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The Structure and Function of Gut Microbiomes of Two Species of Sea Urchins, *Mesocentrotus nudus* and *Strongylocentrotus intermedius*, in Japan

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Sea urchin is an indicator of coastal environmental changes in the global warming era, and is also a model organism in developmental biology and evolution. Due to the depletion of wild resources, new aquaculture techniques for improving stocks have been well studied. The gut microbiome shapes various aspects of a host's physiology. However, these microbiome structures and functions on sea urchins, particularly *Mesocentrotus nudus* and *Strongylocentrotus intermedius* which are important marine bioresources commonly found in Japan, have not been fully investigated yet. Using metagenomic approaches including meta16S and shotgun metagenome sequencings, the structures, functions, and dynamics of the gut microbiome of *M. nudus* and *S. intermedius*, related to both habitat environment and host growth, were studied. Firstly, a broad meta16S analysis revealed that at the family level, *Psychromonadaceae* and *Flavobacteriaceae* reads (38–71%) dominated in these sea urchins, which is a unique feature observed in species in Japan. *Flavobacteriaceae* reads were more abundant in individuals after rearing in an aquarium with circulating compared to one with running water. *Campylobacteraceae* and *Vibrionaceae* abundances increased in both kinds of laboratory-reared sea urchins in both types of experiments. 2-weeks feeding experiments of *M. nudus* and *S. intermedius* transplanted from the farm to laboratory revealed that these gut microbial structures were affected by diet rather than rearing environments and host species. Secondly, further meta16S analysis of microbial reads related to *M. nudus* growth revealed that at least four Amplicon Sequence Variant (ASV) affiliated to *Saccharicrinis fermentans*, which is known to be a nitrogen (N_2) fixing bacterium, showed a significant positive correlation to the body weight and test diameter. Interestingly, gut microbiome comparisons using shotgun metagenome sequencing of individuals showing higher and lower growth rates revealed a significant abundance of "Nitrate and nitrite ammonification" genes in the higher-grown individuals

under the circulating water rearing. These findings provide new insights on the structure-function relationship of sea urchin gut microbiomes beyond previously reported nitrogen fixation function in sea urchin in 1950s; we discovered a nitrate reduction function into ammonium for the growth promotion of sea urchin.

Keywords: sea urchin, gut, microbiome, *Mesocentrotus nudus*, *Strongylocentrotus intermedius*

INTRODUCTION

Sea urchin is an important aquatic resource worldwide. However, its global production has been decreasing since the 1990s (Stefánsson et al., 2017). *Strongylocentrotus intermedius* and *Mesocentrotus nudus* are important fishery resources in many Asian countries. *S. intermedius* is found on the intertidal and subtidal rocky seabed in the northern region in the Pacific Ocean, the Sea of Japan, the Korean peninsula, northeastern China, Sakhalin, and Vladivostok (Agatsuma, 2013). *M. nudus* is found on the intertidal and subtidal seabed and is distributed from Dalian in China to Primorskyi Kray in Russia and Japan (Agatsuma, 2013; Takagi et al., 2019; Ding et al., 2020). As a result of the great efforts of pioneer biologists, seed production of both species has been established, currently on a practical level in Japan. Sea urchin has been used for over a hundred years as a model organism in developmental biology research (Wilson, 1895). Knowledge of sea urchins in the field of biology has expanded to include (1) the effects of toxic substances on their immune system, reproduction, and development (Nobre et al., 2015; Brown et al., 2020; Pikula et al., 2020; Rendell-Bhatti et al., 2021), (2) the gene expression involved in sea urchin fertilization and development stages (Li et al., 2020; Wessel et al., 2021; Cui et al., 2022), (3) the nervous system (Wood et al., 2018; Martín-Durán and Hejnol, 2021; Formery et al., 2021), and (4) sea urchin genomes (Sodergren et al., 2006; Kudtarkar and Cameron, 2017; Kinjo et al., 2018; Warner et al., 2021). Sea urchins have also been studied in various aspects related to the impact of current changing environments, such as ocean acidification and global warming to their development and growth (Dworjanyn and Byrne, 2018; García et al., 2018; Zhao et al., 2018; Houlihan et al., 2020).

Sea urchin has been studied for a long time as fisheries resources and model organisms of biology. However, there have been a few studies on general views of their gut microbes' structure, function, and dynamics, particularly using individual-level metagenome approaches (Hakim et al., 2015, 2016, 2019, Yao et al., 2019; Faddetta et al., 2020; Miller et al., 2021). The abundances of reads assigned to phyla *Bacteroidetes* and *Proteobacteria* were observed in the gut of *Tripneustes gratilla*, and those assigned to phyla *Fusobacteria* and *Proteobacteria* in *Diadema setosum* and *Stomopneustes variolaris* (Yao et al., 2019), and the order *Vibrionales* was abundant in wild American green sea urchin (Hakim et al., 2016). Compared to the important roles of gut microbiota in various animals, including humans (Sharma et al., 2019; Youngblut et al., 2019; Fong et al., 2020; Fan and Pedersen, 2021; Morais et al., 2021; Tang et al., 2021), knowledge of gut microbes of sea urchin is behind that of other animals. Only the roles of nitrogen-fixing microbes in the gut of sea urchins have been discussed since the 1950s to answer why C/N rich diet provides nutrition to sea urchins (Mann, 1977).

In this study, we applied a non-destructive individual methodology (Yamazaki et al., 2016) to sea urchin species, *S. intemedius* and *M. nudus*, in Japan for the first time, (1) to examine the external factors, e.g., environments and diets, affecting the structure of the sea urchin gut microbiome, and (2) to explore the function of those gut microbiomes and specific microbes contributing to host growth by monitoring fecal microbiome changes. Here, we report new insights into the gut microbiome's structure, function, and dynamics of two sea urchin species in Japan.

MATERIALS AND METHODS

Samples Collection

M. nudus (n = 13) cultured at the Esan Seedling Center (ESC), Hakodate, and *S. intermedius* (n = 4) cultured at the Toi Seedling Center (TSC), Hakodate, in 2014 and 2015, were used in these individual microbiome studies. Both ESC and TSC were also designated to be Farm below. The procedure for rearing the two species of sea urchin larvae was carried out based on Sakai et al. (2003). Wild individuals of *M. nudus* (n = 4) and *S. intermedius* (n = 4) collected at Menagawa, Hakodate, in 2015 were also used as a comparison. We did not need a specific authorization to handle wild animals' feces. The seawater samples taken from sea urchin rearing in both running and circulation-water system experiments were also used.

Rearing Experiments

Two types of rearing experiments were performed to understand (1) factors affecting the shape of the gut microbiome (Experiment 1; Exp. 1) and (2) microbiome functions affecting the host growth (Experiment 2; Exp. 2) (**Supplementary Figure 1**).

Experiment 1 (Exp. 1)

Nine individuals, including the two species *M. nudus* and *S. intermedius* were used for this experiment. Five specimens of *M. nudus* (test diameter 24.43 ± 6.69 mm, weight 8.67 ± 5.91 g) were collected at the farm ESC. They were fed with microalgae throughout the larval stage and subsequently a brown alga, *Saccharina japonicus*, at juvenile stage until collection time. Four *S. intermedius* (weight 2.88 ± 0.76 g) were obtained from the farm TSC, and were fed with microalgae until that collection. After feces collection on these farms, the sea urchins were transplanted to the laboratory of Hakodate

Fisheries Experiment Station (HFES), which is equipped with a running-water rearing system and reared for 2-weeks. At the HFES laboratory, an ambient natural seawater temperature of approximately 15°C was used to feed sea urchins individually in a caged 2 L polyethylene terephthalate (PET) aquarium created with water exchange holes with full feeding of fresh *S. japonicus* thalli. After 2 weeks of rearing at the laboratory, feces samples were collected. Seawater samples from farm and laboratory aquarium were collected and used for microbiome analyses.

Experiment 2 (Exp. 2)

Eight specimens of *M. nudus* (test diameter 22.25 ± 4.47 mm, weight 4.77 ± 2.13 g) were taken from ESC, and they were transplanted to the laboratory of Hokkaido University, and fed with a fine slice of boiled frozen *S. japonicus* thalli. They were fed for 6 weeks in a circulating-water rearing system maintained at an optimal temperature of 18°C. Every day, half of the water was replaced with fresh artificial seawater. Artificial seawater (SEALIFE, Marine Tech, Japan) was used. Feces and seawater samples were collected at the farm ESC. They were also collected bi-weekly after start rearing in the laboratory.

Collection of Feces and Seawater

Feces and seawater samples for microbiome analyses were collected according to Yamazaki et al. (2016). In brief, feces collections on-site and at the farm HFES were performed inside an instant clean booth, illuminated by ultraviolet light for 15 min (GL-15, Panasonic, Japan). Sea urchin individuals were cleaned using filter-sterilized seawater and moved individually into sterile beakers with filter-sterilized seawater until feces were released. The filter-sterilized seawater was prepared as follows; the natural seawater taken from a coastal area at a depth of 3–4 m was transferred to pressure tanks and filtered through a 0.22 μm Sterivex filter (Sterivex-GV Sterile Vented Filter Unit 0.22 μm, EMD, Millipore, United States) under positive pressure using filtered (0.22 μm) high purity N_2 gas. Feces was collected into 1.5 mL tubes using adopted 5 mL pipette tips. Feces collected on-site and at the farm HFES were immediately frozen in a sterilized 1.5 mL tube on dry ice, transferred to our lab, and kept at –80°C until the DNA extraction was performed. A total of 5 L of the seawater was collected and filtered through a 0.22 μm Sterivex filter. The Sterivex filter was filled with a SET buffer (sucrose 20%, EDTA 50 mM, Tris-HCl 50 mM) and rapidly frozen on dry ice, and kept at –80°C until the DNA extraction was performed.

Microbial DNA Extraction

According to the manufacture's protocol, the microbial DNA extraction of sea urchin feces was performed using the NucleoSpin Soil Kit (MACHEREY-NAGEL, Germany). According to the modified manufacture's protocol, the microbial DNA extraction from seawater was performed using the NucleoSpin Tissue Kit (MACHEREY-NAGEL) (Yamazaki et al., 2016). We used 20% SDS and proteinase K (20 mg/mL) for pre-lysis instead of buffer T1 and proteinase K. We also used 1 mL buffer B3 instead of 200 μ L.

Meta16S Sequencing and Downstream Analyses

PCR amplification and amplicon sequencing of the V1-V2 region on 16S rRNA gene were also performed according to Yamazaki et al. (2016) with minor modifications. Amplified PCR products using 27Fmod with barcode sequences and 338R primers were sequenced using MiSeq (Illumina, United States). Data was further qualified by the removal of reads with average quality values below 25. Filter-passed reads were filed as FASTA for downstream analysis after trimming off both primer sequences.

The sequence data of 16S rRNA genes was analyzed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) (Bolyen et al., 2019) version 2019.1. Sequence denoise, dereplication, and chimeras filtering were performed using the DADA2 pipeline (Callahan et al., 2016). During the "dada2" pipelines, the representative Amplicon Sequence Variant (ASV) sequences, consist of reads with 100% similarity, were constructed. ASVs were assigned taxonomic status through the "q2-feature-classifier" plugin (Bokulich et al., 2018) with the Greengeenes (version 13.8) database trained by Naïve Bayes methods. ASVs assigned to chloroplast or mitochondria were removed from the dataset through the "filter-features" plugin. Using this dataset, further analyses were conducted. For beta diversity, we performed weighted and unweighted UniFrac analysis (Lozupone et al., 2011) and visualized them in a PCoA plot based on a phylogenetic tree generated from the Greengenes database through the FastTree pipeline. To verify if specific ASVs abundance correlated to sea urchin body weight, we performed multiple Person correlations, and p-values were adjusted using the Holm (1979) method. We searched for highly ($r > 0.6$) and significantly (correlated $p < 0.05$) correlated ASVs against the bodyweight and test diameter of the sea urchins. ASVs that occurred in only one sample were removed from this analysis. Growth rate was calculated by Excel software. Meta16S sequences retrieved from the public database were also included in understanding the overview structure of the gut microbiome of sea urchin worldwide; SRP062365 (Hakim et al., 2015), SRP076869 (Hakim et al., 2016), and PRJNA504890 (Hakim et al., 2019).

Shotgun Metagenomic Sequencing and Analysis

Shotgun metagenome sequencing using the Illumina platform was performed on the high and low growth individuals based on their growth rate. Analysis of correlation coefficient used Excel software using the standardized number of leads and the body weight and test diameter of *M. nudus* was performed. The ASVs with an absolute correlation coefficient greater than 0.6 were defined as bacterial communities that correlated with sea urchin growth.

Individuals of *M. nudus* with high growth rates and low growth rates were selected, and the DNAs from fecal samples at both 4th and 6th weeks of rearing were metagenomically sequenced. The shotgun metagenome sequencing was performed on the HiSeq platform by Hokkaido System Science, Co., Ltd., Sapporo, Japan. The DNA quality and quantity were

estimated using NanoDrop, Qubit Fluorometer, and Agilent 2200 TapeStatin System. Using TruSeq Nano DNA LT Sample Prep Kit, genomic DNA was fragment and insert DNA of 350 bp was selected and connected adaptor sequences. Sixty nanograms of each DNA sample were performed on the HiSeq platform. Sequences showing low fluorescence purity were removed.

The FASTQ format sequence data set was uploaded to MG-RAST server version 3.5. For QC, after dereplication was performed using the DRISEE method, low quality ($25 < QV$) sequences were removed with Dynamic Trim. The encoded gene region of protein/rRNA was predicted using reads that passed QC according to the "FragGeneScan" algorithm. The predicted proteins were clustered (similarity $\geq 90\%$) by BLAST analysis using UCLUST. Sequence assignment was performed in MG-RAST server under conditions excluding the following: expected value $\leq 1 \times 10^{-5}$, minimum identity $\leq 60\%$, base pair ≤ 15, amino acid minimum alignment. Taxonomic annotation was performed using the NCBI database, and the functional annotation was performed using the SEED database (Overbeek et al., 2014). Post-assignment metagenomic sequence data was analyzed using MG-RAST tools and STAMP v2.0.9 software (Parks et al., 2014). To test if the abundance of each functional feature of the high and low growth sea urchin differed, Fisher's exact test with Newcombe-Wilson confidence interval calculation method and Storey's FDR for multiple test correction method was used with STAMP software. $p < 0.05$ was considered statistically significant. To visualize metagenomic results, profile bar plots and extended error bar plots were generated at Subsystem Levels 1 and 3. To plot data, results were filtered by q-value (< 0.05) and effect size (> 0.1 or > 0.2) (Yadav et al., 2015). To investigate the taxonomic affiliation of the significantly different functional features between the high and low growth sea urchin, we used the MG-RAST workbench tool implementing KEGG Orthology and GenBank database to annotate with functional features.

RESULTS

Total Sequence Reads, Quality Trimming and Amplicon Sequence Variant Designation

Meta16S Sequence

In Exp. I, a total of 239,928 raw reads from 17 samples [*M. nudus* ($n = 6$), *S. intermedius* ($n = 8$) and rearing seawater ($n = 3$)] were produced (**Supplementary Table 1**). After QC, eukaryotic-derived reads were removed from these subjects to obtain 153,084 reads (131,198 reads and 21,886 reads from sea urchin and seawater samples, respectively). The total number of ASVs obtained from 17 samples was 1,250.

From eight wild sea urchin samples, we obtained a total of 276,858 raw reads (*M. nudus* $n = 4$, *S. intermedius* $n = 4$). After trimmed and removed eukaryotic reads, 96,332 qualified reads from the sea urchin samples (31,857 and 64,475 reads from *M. nudus* and *S. intermedius*) were produced. A total of 147,224, and 309 ASVs from *M. nudus*, *S. intermedius*, and wild animals have been observed (**Supplementary Table 2**).

In Exp. II, a total of 1,223,407 raw reads were obtained from a total of 36 samples [*M. nudus* samples ($n = 32$) and rearing seawater ($n = 4$)]. After QC, reads derived from eukaryotic microorganisms were removed, and 880,046 reads (775,774 reads and 104,272 reads, respectively, from sea urchin and seawater samples) were obtained. The total number of ASVs obtained from 36 samples were 1,613 (**Supplementary Table 2**).

Shotgun Metagenome Sequence

From the high growth individuals reared, 4,435,332 and 2,268,127 reads were obtained at the 4th and 6th weeks, respectively. After quality filtering, 3,848,669 reads (4th week) and 2,188,052 (6th week) were used for MG-RAST annotation, and a total of 3,329,288 and 1,707,418 reads were annotated from the high growth individuals reared for each sample. From the low growth individuals reared, 3,346,079 and 3,192,106 reads were obtained at the 4th and 6th weeks, respectively. After Quality filtering, the remaining 3,254,461 reads (4th week) and 3,117,838 (6th week) were used for MG-RAST annotation. A total of 2,560,781 and 2,306,340 were annotated from the low growth individuals at the 4th and 6th weeks, respectively (**Supplementary Table 3**).

Overall Structure of Gut Microbiome of Sea Urchins *Mesocentrotus nudus* and *Strongylocentrotus intermedius* in Japan

Compared to previously reported data for American green sea urchin (*Lytechinus variegatus*) (Hakim et al., 2015, 2016) and purple sea urchin (*Strongylocentrotus purpuratus*) (Hakim et al., 2019) gut microbiome, we did not find any apparent differences in average dominance of sea urchin microbiome in Japan from those of American species green and purple sea urchins at phylum-level, which consisted of more than 80% *Proteobacteria* and *Bacteroidetes* (**Figure 1**). However, relative abundances of *Proteobacteria* and *Bacteroidetes* fluctuated (more than 15% standard deviation) in relation to each other depend on rearing conditions those of *Proteobacteria* decreased in *M. nudus* under circulated water aquarium, and those of *Bacteroidetes* showed the opposite results (**Figure 1A** and **Supplementary Figure 2**).

Key members of each sea urchin microbiome could be more clearly defined below class level at the family level (**Figures 1B–D**, **2** and **Supplementary Figures 3**, **4**). Families *Psychromonadaceae* and *Flavobacteriaceae* dominated 38–71% on sea urchins in Japan under all rearing conditions. Meanwhile, these two families dominated between 60 and 67% in wild sea urchins, whereas in seawater around 15–31%. The species of *S. purpuratus* and *L. variegatus* were dominated by 41% and less than 5.1% of *Psychromonadaceae* and *Flavobacteriaceae*, respectively. Only *Psychromonadaceae* was present in wild *S. purpuratus*. The relative abundances of families *Campylobacteraceae* and *Vibrionaceae* were affected by rearing conditions. *Campylobacteraceae* abundances increased in laboratory aquarium conditions in two species of sea urchins in Japan and *L. variegatus*. *Vibrionaceae* abundances increased in *M. nudus* in the circulated water aquarium and *L. variegatus*

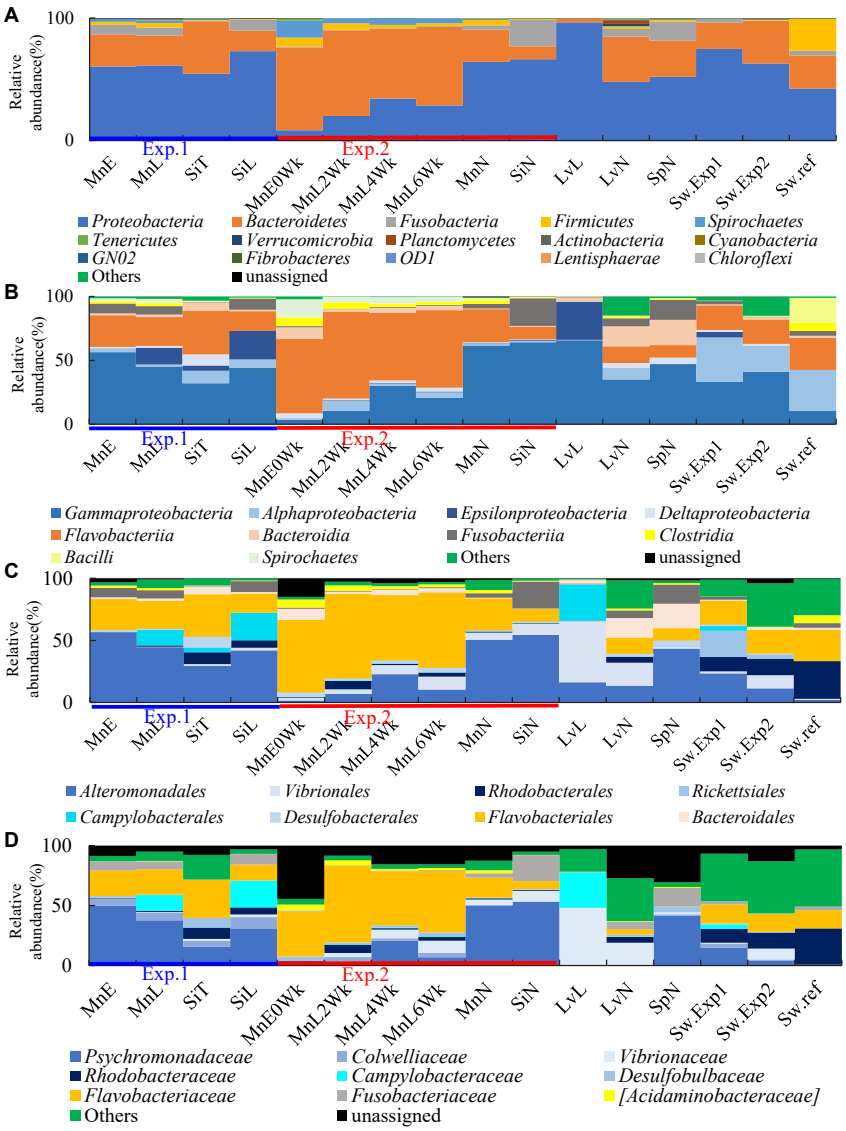

FIGURE 1 | Average microbial community structure of sea urchin gut microbiome. **(A)** Phylum **(B)** Class. **(C)** Order. **(D)** Family. Mn, *M. nudus*; Si, *S. intermedius*; Lv, *L. variegatus*; Sp, *S. purpuratus*; E, the Esan Seedling Center; T, the Toi Seedling Center; N, Natural; L, Laboratory; 0Wk, Zero week; 2Wk, two week; 4Wk, four week; 6Wk, six week; Sw.Exp1, Seawater Experiment 1; Sw.Exp2, Seawater Exp. 2; Sw.ref, Seawater reference.

in the laboratory aquarium. Abundance of *Rhodobacteraceae* (around 5–10%) was rather high in *S. intermedius* both in wild and laboratory reared individuals. *Fusobacteriaceae* was found in small quantities and was detected 2– 20% in all sea urchin species and under all rearing conditions (**Figure 1D**).

The gut microbiome of *S. intermedius* fed microalgae on the farm resembled those of *M. nudus* fed kelp thallus both in the farm and laboratory (**Figures 1**, **3**). In detail, dominant microbial taxa (>5%) of the gut microbiome of *S. intermedius* in the farm, laboratory, and in wild conditions consisted of *Psychromonadaceae* (16, 31, and 53%, respectively), *Flavobacteriaceae* (32, 13, and 7%), *Rhodobacteraceae* (10, 6, and 1%), and *Colwelliaceae* (5, 9, and 0.2%). *Fusobacteriaceae*, *Campylobacteraceae,* and *Vibrionaceae* fluctuated more in the *S. intermedius* gut microbiome. Similarly, those of the gut microbiome of *M. nudus* mainly consisted of *Psychromonadaceae* (38–50%), *Flavobacteriaceae* (17–21%), and *Fusobacteriaceae* (4–8%). As mentioned above, *Psychromonadaceae/Flavobacteriaceae/Vibrionaceae* ratio dynamically fluctuated in *M. nudus* in a recirculating-water system; 1:41:0.4 at the start of rearing were 1:7.3:1.4 at the end of 6th week rearing.

Factors Affecting Sea Urchin Gut Microbiome

Two sea urchins of different species were transplanted to a laboratory aquarium from farms to assess what factors, e.g.,

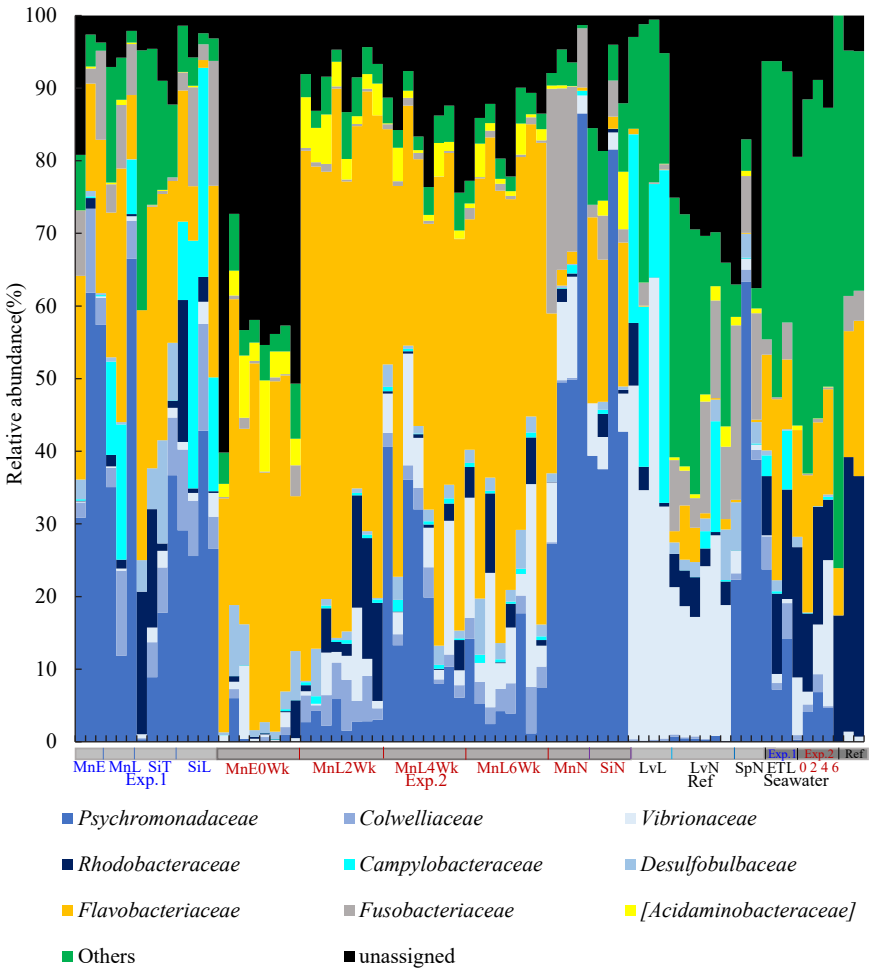

FIGURE 2 | Family-level individual microbial community structure of sea urchin gut microbiome. Mn, *M. nudus*; Si, *S. intermedius*; Lv, *L. variegatus*; Sp, *S. purpuratus*; E, the Esan Seedling Center; T, the Toi Seedling Center; N, Natural; L, Laboratory; 0Wk, Zero week; 2Wk, two week; 4Wk, four week; 6Wk, six week; Sw.Exp1, Seawater Experiment 1; Sw.Exp2, Seawater Exp. 2; Sw.ref, Seawater reference.

diets, rearing environments, and host species affect the shaping of the gut microbiomes of sea urchin in Exp. 1. PCoA plot based on unweighted UniFrac revealed a grouping of both gut microbiomes of *M. nudus* in farm and laboratory after 2 week feeding trials. Three of four individual gut microbiomes in the laboratory reared *S. intermedius* were shifted to the group of *M. nudus*, and the other one was likely to be closer to those of *M. nudus* (**Figure 3**).

Amplicon Sequence Variants Correlated to Hosts' Body Weight and Shell Length

A total of seven ASVs were found in the gut microbiota of sea urchins reared in a circulating aquarium in Exp. 2, showing a positive correlation with host test diameter and body weight (**Figure 4** and **Table 1**). The five ASVs identified correlated with the body weight of sea urchin ($r > 0.6$) with statistical significance ($p < 0.05$) (**Figures 4A,B,D**, and **Table 1**). Three of the five ASVs (BW1, BW2, and BW4) with a host weight gain and correlation coefficient of 0.6 or higher and a significant correlation coefficient ($p < 0.05$) were closest to *Saccharicrinis fermentans* sequence with 91.2–91.5% similarities (**Table 1**). The other two ASVs (BW3 and BW5) had the sequences closet to *Kiritimatiellaeota* bacterium S-5,007 (89.7% similarity) and *Saccharicrinis carchari* (88.2% similarity), respectively. Moreover, two ASVs correlated with test diameter (DM1, ASV had positive and DM2, ASV negative correlation) ($r > 0.6$, $r < -0.6$) with statistical significance ($p < 0.05$). One ASV (DM1) showed a positive correlation had the sequence closest to *S. fermentans* sequences (91.5% similarity) (**Table 1**). One ASV (DM2) showed a negative correlation with the sequences closest to *Flaviramulus ichthrvoenteri* sequence (88.6% similarity).

Functional Analysis of Sea Urchin Gut Microbiome

Shotgun metagenome analysis of gene function subsystem level 1 (**Supplementary Figure 5**) and subsystem level 3 (**Figure 5**) between each functional genes showed significantly different proportion at both 4th and 6th week. The comparison of

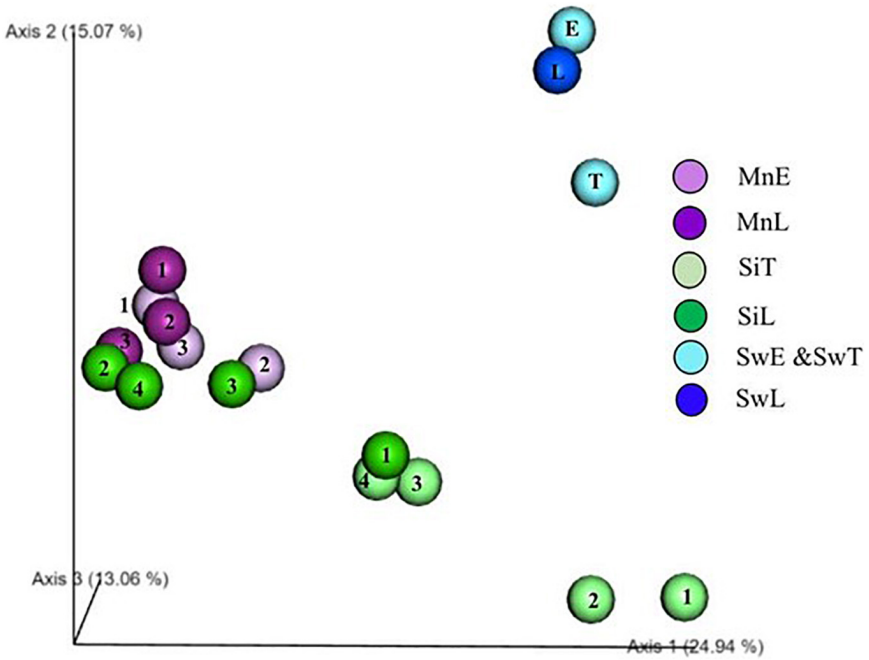

FIGURE 3 | PCoA plot of sea urchin microbiome. Unweighted Unifrac 3D PCoA plot. The numbers (1, 2, 3, 4) in symbol indicates individual number of sea urchin samples. Mn, *M. nudus*; Si, *S. intermedius*; E, the Esan Seedling Center; T, the Toi Seedling Center; L, Laboratory; Sw, Seawater.

functional genes between bacteria with higher and lower growth rates found abundance differences in more than 34 functional genes (**Supplementary Table 4**). The two highest genes occupied more than 15% on the relative proportion on functional genes level were a nitrite reductase [NAD(P)H] large subunit (EC 1.7.1.4) and periplasmic nitrate reductase precursor (EC 1.7.99.4), that affiliated to "nitrate and nitrite ammonification." We observed a rising trend in bacterial abundance between the 4th and 6th weeks (**Figure 5**), which correlates with the existence of potential genes involved in the growth of the sea urchin *M. nudus*. These potential genes were identified as belonging to the order *Vibrionales*.

DISCUSSION

Landmark findings on the reproductive physiology, development, and body structure of sea urchins are available (Ernst, 1997; Sodergren et al., 2006; Lawrence, 2013), however, knowledge on the structure and function of the gut microbiome, which is likely to influence host physiology and development, is extremely limited. N_2-fixation (Guerinot and Patriquin, 1981) and amino acid supply (Fong and Mann, 1980) reported in 1980s were the most recent representative findings on the functional aspects of the sea urchin microbiomes using classical feeding experiments. Gut microbial community studies of sea urchin have recently re-emerged using next-generation sequencing technology in contributing to accumulated knowledge; (1) the microbiome of purple sea urchin in European *Paracentrotus lividus* differs in each part of the gut tract (Meziti et al., 2007), (2) the gut microbiota of the American species of green sea urchin *L. variegatus* may complement host metabolism (Hakim et al., 2016), (3) the gut microbiome of the European species of purple sea urchin *P. lividus* inhibits the growth of microbes introduced from the environment and may play an important role in gut microbiota homeostasis (Laport et al., 2018), (4) the diversity and function of the gut microbiome of the American species of purple sea urchin may be reduced due to the increase in seawater temperature associated with global warming (Brothers et al., 2018), (5) geographical factors may have a more significant influence than diet on the formation of the symbiotic microbiome of larvae of the humpback sea urchin (Carrier et al., 2019), and 6) the possibility that sea urchin in the family *Shizadteridae* continue to have a symbiotic relationship with common gut bacteria even before species divergence (Ziegler et al., 2020). However, our understanding of the structure and function of the gut microbiota in sea urchin in Japan, such as *M. nudus* and *S. intermedius*, is behind other sea urchin species. It is necessary to expand our knowledge for further innovation of effective seed production technology for these sea urchin species in Japan. Even in marine invertebrates, fine-scale gut microbiome studies using individual microbiome approaches, which can monitor microbiome changes structurally and functionally without sacrificing individual animals, have revealed unexpected contributions of the gut microbiome to host growth and physiology of sea cucumber, which belong in the same phylum as sea urchin (Yamazaki et al., 2016). Therefore, for the first time we applied these individual microbiome approaches to expand our knowledge in finding new associations between the sea urchin host and its gut microbiomes.

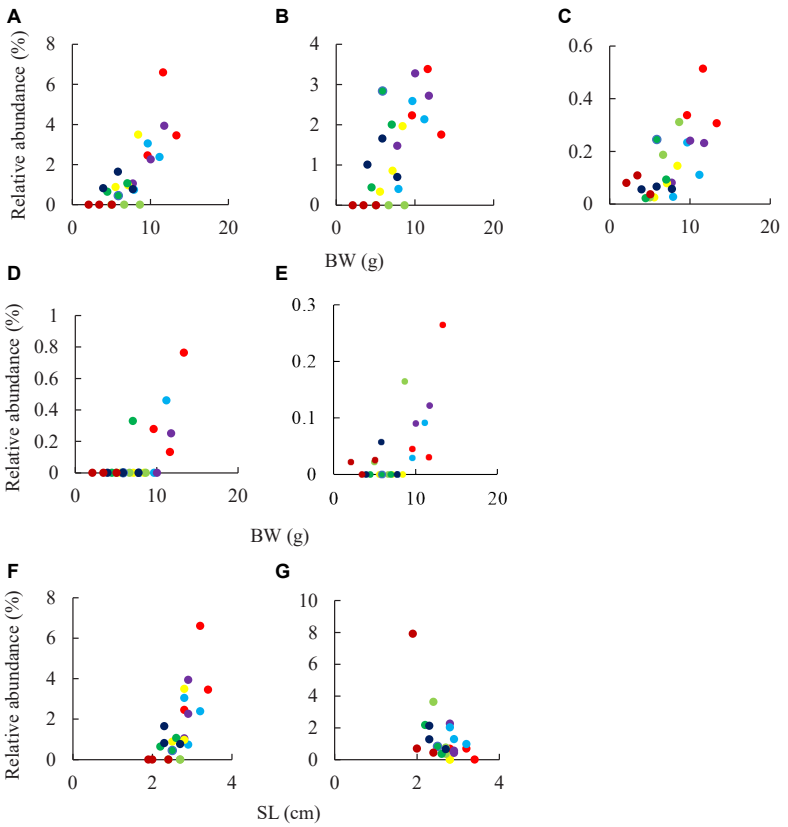

FIGURE 4 | Correlation between ASV abundance and host growth in Exp. 2. Same colors showed the data collected from same samples number at different time point. BW, hosts' bodyweight; SL, hosts' diameter. **(A)** BW1, **(B)** BW2, **(C)** BW3, **(D)** BW4, **(E)** BW5, **(F)** DM1, and **(G)** DM2, see **Table 1** for details. Sample numbers: 1, 2, 3, 4, 5, 6, 7, and 8.

TABLE 1 | ASVs correlated to growth of the sea urchin *M. nudus* (Exp. 2).

ASV ID	ID	Closest taxa	Identity (%) of the 16S rRNA gene sequences	Correlation	*p*-value
7ad7a993096d762eea0f11b6e9b12697	BW1	*Saccharicrinis fermentans*	91.5	0.768726	1.14E–05
45adbed4e7caeb4864617a35df730e45	BW2	*Saccharicrinis fermentans*	91.2	0.668635	0.000355
0acddd2c18bc6b015bf0f83dab2bb20b	BW3	*Kiritimatiellaeota* bacterium S-5,007	89.7	0.66822	0.000359
05b07909be6b43512e1d49e488c795df	BW4	*Saccharicrinis fermentans*	91.2	0.643274	0.000697
c7395fe383e772bb5df6a28ae6e900b1	BW5	*Saccharicrinis carchari*	88.2	0.641202	0.000734
7ad7a993096d762eea0f11b6e9b12697	DM1	*Saccharicrinis fermentans*	91.5	0.628922	0.000995
1292c35cfbc3711fd999120ffa6f9a69	DM2	*Flaviramulus ichthyoenteri*	88.6	–0.68511	0.000221

The increase of *Vibrionaceae* and *Campylobacteriaceae*, in more detail, genus *Vibrio* and *Arcobacter*, respectively, was observed in both *M. nudus* and *S. intermedius* after rearing in running water more than 2 weeks. Increases of these taxa have been reported in studies using American green sea urchin (Hakim et al., 2015, 2016). An increase of more than 20% in *Vibrionaceae* and *Campylobacteriaceae* observed in those reared sea urchin indicates that both bacterial families may be defined as a fluctuated community in the gut microbiome of sea urchin (Hakim et al., 2015, 2016).

By comparing our findings to those of other researchers (Hakim et al., 2015, 2016; Yao et al., 2019), it was determined that seawaters had a negligible effect on the gut microbiome of sea urchins. When two distinct species of sea urchins were transferred from the farm to the laboratory settings, comparable microbiome changes were found in the sea urchin fed with the kelp *S. japonicus* thalli. Our research discovered that diet has a significant effect on the gut microbiota of sea urchin species. It is reasonable to conclude that the rearing environment of sea urchin and host species have minimal impact on gut microbiomes. Our study established that diet significantly influences the gut microbiota of sea urchin species. The similar phenomenon was seen in a variety of species, including humans, indicating that gut

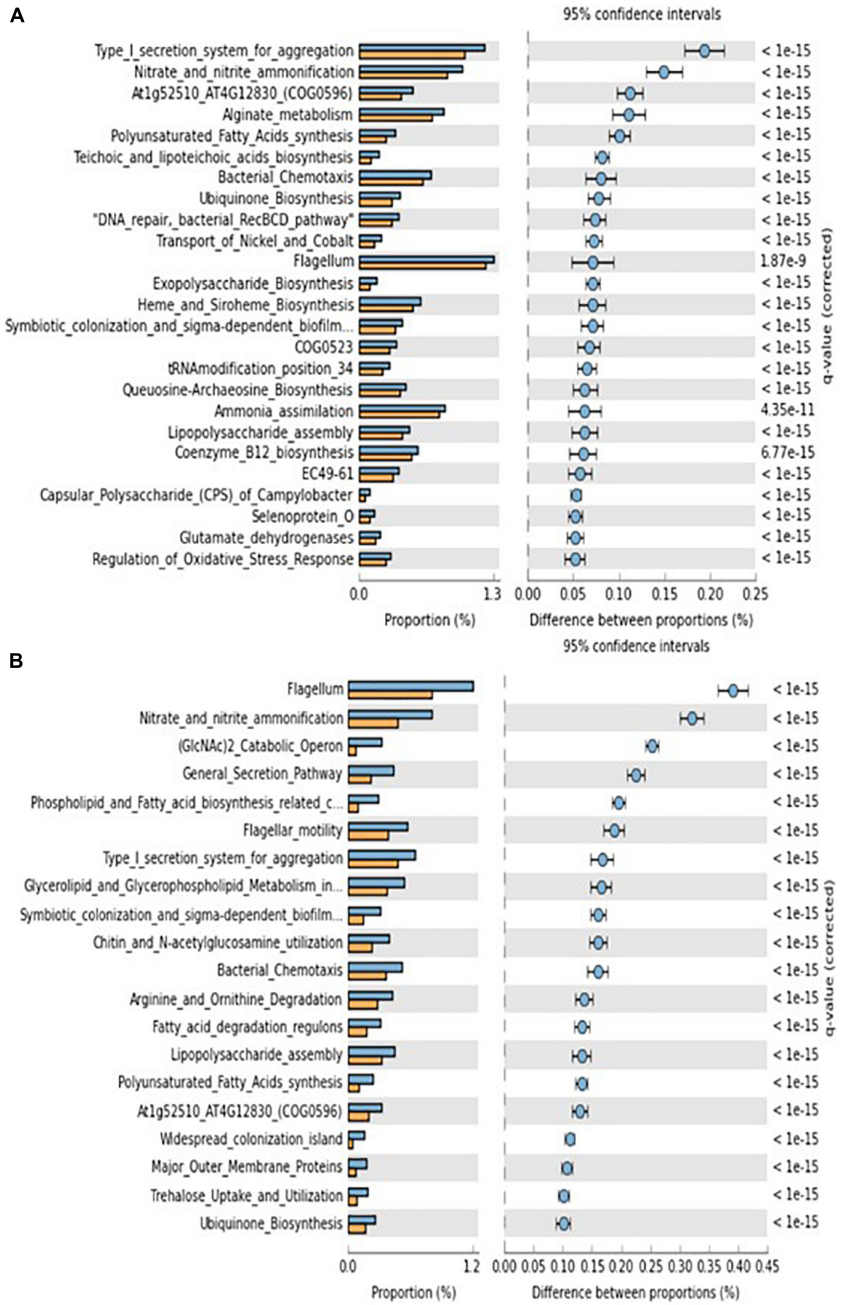

FIGURE 5 | Metagenome gene functions (subsystem level 3) significantly abundant at individuals showing higher lower growth rates. **(A)** 4 weeks and **(B)** 6 weeks. Blue and orange bars indicate higher and lower grown sea urchin individuals, respectively.

microbiota shaping mechanisms are evolutionarily conserved (e.g., Wilson et al., 2020).

Five ASVs showed strong positive correlations with body weight or test diameter in the sea urchin *M. nudus*. Among these ASVs, a bacterium similar to *S. fermentans* was affiliated. This bacterium has been reported to be capable of fermenting amygdalin, glucose, mannitol, and sucrose (Yang et al., 2014). Since the kelp, fed in these studies, contained relatively high amounts of mannitol, it may contribute to the growth of the host through fermentation of the diet ingredients. In addition, *S. fermentans* is also known to possess a N_2-fixing ability (Inoue et al., 2015). Guerinot and Patriquin (1981) reported the possibility that N_2-fixing bacteria exist in the digestive tract of sea urchins *S. droebachiensis* and play a role in supplying nitrogen sources to the host. Therefore, it is possible that *Saccharicrinis*- like bacteria also contribute to the growth of sea urchins *M. nudus* and *S. intermedius* through their N_2-fixing ability.

The number of functional genes involved in "nitrate and nitrite ammonification" was significantly higher in high-growth individuals at subsystem level 3 when sea urchin reared in a circulating aquarium. "Nitrate and nitrite ammonification" is also called dissimilatory nitrate reduction to ammonification (DNRA), a reaction in which nitrate is converted to ammonium under anaerobic conditions. The genes *nrfE*, *nrfF*, and *nrfG* are required to activate cytochrome c nitrite reductase, NrfA, in *Escherichia coli* (Eaves et al., 1998). One of the functions of NrfA is to produce ammonium in the periplasm, which is transported to the cytoplasm where it is converted to glutamine by an amino acid, for assimilation (Einsle, 2011). The utilization of ammonium produced by bacteria has been reported mainly in plants. In addition to this, recent studies on nitrogen dynamics in the digestive tract of aquatic insects have shown that there may be a pathway by which gut microorganisms take up nitrate-nitrogen from the host digestive tract and convert it to ammonium through DNRA for utilization by the host (Ayayee et al., 2019). Nitrate accumulation is commonly observed in a closed aquarium system, and the concentration reaches to 10 time higher than those determined in the ocean (e.g., Kim et al., 2017). Unfortunately, we did not determine the nitrate concentrations in this study, nitrate accumulation in the circulating aquarium might trigger the dynamics of DNRA by the gut microbiome of sea urchin. We might discover a veiled nitrogen metabolism through the gut microbiome of sea urchin, which could contribute host growths under those aquaculture conditions. Fong and Mann (1980) suggested the existence of gut microorganisms that supply amino acids to sea urchins. Although many studies have shown that N_2-fixing bacteria exist in the gut microbiota of sea urchin and that they may play a role in supplying nitrogen source to the host, few studies have addressed the possibility that other nitrogen cycling pathways may play a role in supplying nitrogen source to sea urchin host. The presence of DNRA-related respiratory cytochrome c nitrite reductase has been reported in several organisms, including *E. coli* and *Vibrio fischeri*, and it is likely that *Vibrionales*, which was found to be a candidate for the *nrfEFG* gene, also possesses it. *Vibrionales*, which were found as a candidate with the *nrfEFG* gene, are also likely to have the gene. In the future, it will be necessary to isolate gut microorganisms and clarify their nitrogen metabolisms that contribute to the nitrogen supply to the host, with a view to their application as probiotics in fisheries.

CONCLUSION

Overall, *Psychromonadaceae* and *Flavobacteriaceae* dominated in gut microbiomes of two species of sea urchins in Japan, *M. nudus* and *S. intermedius*. Diets rather than rearing environments and host species is the most potent factor structuring gut microbiomes of the sea urchins. *S. fermentans* was identified as ASV showing a positive correlation with host shell length and body weight in *M. nudus*, suggesting a link in the symbiotic association through nitrogen fixation. *Vibrionales* possessing genes responsible for nitrate and nitrite ammonification pathway also correlated to the growth of sea urchin species in Japan. Our study showed *M. nudus* and *S. intermedius* could be excellent candidates for studying marine invertebrates' structure and function relationships. Further studies in the utilization of gut microbiomes of sea urchin could allow us to develop sustainable technology of fisheries resources in a changing world.

DATA AVAILABILITY STATEMENT

The 16S rRNA and metagenome data sets generated in this study were deposited in DDBJ/GenBank/ENA database under BioProject accession number PRJDB12341.

AUTHOR CONTRIBUTIONS

MY, JY, SM, and YS did molecular biology experiments and data analysis. MY, JY, and YS collected samples. JY, SM, and TS conceived of the study and coordinated the experiments and data analysis. AH, MY, SM, and TS mainly wrote this manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.802754/full#supplementary-material

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