numbers in the samples were normalized by random subsampling, and diversity indices were calculated using MOTHUR (Schloss et al., 2009). The differences between samples were analyzed using the Kruskal-Wallis test in the IBM SPSS Statistic 24. Results with *P* value < 0.05 were considered statistically significant. The sequencing reads obtained from the study are available in the EMBL SRA database under study number PRJEB21616 (http://www.ebi.ac.uk/ena/data/view/PRJEB21616).

2.6. Whole metagenome sequencing and analysis

The whole metagenome of the microbiome was analyzed after artificial infection to identify the shift in functional genes. The metagenomic DNA extracted from infected and uninfected samples after storage in 4 °C for 0 h, 1 h, 4 h, and 12 h were fragmented using a Covaris M220 sonicator (Covaris, Inc., Woburn, MA, USA). Metagenomic libraries were prepared using an Accel-NGS 2S plus DNA library kit (Swift Bioscience, Ann Arbor, MI, USA), according to the manufacturer's protocol. The size of the metagenomic libraries was verified using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), and the concentration of the libraries was measured using a PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA). Equimolar concentrations of the sample libraries (2 nM) were quantified using a TaKaRa PCR Thermal Cycler Dice Real Time System III (Takara) with Illumina adapter sequence primers. Sequencing was performed using the Illumina MiSeq system (300 bp-paired ends) with four samples multiplexed per run.

For the whole metagenome analysis, the adapters and sequences with short read lengths (150 bp in each pair) and low Q scores (Q < 20) were trimmed using the Nesoni clip tool of the Nesoni high-throughput sequencing data analysis toolset (version 0.127, http://www.vicbioinformatics.com/software.nesoni.shtml). The taxonomic profiles of the trimmed reads for each sample were determined using the MetaPhlAn2 tool, which maps raw sequences onto databases comprising predefined clade-specific maker genes (Segata et al., 2012). The resultant raw counts were normalized for total marker-gene length and outliers, yielding the profiles of clade relative abundance, marker-gene presence/absence, and marker gene abundance (in reads per kilobase per million units; RPKM). Functional metagenome profiling was performed using the HUMAnN2, which utilizes pangenome mapping of sequences to the pangenomes of functionally annotated species determined by taxonomic profiling (Abubucker et al., 2012). In this step, unmapped reads were aligned to the universal protein reference database (UniRef50) using the DIAMOND program (Buchfink, Xie, & Huson, 2015). The resultant functional profiles were obtained in terms of gene family abundance at the community level in RPK units and then normalized for gene length. The resultant UniRef50 IDs were converted to

Table 1
Summary of calculated diversity indices in each sample.

Sampling time	Sample	Analyzed reads	Normalized reads	Observed OTUs	Estimated OTUs (Chao1)	Shannon diversity index
August	Site A_1	55,180	9000	2417	10,345.4	5.80
	Site A_2	77,845	9000	2390	11,825.1	5.86
	Site A_3	24,428	9000	2179	9210.9	5.04
	Site A_4	47,575	9000	3935	23,753.2	7.01
	Site A_5	22,720	9000	3429	15,433.8	6.64
	Site B_1	18,119	9000	3093	13,622.8	6.30
	Site B_2	29,849	9000	2885	12,790.5	6.19
	Site B_3	18,316	9000	2548	10,935.6	5.88
	Site B_4	30,564	9000	2350	9650.4	5.55
	Site B_5	22,008	9000	1687	6527.5	4.85
	Site C_1	209,352	9000	771	2066.0	2.94
	Site C_2	20,217	9000	1962	8008.7	5.17
	Site C_3	16,980	9000	2058	7939.7	5.47
	Site C_4	44,659	9000	1881	7035.8	5.04
	Site C_5	8226	8000	2860	13,764.2	6.12
	Site D_1	9036	9000	2412	10,382.9	5.64
	Site D_2	14,368	9000	2991	14,041.3	6.21
	Site D_3	18,854	9000	3061	14,931.3	6.20
	Site D_4	5643	5000	2285	12,112.1	6.38
	Site D_5	145,356	9000	1174	3784.9	3.78
November	Site A_1	84,374	9000	983	3450.5	4.14
	Site A_2	202,619	9000	936	3149.4	4.18
	Site A_3	139,895	9000	950	3855.1	4.22
	Site A_4	229,840	9000	793	2739.9	3.08
	Site A_5	357,886	9000	634	2088.6	3.21
	Site B_1	74,104	9000	998	3284.9	4.33
	Site B_2	52,527	9000	470	2095.8	3.13
	Site B_3	140,187	9000	814	3146.9	3.72
	Site B_4	101,627	9000	588	2178.8	2.63
	Site B_5	131,538	9000	621	1861.1	2.68
	Site C_1	85,965	9000	853	3178.2	3.52
	Site C_2	96,680	9000	861	3056.4	3.44
	Site C_3	61,247	9000	608	1770.3	3.68
	Site C_4	35,245	9000	586	2049.1	3.27
	Site D_1	47,443	9000	584	1782.5	3.17
	Site D_2	370,119	9000	375	1176.2	2.30
	Site D_3	96,805	9000	370	910.6	2.05
	Site D_4	110,613	9000	420	2262.1	2.72

the KO (KEGG Orthology), based on matching K numbers (http://www.genome.jp/kegg/kegg2.html). The statistical differences between groups were analyzed using the two-sided Welch's test and confidence intervals were calculated using Welch's inverted test in Statistical Analysis of Metagenomic Profiles (STAMP) software (Parks & Beiko, 2010). Results with P value < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Comparison of diversity indices and bacterial cell numbers among samples

A total of 3,258,009 reads (average of 41,965 reads from samples collected in August and 134,373 reads from those collected in November) after trimming were analyzed (Table 1). The number of reads in each sample was normalized to 9000 by random subsampling. The highest number of observed OTUs was detected in the sample site A_4 (3935) in August, whereas the lowest number of observed OTUs was detected in the sample site D_3 (370) in November. The Shannon diversity indices and bacterial loads on the octopuses were compared between samples collected in August and November, as well as between samples from different sites in the same time (Fig. 2). The diversity of microbiota in small octopuses was higher in August samples (5.8 ± 0.9) than in November samples (3.2 ± 0.7; $P < 0.001$). For August samples, the highest diversity of microbiota was detected in site D (6.2 ± 1.0) and the lowest was detected in site C (5.2 ± 1.1). However, the quantified bacterial amounts were found to be different from the calculated diversity (Fig. 2D). The highest amount of bacteria was found in samples from site C (1.18×10^8 copies/g) and the lowest was found in site A (5.83×10^6 copies/g). This result indicated that the amounts of dominant bacteria in octopus microbiota were high in samples from site C. For November samples, the highest diversity of microbiota was detected in site A (4.1 ± 0.5) and the lowest was in site D (2.5 ± 0.4) ($P < 0.05$). The highest bacterial load in octopus was found in samples of site B (7.08×10^7 copies/g) and the lowest was found in site D (5.39×10^6 copies/g). The decreased diversity of octopus microbiota in site D was associated with the decreased amounts of bacteria. Although the diversity was lower in site A at November (4.1 ± 0.5) than August (5.9 ± 0.7), the diversity was highest in this site among all the sites at November; furthermore, the bacterial amount in August (5.83×10^6 copies/g) and November (5.74×10^6 copies/g) were similar. The small changes in diversity and bacterial amount in site A compared to the other sampling sites might have been due to the small changes in seawater temperature between August and November ($\Delta Tm = 6.1$ °C in site A, whereas $\Delta Tm > 8.3$ °C in other sites). This difference might be because that site A was located far from the other sampling sites, and in Korean western coast of the semi-enclosed sea between mainland China and the Korean Peninsula (Fig. 1). The decreased microbial diversity in November was associated with the decreased amounts of bacteria in the other sites. These results showed that the diversity of microbiota and bacterial loads in the octopuses was influenced by environmental factors such as water temperature and location.

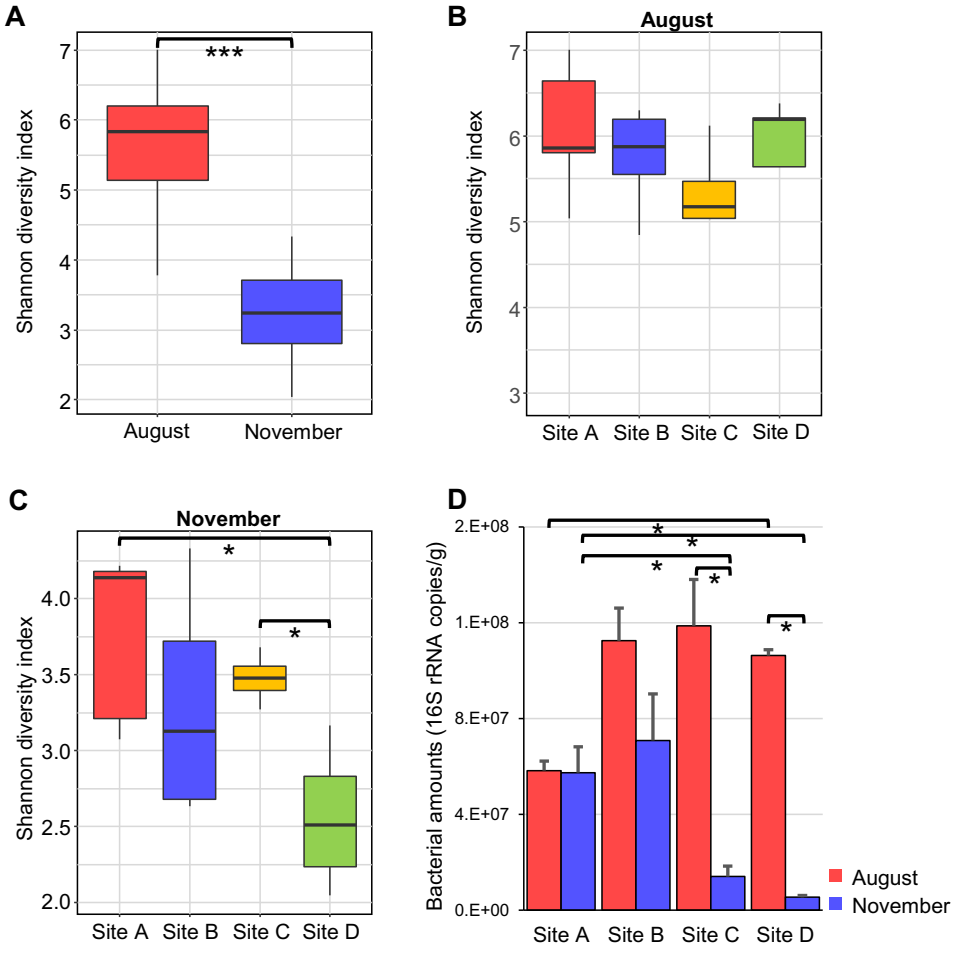

Fig. 2. Comparison of microbial diversity and bacterial amount in small octopuses from different sites and sampling times. (A) Comparison of the Shannon diversity indices between August and November samples. (B) Comparison of diversity indices of samples from different sites in August and (C) November. (D) The amount of bacteria was estimated using quantitative real-time PCR. The average bacterial loads on small octopuses from different sites and sampling times were compared. Error bars indicate the standard deviation. * P value < 0.05, *** P value < 0.001.

3.2. Comparison of microbiota composition in octopuses at the phylum level

The compositions of microbiota in samples collected in August and November were compared at the phylum level (Fig. 3A). *Bacteroidetes* was the predominant phylum (40.4%), followed by *Tenericutes* (23.8%), *Proteobacteria* (18.2%), and *Spirochaetes* (11.6%) in the August samples. The relative abundance of *Proteobacteria* was higher (63.5%), whereas those of *Bacteroidetes* (18.6%), *Tenericutes* (11.3%), and *Spirochaetes* (0.001%) were lower in the November samples than the August ones (P < 0.05). The differences of microbiota were also compared among samples from different sites at the same sampling time (Fig. 3B). In August samples, the relative abundance of *Bacteroidetes* was highest in

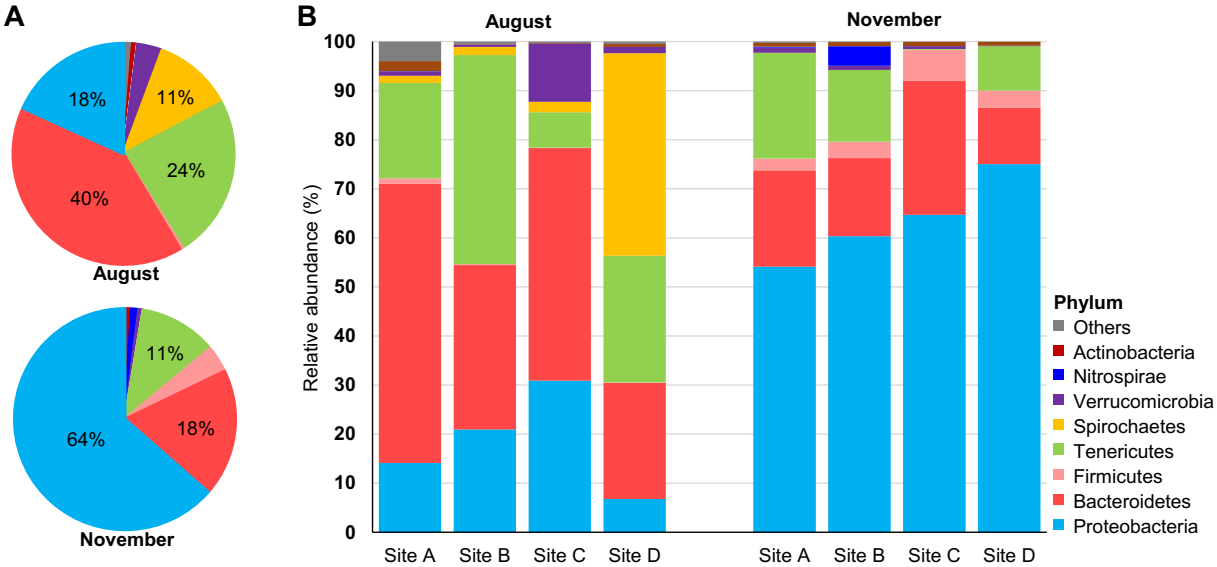

Fig. 3. Comparison of bacterial communities at the phylum level. (A) Average proportion of phyla composition in August and November samples. (B) Comparison of the phyla composition in samples from different sites. Phyla with relative abundance < 1% in each sample were combined to the "others" group.

site A (56.9%). The highest proportion of *Tenericutes* was detected in site B (42.6%) and that of *Proteobacteria* was detected in site C (30.9%). *Spirochaetes* was found to be dominant in site D (41.3%). The relative abundance of *Proteobacteria* was higher in all sampling sites at November. In the November samples, the highest proportion of *Proteobacteria* was detected in site D (75.0%) and that of *Bacteroidetes* was detected in site C (27.4%). The highest proportion of *Tenericutes* was detected in site A (21.6%). The phylum composition of microbiota among sampling sites differed more in August than in November. *Proteobacteria* was the predominant phylum in all the sites in November, whereas the predominant phylum differed among the sampling sites in August. The relative abundance of *Proteobacteria* was lower in samples from site A in November than in August, while that of *Tenericutes* was similar in site A in August and November. The smaller change in phylum composition in site A than other sites was consistent with the smaller changes in diversity indices and bacterial amounts in site A (Fig. 2). In addition, significant changes of bacterial amounts and diversity in site D were likely related to the changes in composition of phyla between August and November in this site. Site D was in the southern coast of Korea, which is far from the other sampling sites (Fig. 1). These results also suggested that the environment influences the microbiota in small octopuses.

3.3. Comparison of microbiota composition in octopuses at the genus level

The relative abundances of frequently detected genera (> 1% of total microbiota in at least one sampling site) were compared using heatmap analysis (Fig. 4). The relative abundances of dominant genera were found to differ based on sampling time and region. Three groups (numbers 1-1, 1-2, and 2 in the heatmap) were different among the samples.

Burkholderia, *Vagococcus*, and *Psychrobacter* within group 1-1 were abundant in the November samples. Some species of *Burkholderia* have been detected in marine sediments, and they can survive in relatively low temperatures and degrade organic compounds such as ploly-chlorinated biophenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Master & William, 1998; Revathy, Jayasri, & Suthindhiran, 2015). *Vagococcus* species have been isolated from marine environments and have been suggested as candidate probiotics for aquaculture (Lawson, Foster, Falsen, Ohlen, & Collins, 1999; Lebreton et al., 2013; Sorroza et al., 2012). *Psychrobacter* species are psychrophilic bacteria that have been detected in various marine hosts (Jung, Lee, Oh, Park, & Yoon, 2005; Lee et al., 2016). Because small octopuses inhabit marine sediments, including tidal flats, these bacteria were abundant in November samples, which were subjected to relatively low water temperatures.

Although genera within group 1-2 were more abundant in November samples than August samples, their proportions differed based on the sampling locations. In November samples, four genera, *Leptospirillum*, *Pseudoalteromonas*, *Pseudofulvibacter*, and *Endozoicomonas*, were more abundant in site B than in other sites. *Leptospirillum* species are iron-oxidizing bacteria that have been isolated from marine sediments (Tyson et al., 2005). *Pseudoalteromonas* have been found associated with eukaryotic hosts in the marine environment; they have been reported to be important for microbe-host interaction with synthesis of biologically active molecules (Holmstrom & Kjelleberg, 1999). *Pseudofulvibacter* species are widely distributed in a number of marine environments, including tidal flat sediments and oil-polluted coastal sediments (Acosta-Gonzalez, Rossello-Mora, & Marques, 2013; Yang, Seo, Lee, Kim, & Kyoung Kwon, 2016). *Endozoicomonas* are symbiont bacteria that associate with many marine hosts; they are known to be involved in the host metabolism, including in carbohydrate cycling (Neave, Apprill, Ferrier-Pages, & Voolstra, 2016). Site B is surrounded by many islands, where organic nutrients can be accumulated (Fig. 1). Thus, the abundances of these four genera may have been influenced by the environment of their habitats.

Although the relative abundances of genera in group 2 were higher in August than in November, the sampling locations also affected them. *Mycoplasma* species are generally host-specific pathogens found on mucosal surfaces; they have been known to infect humans through cutaneous transmission after contact with infected marine mammals (Hunt et al., 2008; Sun, Jones, Dobbs, Bodhankar, & Simecka, 2013). *Balneatrix* species have been found in marine environments and

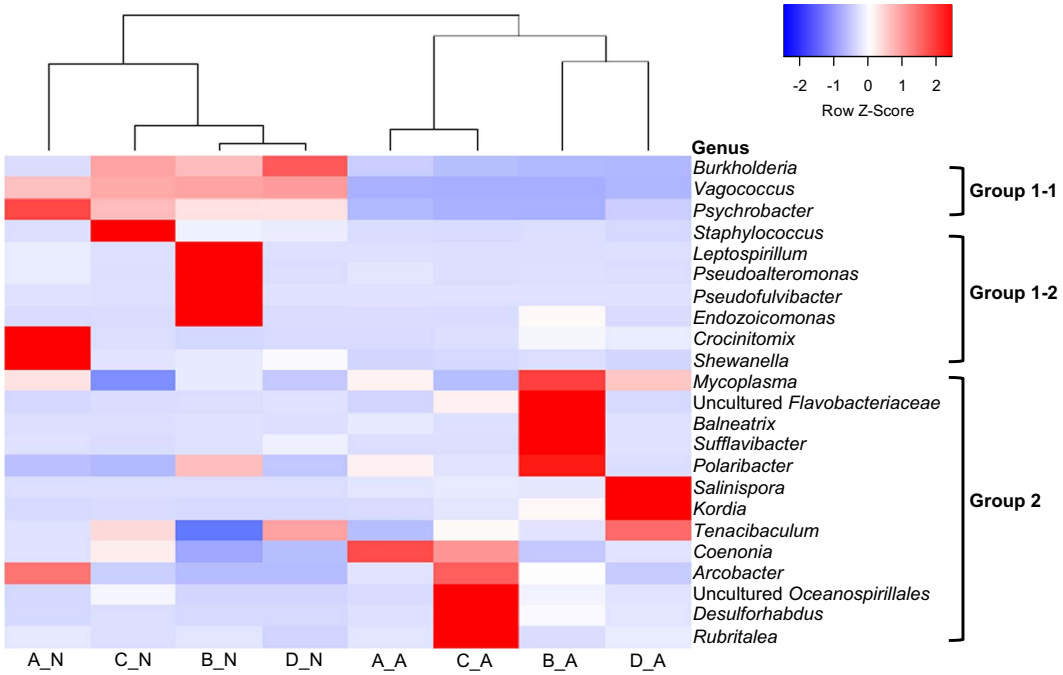

Fig. 4. Heatmap analysis of frequently detected genera in small octopus samples. Clustering was performed using the Spearman rank correlation. Different colors indicate the relative abundance of each genus. Sample names indicate the sampling site and sampling time (for example, A_N; sample collected from site A in November). (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this chapter.)

associated with pneumonia and meningitis (Dauga et al., 1993; Luria, Ducklow, & Amaral-Zettler, 2014). *Sufflavibacter* species have been found in marine environments, and *Polaribacter* species have been reported to degrade algal polysaccharides in the ocean (Kwon, Yang, Lee, Cho, & Kim, 2007; Xing et al., 2015). The abundance of these genera in August samples from site B could also be attributed to the sampling location and the accumulated organic compounds in this site.

Salinispora species are marine bacteria, which have been used as a model for natural product research (Jensen, Moore, & Fenical, 2015). *Kordia* species are also marine bacteria that can degrade macromolecules of bloom-forming microalgae (Sohn et al., 2004). *Tenacibaculum* species are widespread in marine environments and can degrade a variety of biopolymers such as various cellulose derivatives, xylan, agar, and chitin (Frette, Jorgensen, Irming, & Kroer, 2004; Oh, Kahng, Lee, & Lee, 2012). These three genera were abundant in site D in August, and may be associated with higher temperature in this site (28.9 °C) than the others (23.3–26.7 °C).

Coenonia species can cause respiratory diseases in ducks and geese (Vandamme et al., 1999). *Arcobacter* species have been isolated from marine environments and can oxidize sulfide (Wirsen et al., 2002). *Desulforhabdus* species have been detected in marine habitats and can reduce sulfates (Rutters, Sass, Cypionka, & Rullkotter, 2001). *Rubritalea* species have been found in marine environments and can produce squalenes and carotenoids (Yoon, Matsuda, Adachi, Kasai, & Yokota, 2011). These dominant genera were found in site C in the southwestern coast of Korea (Fig. 1). Some species of the dominant genera in August samples were pathogenic, and so, the potential risk of foodborne illness through the consumption of small octopuses might be higher in August than in November. Further studies are needed to better understand the relation of these genera with geochemical factors in sampling locations and the pathogenicity of potential pathogens in the microbiota of small octopuses in August.

3.4. Shifts of microbiota composition in small octopuses infected with V. vulnificus over storage time

We used metagenomics analysis to investigate the effect of *V. vulnificus* infection on the indigenous microbiota in octopus samples. As storage time and temperature influenced the composition of the microbiota, we analyzed samples stored for different periods at different temperatures after infection. Although the number of observed OTUs was similar after 12 h of storage at both of 4 °C and 27 °C in infected and non-infected samples, it was relatively higher in infected samples than non-infected samples (303.5–381 OTUs in infected samples and 263–277 OTUs in non-infected samples) (Fig. 5). The changes in the Shannon diversity index were similar to the changes in the number of OTUs. The bacterial amounts on the octopuses decreased after 12 h of storage at all temperatures studied. However, the decreasing amount of bacteria was small in the infected samples, and the bacterial loads in the infected samples (1.17×10^6 copies/g) were relatively higher than those in the non-infected samples (8.37×10^5 copies/g) ($P < 0.05$). These results indicated that the diversity of microbiota was higher in infected samples than in non-infected samples and this change was associated with the amounts of bacteria in the samples.

The compositions of the microbiota in samples in different conditions were compared (Fig. S1). The compositions of the microbiota varied based on temperature and storage time, since the indigenous microbiota on each sample at 0 h was different. However, *Tenericutes* and *Proteobacteria* were the predominant phyla in all samples, and the shifts in the microbiota of non-infected and infected samples at 4 °C and 27 °C were similar. Although the relative abundance of *Tenericutes* and *Proteobacteria* decreased over time ($P < 0.05$) under most of the conditions studied, the change in the proportion of *Proteobacteria* was smaller in infected samples (average 5.7% change) than in non-infected samples (average 11.7% change). The proportion of *Proteobacteria* increased only in infected samples at 4 °C after 12 h of storage (19.4% at 0 h and 31.1% at 12 h). The relative abundance of *Verrucomicrobia* increased after 12 h of storage in both non-infected and infected samples at 27 °C (0.4% at 0 h and 34.9% at 12 h). *Mycoplasma* was the predominant genus within *Tenericutes*, and *Psychrobacter*, *Bradyrhizobium*, *Arcobacter*, and uncultured *Oceanospirillales* were the dominant genera within *Proteobacteria*. *Rubritalea* was dominant within *Verrucomicrobia*; the proportion of this genus increased after 12 h of storage at 27 °C. A higher abundance of *Rubritalea* was also observed in site C in August (Fig. 4). Thus, the increase in the proportion of this genus was associated with relatively high temperatures in the storage model experiments. The proportion of *Vibrio* was relatively low in all the samples, and its relative abundance increased only in infected samples after 12 h of storage at 27 °C due to the increased proportion of *V. vulnificus* (Fig. S2). The optimum temperature for the growth of the *Vibrio* genus ranges from 17 to 35 °C, and high densities of *V. vulnificus* have been detected in oysters at temperatures above 26 °C (Motes et al., 1998). *Vibrio* spp. did not detect in the microbiota of small octopuses in August or November (Fig. 4). Therefore, the proportion of *V. vulnificus* on small octopuses increased at 27 °C only because of the infection and the temperature. These results indicated that the potential risk of foodborne illnesses by *V. vulnificus* increased after 12 h of storage at above 26 °C.

3.5. Shifts of functional genes in the microbiota of octopuses infected with V. vulnificus

Some changes were detected in the microbiota, and the overall trend of these changes was similar in non-infected and *V. vulnificus*-infected small octopuses. However, the potential function of the microbiota could be different in the non-infected and infected samples. Therefore, we analyzed the potential functional changes in the microbiota using the whole metagenome. In general, small octopuses are stored in the market in ice and transported to consumers at 4 °C. We therefore analyzed the whole metagenome of the microbiota in the non-infected and infected samples at 4 °C. A total of 60,630,356 sequences were obtained, and 48,578,362 sequences (average of 6,939,766 sequences per sample) were analyzed after the trimming process. The overall shifts in the KO were compared at the highest category (Fig. S3). The different functional categories between non-infected and infected samples were not detected ($P > 0.05$). The relative abundance of all the categories increased over time in both non-infected and infected samples. Fifty-two KO categories were significantly different over storage time (Table S1). We then compared the major virulence genes of *V. vulnifucus* in the non-infected and infected samples, based on the data obtained from the virulence factors database (http://www.mgc.ac.cn/VFs). Genes related to adherence, motility, and toxicity were detected in the whole metagenome sequences (Fig. 6). The relative abundance of the adherence genes was slightly higher in the non-infected samples after 1 h, whereas that of motility genes was lower for 1 h and 4 h of storage time. The overall changes in the abundance of adherence and motility genes were similar in the infected and non-infected samples ($P < 0.05$ in infected samples). However, the relative abundance of motility genes showed a remarkable decrease at 4 h, while that of adherence genes increased at 4 h in infected samples ($P < 0.05$). These results suggested that the indigenous microbiota with motility genes was decreased and microbiota with adherence genes was increased in the samples during the early storage time; however, the composition of the microbiota changed at 12 h, as shown in Fig. S1. Therefore, the relative abundance of genes related to adherence decreased, while that of genes related to motility increased at 12 h. The changes in the abundance of genes related to motility and adherence in infected samples at 4 h might be attributed to the effect of infection by the *V. vulnificus*. However, the relative abundance of motility and adherence genes was similar in non-infected and infected samples after 12 h of storage. This result indicated that the *Vibrio* could not colonize the octopus after 12 h at 4 °C, as is evident from the changes in the proportion of *Vibrio* (Fig. S2). Thus, the indigenous microbiota might

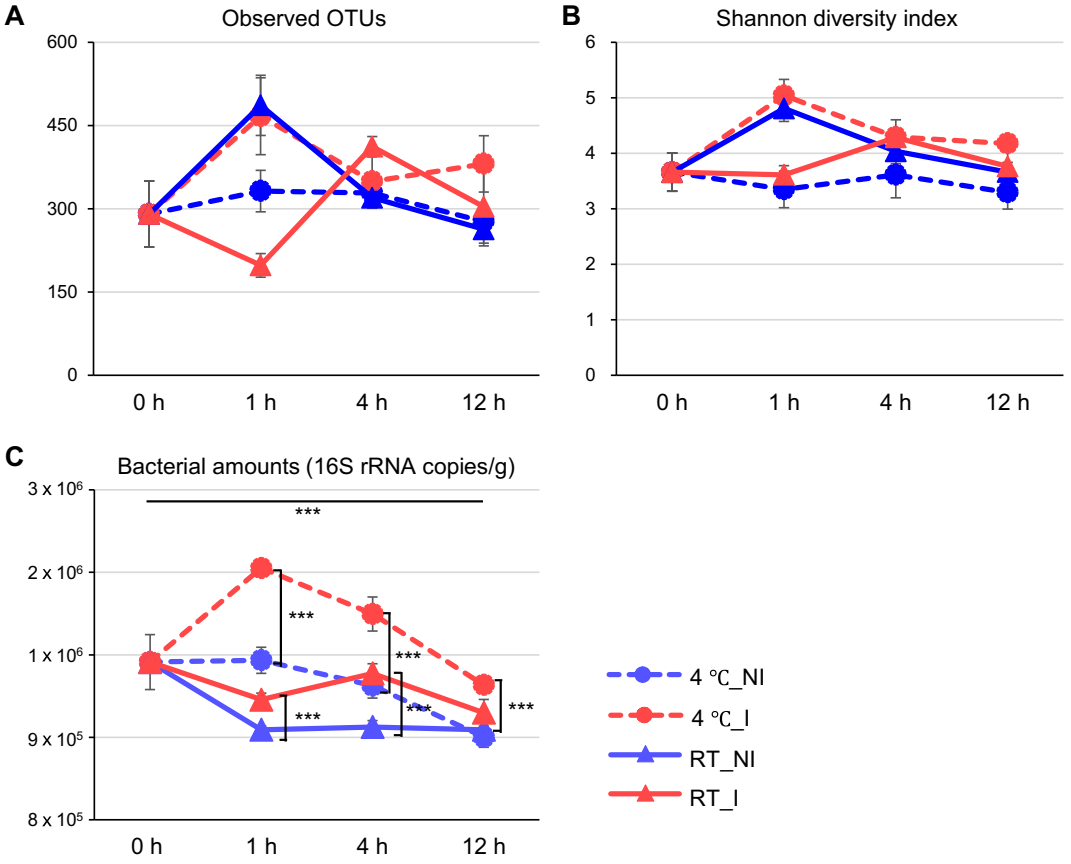

Fig. 5. Comparison of (A) the number of observed operational taxonomic units (OTUs), (B) the Shannon diversity index, and (C) the amount of bacteria in non-infected small octopuses stored at 4 °C (4 °C _NI, blue circle), *V. vulnificus*-infected octopuses stored at 4 °C (4 °C_I, red circle), non-infected octopuses at 27 °C (RT_NI, blue triangle), and infected octopuses stored at 27 °C (RT_I, red triangle). *** P value $<$ 0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

attach to the octopus body and compete with *Vibrio* for colonization after 1 h of storage. Although the relative abundance of toxicity genes was higher in infected samples than non-infected samples, the shifts in the genes related to toxicity were similar in non-infected and infected samples over time ($P > 0.05$). ATP-putative hemolysin was detected only in infected samples and hemolysin A was detected in non-infected samples as well. However, the origin of these toxicity genes could not be identified due to the limited genomic information available on public databases. These results indicated that *V. vulnificus* could not colonize the small octopus, particularly at 4 °C, due to the presence of indigenous microbiota at the early stages of storage. Further studies on the toxicity of the detected toxin genes are needed to understand the

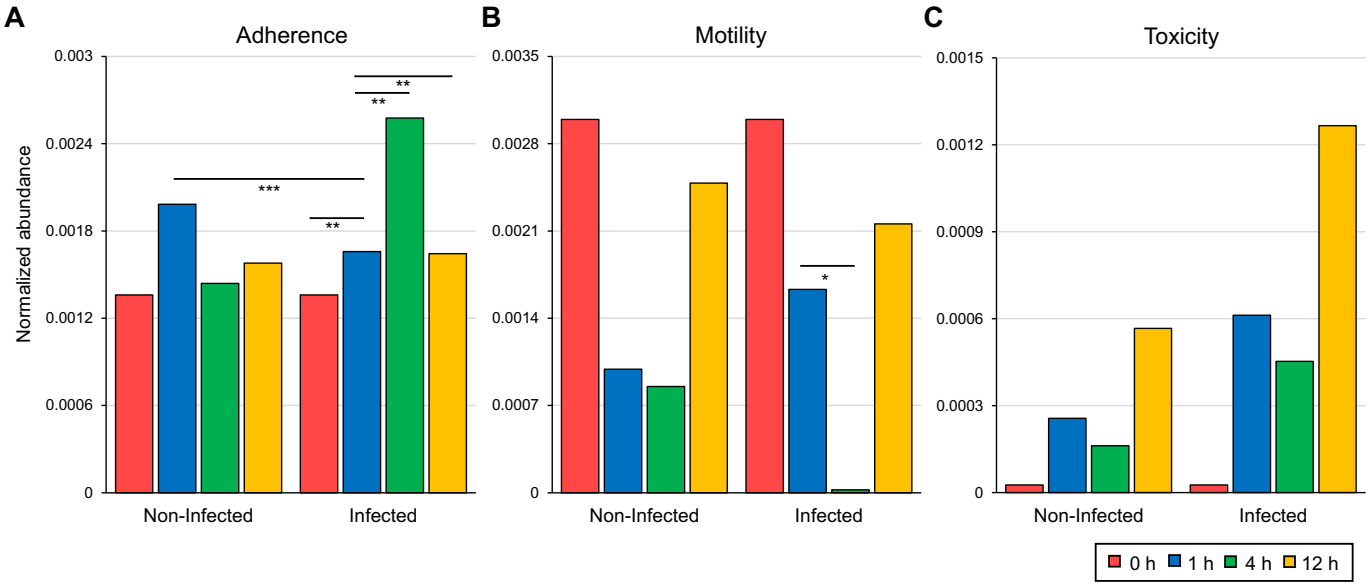

Fig. 6. Comparison of normalized proportions of metagenomic genes related to (A) adherence, (B) motility, and (C) toxicity categories in non-infected and *V. vulnificus*-infected octopuses stored at 4 °C during storage time. * P value $<$ 0.05, ** P value $<$ 0.01, *** P value $<$ 0.001.

potential risk of changes in the microbiota during storage for food processing of small octopus products.

4. Conclusions

The microbiota of the small octopuses collected in August and November from different sampling sites in Korea was analyzed. *Bacteroidetes*, *Proteobacteria*, *Tenericutes*, and *Spirochaetes* were the predominant phyla in the microbiota, and the composition of the microbiota differed, based on the region and collection time. August samples showed higher diversity and bacterial loads, likely due to the higher water temperature, and potential pathogens were more abundant in this period. The microbiota of the small octopus was influenced by the sampling region, since small octopus inhabit marine sediments such as tidal flats, where organic compounds accumulate from nearby regions. Although the microbiota of octopuses changed over storage time, the shifts in its composition were similar in non-infected and infected octopuses in the artificial infection experiment. In addition, the shifts in functional genes, including virulence factors, were similar in non-infected and infected octopuses. The colonization of small octopuses by *V. vulnificus* could be inhibited by the indigenous microbiota during storage. These results in present study could not be generalized the microbiota and the changes of the indigenous microbiota by *V. vulnificus* infection in the small octopus due to the relatively small number of samples and selected sites. Further studies are needed to better understand the mechanisms and toxicity of microbiota of the octopuses. However, the small octopus was obtained from the highest consumed sites and seasons, and the shifts of indigenous microbiota in artificial infection experiment were analyzed with 32 small octopus samples from the same sampling site and season. The changes of microbiota composition and functional genes were similar in 24 small octopus samples. Therefore, these results can show the possible changes of indigenous microbiota by *V. vulnificus* infection in the most consumed small octopus. In addition, this is the first report on microbiota of small octopuses and can be used as a guide to better manage octopus products and prevent food poisoning by octopus consumption.

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