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Characterization of Microbiomes Associated With the Early Life Stages of Sea Cucumber *Apostichopus japonicus* Selenka

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There is a lot of evidence indicating pioneer microbes in early life having various effects on later host biology. Because of the influential phylogenetic position of sea cucumber, which is a deep branching clade in Deuterostomia, the attention on the microbiome in sea cucumber has been increasing. Although microbes in sea cucumber have been reported in several studies, there is a lack of knowledge regarding the pioneer microbiota in the early life stages of sea cucumber. In this study, microbiota changes during the larval development of sea cucumber were assessed using a laboratory rearing system. Microbial community structure was likely to be related to the developmental stage and significant alterations were detected in the late auricularia stage. The relative abundances of *Oceanospirillales*, *Alteromonadales,* and *Rhodobacterales* significantly varied after gut formation. A total of 257 strains were isolated from larval developmental stages of sea cucumber and affiliated to 124 ASVs in the metagenomic analysis. This data demonstrates for the first-time dynamic changes of sea cucumber microbiota in the developmental stages in early life.

Keywords: pioneer microbiome, gut microbiome, holobiont, sea cucumber, *Apostichopus japonicus*

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INTRODUCTION

Since the introduction of holobiont and hologenome concept, comprehensive studies on how holobiont assembly starts, in particular, which kinds of microorganisms first colonize when establishing the holobiont have been the subject of discussion. These first colonizers are called pioneer microbes, and have been widely studied in Deuterostomia animals with a view to investigating their composition and function in holobionts (Wopereis et al., 2014; Arrieta et al., 2015; Schokker et al., 2015; Gensollen et al., 2016). In humans, Therodor Escherich (1857–1911) was the pioneer in the progress of understanding human gut microflora, demonstrating the relationship between intestinal bacteria and physiology of digestion in infants (Escherich, 1886; Bettelheim, 1986; Shulman et al., 2007). Escherich's observations have been strengthened by the evidence that early life microbiota plays an important role in shaping the immune system (Gomez de Agüero et al., 2016; Korpela and de Vos, 2018), and in decreasing risks in developing allergic diseases (Koenig et al., 2011; Arrieta et al., 2014; Gensollen et al., 2016), with the development of modern sequencing technology and metagenomics today. A recent study in infant gut microbiome indicated there are dynamic changes in early life microbiome during the first year after birth,

and the newborns' gut microbiome gradually develops and resembles adult-like microbiomes with *Firmicutes* and *Bacteroidetes* as the most abundant taxa, followed by *Actinobacteria* and *Proteobacteria* (Bäckhed et al., 2015).

As many studies already indicate that pioneer microbiome in early life is crucial in Deuterostomia, an increasing number of studies focus on the influence of pioneer microbes on host biology which could then be used to benefit agriculture and aquaculture. Pioneer microbes' colonization in bird chicks showed an impact on the day of hatching through alteration of intestinal proteome which affects gastrointestinal tract immunity and cellular development (Wilson et al., 2019). The pioneer gut microbiota of tilapia larvae varied between individuals in different rearing environments (Giatsis et al., 2014). Although many previous studies have suggested the importance of early life microbiota in host biology, the structure and function of pioneer microbiota in marine Deuterostomia invertebrate has not been fully elucidated yet.

Sea cucumber (Echinodermata, Holothuroidea) is a marine invertebrate within Deuterostomia linage, and is a worldwide popular fishery and aquaculture resource, especially in Asian regions, due to its nutritive and medicinal value. In Asian region, sea cucumber has high commercial value and 200,000 tons a year in China and 6,500 tons a year in Japan are produced, which show that *Apostichopus japonicus* Selenka is one of the most consumed species (Ministry of Agriculture and Rural Affairs Fishery Administration, 2003-2012; MAFF, 2020). However, the fisheries of wild *A. japonicus* have declined worldwide and the animal has been placed on the red list of endangered species. Wild *A. japonicus* in China had already been reported as being overfished in the 1950s (Zhang and Liu, 1984; Hamel and Mercier, 2013). The huge demands for sea cucumber in recent decades has resulted in overfishing and over-exploitation in more than 70% of regions across the world (González-Wangüemert et al., 2018). Many studies have recommended regulation and management of sea cucumber fisheries and to develop restocking programs of overfished species in various regions including Brazil, Mexico, the Philippines, the Mediterranean, and Northeast Atlantic (González-Wangüemert et al., 2018; Jontila et al., 2018; Rogers et al., 2018; Gamboa-Álvarez et al., 2020). Due to the growing commercial demand for sea cucumber and decreasing wild stocks, aquaculture of sea cucumber has emerged and developed. *A. japonicus* is one of the most popular species in sea cucumber aquaculture, and artificial breeding was first attempted in 1937 and total output reached 170,830 tons in China, 2012 and 6,611 tons in Japan, 2019 (Inaba, 1937; Ministry of Agriculture and Rural Affairs Fishery Administration, 2003-2012).

In order to develop sea cucumber aquaculture, larval development of sea cucumber have been intensively studied (Chen and Chian, 1990; Sewell and McEuen, 2002; Ramofafia et al., 2003; Hu et al., 2010; Soliman et al., 2013). There are six major developmental stages in the early life of *A. japonicus* after the egg is fertilized; blastula, gastrula, auricularia, doliolaria, pentactula, and juvenile stages (Soliman et al., 2013; **Supplementary Figure 1**). Fundamental organs (buccal cavity, esophagus, intestine, cloaca, ciliary bands) appear in the auricularia stage and mature in the juvenile stage within 17 days on average after fertilization under optimal conditions. Primary tentacles and primary podium are used for attaching to habitat in the pentactula stage and then fully develop to tentacles and foot tube in the juvenile stage. Although some studies indicate food, culture conditions and bacterial control may relate to growth and survival rates of sea cucumber larvae, no certain links have been confirmed (Ramofafia et al., 2003; Hu et al., 2010).

Sea cucumber microbiome has been vigorously studied in recent decades in the aspects of diseases and host physiology. Rotting-edges syndrome caused by *Vibrio lentus* (Zhang et al., 2010) and stomach ulcer disease caused by *Vibrio splendidus* (Wang et al., 2006) occur in the auricularia to doliolaria stage. Off-plate syndrome is a serious disease caused by several bacteria that rapidly spreads and has a high mortality rate during the pentactula stage (Zhang et al., 2009). Probiotics have also been studied as potentially improving growth and stimulating the immune system in juvenile sea cucumbers (Ma et al., 2019). Intestinal microbial community composition altered by dietary supplementation benefits the growth of sea cucumber (Yang et al., 2015b). *Rhodobacterales* retaining PHB metabolism genes may play a key role in cultured sea cucumber growth (Yamazaki et al., 2016). Molecular ecological network analysis also indicates the stability of the intestinal community ecosystem is improved by probiotics and florfenicol has a positive impact on sea cucumber growth (Yang et al., 2017). All these studies suggest that microbiome in sea cucumber larvae development might be a crucial factor in developing seed production for sustainable aquaculture, however, there is a lack of knowledge of the structure and function of pioneer microbes of sea cucumber larvae and the dynamic changes during the larval development.

In this study, we set up a laboratory rearing system and performed Meta16S analysis in order to assess the changes to the microbiome during the larval development of sea cucumber and to detect the pioneer microbiota in the early developmental stages. Our results demonstrate that detection of pioneer microbiome in *A. japonicus* and stepwise changed microbiota that significantly related to organogenesis and larval development.

MATERIALS AND METHODS

Sample Collection and Rearing Conditions Under Laboratory Conditions

Fertilized eggs of the sea cucumber *A. japonicus* were prepared at 18.7°C at a farm in Hokkaido Aquaculture Promotion Corporation Kumaishi Branch, Japan (42.12574, 139.99966) on 11:00 am 5 August 2019. These eggs were transferred to the laboratory of Microbiology, Faculty of Fisheries Sciences, Hokkaido University whilst being kept at 18°C for 2 h, and then used immediately for experiments. Density of the fertilized eggs was set at 7,500 eggs/L in an 8 L volume aquarium after manual counting of these eggs in 0.1 mL seawater using a microscope (AxioImager Z2, Zeiss, Oberkochen, Germany) and reared at 18°C. The aquarium was prepared using a

sterilized 8 L glass bottle (Ishizuka glass Co. Ltd., Aichi, Japan) set in an incubator (MLR-352-PJ, PHC Corp., Tokyo, Japan). Each bottle was filled with 7.5 L natural filtrated seawater using a 50 μm mesh cartridge filter (SWP50P10, AS One, Osaka, Tokyo) used in the Kumaishi farm. Each 80 mL (7,500 eggs) of fertilized egg suspensions, corresponding to the final egg density of around 1 egg/mL, was added to each bottle, and rearing was started with aeration (SPP-25GA, Techno Takatsuki Co., Ltd., Osaka, Japan). Feeding started at 48 h after fertilization (on 7 August 2020), when early auricularia morphogenesis was observed in over 80% of individuals. A commercially available diatom, *Chaetoceros gracilis* (Hakodate Fisheries Research, Japan), was fed to the sea cucumber larvae daily.

Subsampling of Sea Cucumber Larvae and Microbes in Rearing Water

Sea cucumber larvae at five major developmental stages, gastrula, early and late auricularia, doliolaria, and pentactula after being confirmed by a microscopic observation (**Supplementary Figure 1**) were used for characterization of microbiomes and microbial isolations. Five liters of rearing seawater including sea cucumber larvae were filtered using a sterilized 40 μm nylon mesh (Falcon Cell Strainer, Durham, NC, United States), and washed once using 0.22 μm filter-sterilized seawater. Sterivex filter (SterivexTM-GV Sterile Vented Filter Unit 0.22 μm, EMD Millipore, Billerica, MA, United States) was used to prepare this sterilized seawater. The larvae on nylon filters were immediately frozen at −80°C until DNA extraction.

To prepare microbial fractions in rearing water, five liters of rearing water after passing through nylon mesh was filtered through a 0.22 μm Sterivex filter by positive pressure using filtered (0.22 μm) N_2 gas. These Sterivex filters were preserved at −80°C until DNA extraction.

Microbial DNA Extraction and 16S rRNA Gene Sequencing

Microbial DNA extraction from sea cucumber was performed using the NucleoSpin Soil Kit (MACHEREY-NAGEL, Düren, Germany), according to the manufacturer's protocol. Microbial DNA extraction from seawater was performed using the NucleoSpin Tissue kit (MACHEREY-NAGEL), according to the modified manufacturer's protocol. In brief, seawater samples were heated at 55°C for 1 h to add an active cell lysis process in TE buffer (10 mM Tris–HCl, 1 mM EDTA) containing 20% SDS and proteinase K (20 mg mL^{-1}) instead of buffer T1. In the third step Lyse Sample, 1 mL buffer B3 was used instead of 200 μL amount of the buffer.

The hypervariable V1-V2 region of the 16S rRNA gene was amplified by PCR with barcoded 27Fmod and 338R primers with Illumina adaptor sequences (Yamazaki et al., 2019). PCR amplicons were purified using AMPure XP magnetic purification beads (Beckman Coulter, Brea, CA, United States), and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies Japan). Equal amount of each PCR amplicon was mixed and then sequenced using MiSeq Reagent Kit v3 (600-cycles) with the MiSeq Illumina platform. Based on sample specific barcodes, obtained reads were assigned to each sample.

Meta16S Analysis

The paired-end sequence data with quality scores (i.e., Fastq files) was analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME 2, version 2018.11) (Bolyen et al., 2019). Quality controls (e.g., trimming primers and denoising sequences, removing chimeric sequences) and merging paired-end sequences were performed using DADA2 (Callahan et al., 2016). Reads with 100% similarity constituted an amplicon sequence variance (ASV). Unlike the method to cluster sequences into operational taxonomic units (OTUs) with fixed threshold (usually 97%), this quality control method using DADA2 allows us to detect even a single nucleotide difference. ASVs were assigned to taxonomy using the Naive Bayes classifier and Greengenes database. Using subsampled reads, unweighted UniFrac distances as beta-diversity were calculated and visualized in prinicipal coordinate analysis (PCoA) plots (Lozupone et al., 2011). Significant differences of unweighted UniFrac distance were tested by permutational multivariate analysis of variance (PERMANOVA) (FDR-corrected $p < 0.05$). The phylogenic tree was generated by FastTree (Price et al., 2009). *Z*-score was calculated by genefilter package in R (Gentleman et al., 2021). R studio software (Version 1.2.5019) was used for heatmaps construction, *Z*-score calculation by genefilter package (Gentleman et al., 2021) and statistical significance of *t*-test to pick up the key ASVs.

Isolation and Identification of Microbes From Early Life Stages

Sea cucumber larvae at each life stage were also used for microbial isolations. Larvae were filtered with 40 μm nylon mesh (Falcon Cell Strainer, Durham, NC, United States), washed once with sterilized seawater, and then homogenized in 1 mL filter-sterilized natural seawater for 60 s manually. A 10-fold serial dilution of these homogenates was prepared using a filter-sterilized natural seawater, and then the dilutions were cultured on 1/5 strength ZoBell 2216E agar plate (0.1% polypeptone, 0.02% yeast extract, 1.5% agar, 75% natural seawater collected at Kumaishi farm) at 18°C. After calculating viable bacterial counts, ca. 30 bacterial colonies per plate were purified using the same agar plate.

The16S rRNA gene sequences of each isolate was amplified by colony PCR using GoTaq Green Mater Mix (Promega, Madison, WI, United States), 27F primer (20 pmol), 1492R primer (20 pmol) with the following thermal profile; initial denaturation at 96°C for 3 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min, and final extension at 72°C for 7 min. PCR products of ∼1500 bp were visualized by electrophoresis with 1% agarose gels. PCR products were purified using a Wizard SV Gel and PCR clean-up system (Promega). PCR amplicons were directly sequenced by Hokkaido System Science (Sapporo,

Japan). Sequences were assembled using ChromasPro (Version 2.1.8, Technelysium Pty. Ltd., Australia). Almost complete sequences of 16S rRNA gene of 257 isolates were obtained in this study. In order to determine the phylogenetic position of isolates, the sequences were aligned using Clustal X (Version 2.1) (Larkin et al., 2007) with the top five most similar sequences indicated by megaBLAST and sequences of type strains retrieved from RDP release 11 as reference sequences (Cole et al., 2014). The phylogenetic tree was reconstructed using Maximum-Likelihood algorism with MEGA X (Version 10.1.7) after performing the best model selection, and was further edited by an online tool Interactive Tree of Life (Letunic and Bork, 2019).

Comparison of Sequences Obtained From Isolates and Meta16S Sequences

Integration of Meta16S sequences and 16S rRNA gene sequences of isolates were performed using QIIME2. Identification and taxonomic analysis of representative were also performed during the comparison. Dominate ASVs were identified and isolated and after combination with identification of isolated strains sequenced by 16S rRNA sequencing, taxonomic analysis reach to both gene and species level.

RESULTS

Microbial Community Structure Dynamics During Sea Cucumber Developmental Stages

A total of 974,753 Meta16S sequence reads were obtained from sea cucumber and seawater samples collected from fertilized eggs (FE), gastrula (GL), early auricularia (EA), late auricularia (LA), pentactula (PT), and juveniles (JN) (**Supplementary Table 1**). Reads passed quality control and removed eukaryotic reads (e.g., mitochondria and chloroplast) were used for microbial diversity analyses and taxonomic assignments; 106,713 and 134,940 qualified reads were generated from sea cucumber samples and seawater samples, respectively (**Supplementary Table 1**).

Unweighted UniFrac distance analysis revealed the dynamic changes of microbiotas associated with host development. The 2D PCoA plot based on unweighted UniFrac analysis obtained sea cucumber larvae and rearing seawater samples at each developmental stage indicates that (1) microbiotas between seawater and sea cucumber were not grouped, (2) microbiota between FE, GL, and EA juveniles were grouped but FE, GL, and EA seawater microbiotas were not, and (3) microbiota between PT and JN sea cucumber larvae were grouped and LA, PT, and JN seawater microbiota were grouped (**Figure 1**). Microbial community of sea cucumber samples according to the PCoA plot reveals a large variation between early and late auricularia stages, the time point before and after gut developed. The UniFrac distance matrix also showed similar tendency in microbiota grouping between sea cucumber and seawater (**Figures 2B,C**). A decreased alpha diversity based on Shannon index and increased beta diversity was observed after gut development (**Figures 2A,D**), indicating a less complex but more dissimilar microbial community after the digestive system had developed.

Characterization of Microbial Community Structure at Early Life Stages of *Apostichopus japonicus*

A total of 1,440 ASVs were obtained and further affiliated to 75 bacterial orders using a similarity threshold of 99% sequence identity; 144, 219, 157, 120, 327, and 254 ASVs, and 29, 30, 19, 28, 39, and 35 bacterial orders, were affiliated in FE, GL, EA, LA, PT, and JN stage, respectively (**Supplementary Table 2**). *Oceanospirillales* and *Alteromonadales* were the most abundant in FE, GL, and EA stages with relative abundance ranging from 24.1 to 29.9% and 32.6 to 46.2%, respectively (**Figure 3** and **Supplementary Table 2**). However, abundances of *Oceanospirillales* dramatically decreased to 3.2% in the LA stage and gradually increased in the PT (9.8%) and JN (49.1%) stages. Such a fluctuation in abundance was also observed in *Alteromonadales*, 32.5–46.2% in the FE to EA stages, but those decreased after EA (14.8, 10.6, and 6.3% in LA, PT, and JN stage, respectively) (**Figure 3** and **Supplementary Table 2**). *Vibrionales*, *Pseudomonadales,* and *Rhodobacterales* were the third most abundant taxa in FE, GL, and EA stages before gut organogenesis. *Vibrionales* was detected in FE (7.2%), GL (13.6%), and EA (12.1%) stages than LA (4.7%), PT (1.0%), and JN (0.3%). Likewise, *Pseudomonadales* was at FE (7.7%), but relative lower amounts in GL (4.6%), EA (2.4%), LA (5.0%), PT (1.0%), and JN (0.3%). In contrast, *Rhodobacterales* was observed in LA (26.0%), PT (37.0%), and JN (22.8%) with higher fractions than in the early three stages in FE (4.9%), GL (7.0%), and EA (9.8%). Relative abundance of *Flavobacteriales* was much lower in early stages of FE (3.6%), GL (2.1%), and EA (1.5%) but increased in LA (25.5%), PT (14.2%), and JN (3.4%).

On the other hand, the microbial community in seawater is significantly different from those associated with sea cucumber hosts (**Figure 2B**). A total of 232, 84, 139, 277, 163, and 69 features were detected in fertilized egg rearing seawater (FESW), gastrula seawater (GLSW), early auricularia seawater (EASW), late auricularia seawater (LASW), pentactula seawater (PTSW), and juvenile seawater (JNSW), respectively (**Figures 3**, **4**). *Alteromonadales* and *Oceanospirillales*, which were dominant at early three stages of sea cucumber samples, were rarely detected at FESW (1.3%, 1.7%), but significantly increased to become the second and third most abundant at GLSW (20.3%, 7.1%), followed by rising to the first and second most abundant at EASW (50.1%, 19.0%). Similar to sea cucumber samples, the abundance of *Oceanospirillales* also dropped in LASW (5.3%) but increased in PTSW (46.2%) and appeared to be present in JNSW (87.7%). The abundance of *Alteromonadales* in LASW (21.3%) decreased below a detectable limit (0.1%) in JNSW. *Vibrionales* and *Pseudomonadales* were also abundant in GLSW (5.0%, 4.3%) and EASW (3.7%, 10.7%), but relatively low in later stage PTSW (0.2%, 0.7%) and undetectable in JNSW. *Rhodobacterales* increased in later stages LASW and PTSW but limited at FE, GL and EA stage, during the host development progress. Different from the host bacterial community, *Flavobacteriales* was rarely

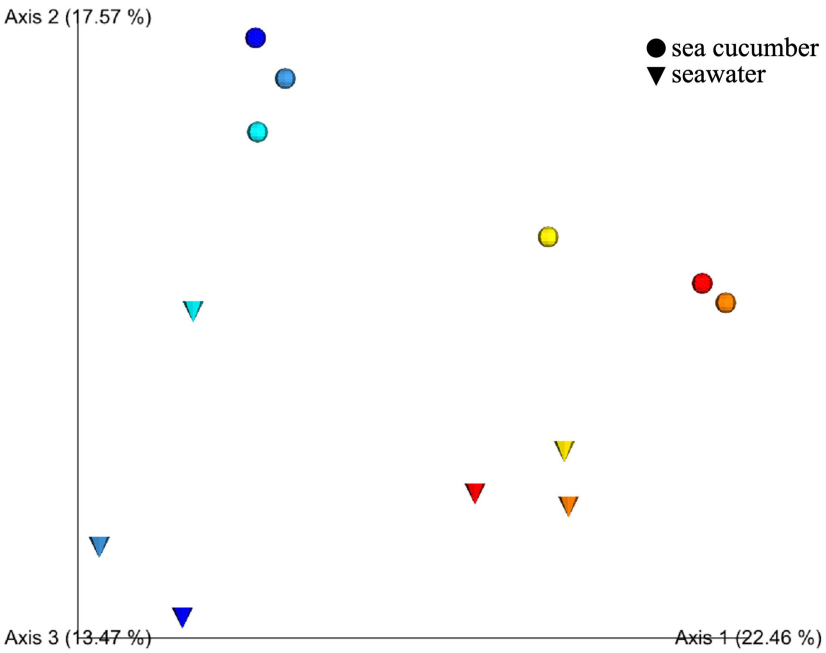

FIGURE 1 | Prinicipal coordinate analysis (PCoA) plot based on unweighted UniFrac distances obtained by comparison of microbiotas at each developmental stage of sea cucumber larva. Blue, fertilized egg; light blue, gastrula; cyan, early auricularia; yellow, late auricularia group; orange, pentactula group; red, juvenile. Circle and cone indicated microbiota from sea cucumber and rearing seawater, respectively.

detected in seawater samples, ranging from 0.3 to 8.5%. SAR11 clade, belonging to class *Alphaproteobacteria*, was observed in all samples of seawater, in particular, rich in FE and GL seawaters with 78.2 and 48.0% frequency, but low relative abundance in sea cucumbers (0.3% in GL) (**Figure 3** and **Supplementary Table 2**).

A further family level taxonomic distribution and heatmap based on relative abundance of top 50 abundant bacterial group revealed that (1) bacterial groups such as *Alteromonadaceae* (*Alteromonadales*), *Alcanivoracaceae* (*Oceanospirillales*), *Pseudoalteromonadaceae* (*Alteromonadales*), *Oleiphilaceae* (*Oceanospirillales*) first appeared and were abundant at FE stage, (2) *Rhodobacteriaceae* (*Rhodobacterales*), *Flavobacteriaceae* (*Flavobacteriales*), and *Marinobacteraceae* (*Alteromonadales*) increased from LA stage, and (3) *Colwelliaceae* (*Alteromonadales*) and *Halieaceae* (*Cellvibrionales*) increased at PT stage (**Figures 4**, **5**). Moreover, JN represents a unique microbiota compared to other life stages; *Nitrincolaceae* (*Oceanospirilales*), the population showed low levels in other life stages (0–7.3%), was significantly dominant in PT with 47.8% relative abundance. *Rhodobacteriaceae* (*Rhodobacterales*) was still the second most abundant family in JN, which appeared to be the most abundant family from the LA stage (22.8–37.0%) (**Figures 4**, **5**).

Dynamics profiling based on ASVs containing more than 500 reads revealed that 14 key ASVs significantly changed at different developmental stages in sea cucumber larvae (**Figures 6**, **7** and **Supplementary Figure 2**); these were assigned into 5 bacterial families, 4, 2, 4, 2, and 2 ASVs were assigned to *Nitrincolaceae* (*Oceanospirillales*), *Alcanivoracaceae* (*Oceanospirillales*), *Rhodobacteriaceae* (*Rhodobacterales*), *Flavobacteriaceae* (*Flavobacteriales*), and *Alteromonadaceae* (*Alteromonadales*). The ASVs assigned to *Nitrincolaceae* and *Rhodobacteraceae* showed different variation trends with corresponding seawater, but the relative abundance of them changed at specific developmental stages in sea cucumber larvae (**Figure 8**). In addition, 2 ASVs of ASV0013 and ASV0051 belonging to *Alteromonadaceae* and *Cellvibrionaceae*, respectively, were defined as core microbiome that present in sea cucumber 's microbiome during whole developmental process, which also detected in rearing seawater (**Supplementary Figure 3**).

Isolation of Key Bacterial Strains During the Larval Development of *Apostichopus japonicus*

A total of 257 strains were isolated from sea cucumber specimens and assigned to 45 species belonging to 32 genera, in which 89.1% of isolates were assigned to known species and 100% of isolates were assigned to known genera (**Figures 8**, **9** and **Supplementary Table 4**). A total of 42, 34, 22, 53, 37, 57, and 13 isolates were isolated from FE, GL, EA, LA, PT, JN, and fertilized egg rearing seawater (NSW), respectively, being counted up 10^2 to 10^4 CFU/g sample (**Supplementary Table 4**). A total of 20 of 42 isolates collected from FE specimen were identified as *Pseudoalteromonas* belonging to *Alteromonadales*, followed by *Alteromonas* (n = 7), *Vibrio* (n = 4), *Idiomarina* (n = 4), *Pseudomonas* (n = 3), *Marinobacter* (n = 2), *Paraglaciecola* (n = 1), and *Shewanella* (n = 1). *Marinobacter* (n = 14) also belonged to *Alteromonadales* was the most abundant genera in isolates collected from GL specimen, followed by *Pseudoalteromonas* (n = 9), *Alteromonas*

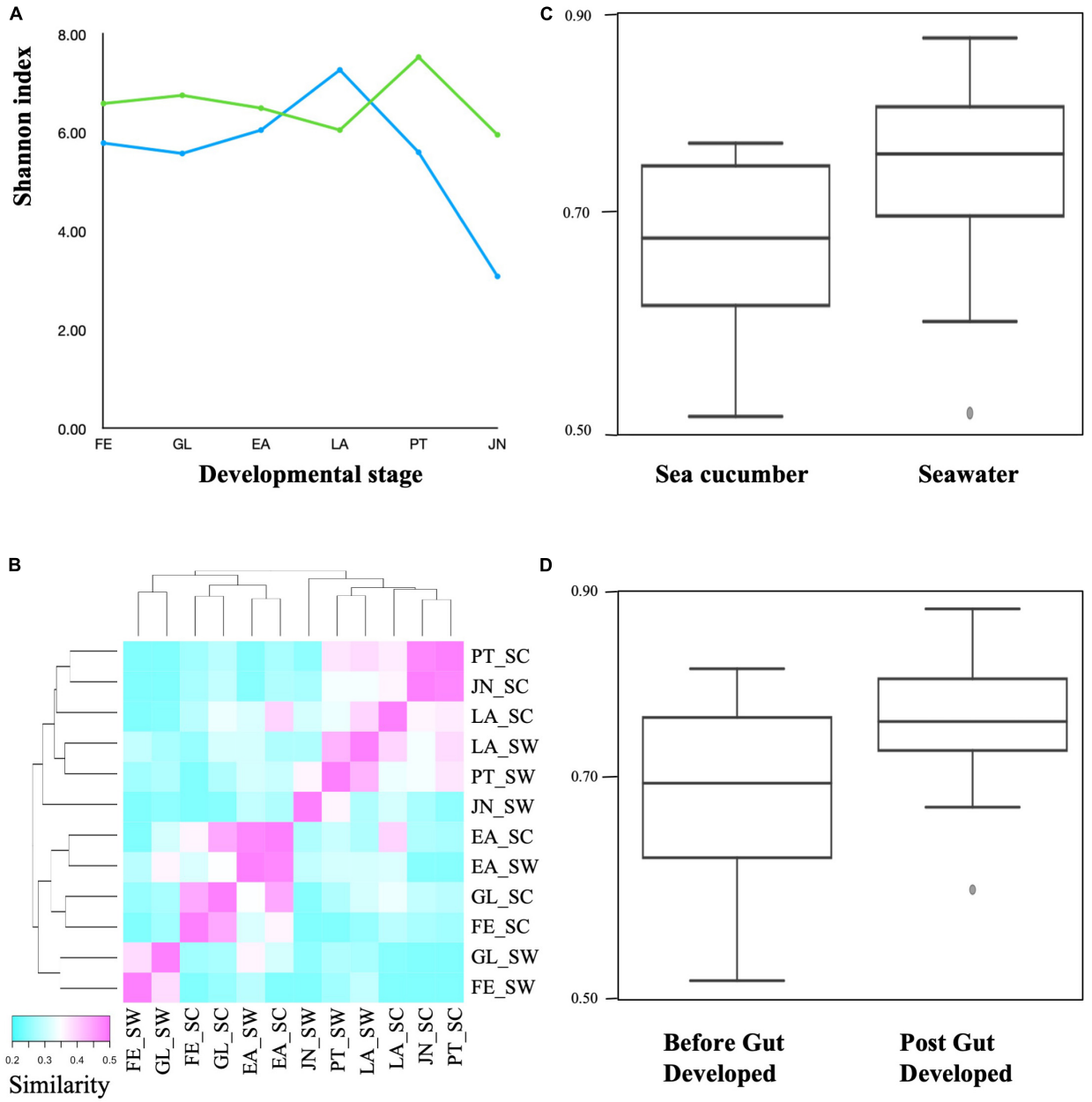

FIGURE 2 | Alpha and beta diversity among sea cucumber and seawater samples. **(A)** Shannon index. Green, sea cucumber samples; blue, seawater samples. **(B)** Heatmap based on unweighted UniFrac distance ($p < 0.05$, $q < 0.05$). Scale represent similarity within samples. FESC, Fertilized egg; GLSC, Gastrula; EASC, Early auricularia; LASC, Late auricularia; PTSC, Pentactula; JNSC, Juvenile; FESW, Fertilized egg rearing seawater; GLSW, Gastrula rearing seawater; EASW, Early auricularia rearing seawater; LASW, Late auricularia rearing seawater; PTSW, Pentactula rearing seawater; JNSW, Juvenile rearing seawater. **(C)** Unweighted Unifrac distance plot between sea cucumber and seawater ($p < 0.05$, $q < 0.05$). **(D)** Unweighted Unifrac distance plot between samples before and post-gut developed ($p < 0.05$, $q < 0.05$).

(n = 4), *Vibrio* (n = 4), *Neptunicella* (n = 1), *Sulfitobacter* (n = 1), and *Bacterioplanes* (n = 1). The most abundant genera in EA isolates were *Alteromonas* (n = 11) and *Pseudoalteromonas* (n = 4), both of them belonging to *Alteromonadales*, subsequently *Marinebacter* (n = 2), *Vibrio* (n = 2), and *Pseudomonas* (n = 1) were also obtained in EA. In LA isolates, 43 of 53 isolates were assigned to *Pseudoalteromonas*, which is the significant dominant genera compared to other genus *Marinobacter* (n = 4), *Alteromonas* (n = 3), and *Pseudomonas* (n = 2).

The composition of isolates showed a variation between LA and PT stages, which *Pseudoalteromonas*, *Marinobacter,* and *Alteromonas* belonging to the order *Alteromonadales* were the most abundant genera in FE to LA but absent in PT (**Figure 9**). In addition, taxonomic diversity of isolates was richer in later

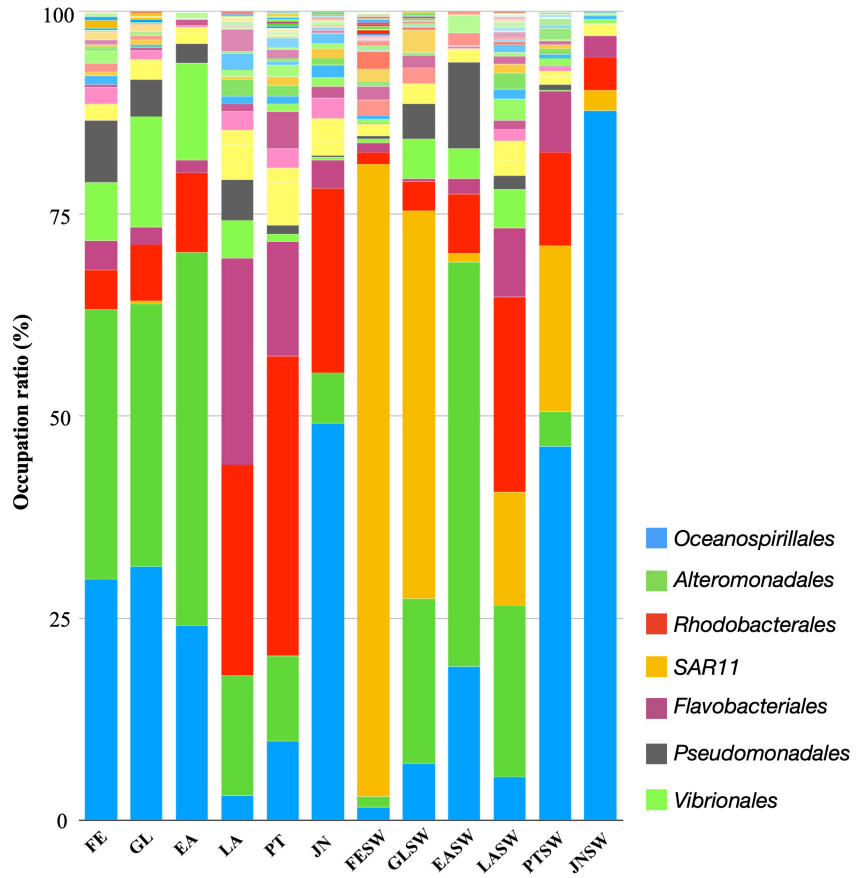

FIGURE 3 | Order-level taxonomic distribution among sea cucumber and seawater samples. Bars represent the relative percentage of each bacterial orders. FE, Fertilized egg; GL, Gastrula; EA, Early auricularia; LA, Late auricularia; PT, Pentactula; JN, Juvenile; FESW, Fertilized egg rearing seawater; GLSW, Gastrula rearing seawater; EASW, Early auricularia rearing seawater; LASW, Late auricularia rearing seawater; PTSW, Pentactula rearing seawater; JNSW, Juvenile rearing seawater.

stages such as PT and JN compared to earlier life stages. Likewise, *Thalassobius* (n = 9) and *Shimia* (n = 8) belonging to the order *Rhodobacterales* were the most abundant genera in PT, followed by *Pseudomonas* (n = 3), *Arenibacter* (n = 3), *Phaeobacter* (n = 3), *Muricauda* (n = 2), *Amphritea* (n = 2), and other 6 isolates assigned to 6 different genera. On the other hand, *Rhodobacterales* and *Alteromonadales* showed a higher possibility to be isolated in JN samples; a total of 20 of 57 isolates in JN were assigned to *Marinobacter* belonging to *Alteromonadales*; 7, 5, and 4 isolates were assigned to *Phaeobacter*, *Shimia,* and *Thalassobius* belonging to *Rhodobacterales*, respectively. The other isolates in JN were assigned to genera *Muricauda* (n = 3), *Alteromonas* (n = 3), *Neptunicella* (n = 3), *Aliiroseovarius* (n = 3), *Arenibacter* (n = 2), *Tropicibacter* (n = 2), *Pseudoalteromonas* (n = 2), *Pseudophaeobacter* (n = 1), *Williamsia* (n = 1), and *Spongiispira* (n = 1). Isolates from NSW were relatively unique compared to the sea cucumber samples. Genera *Sphingomonas*, *Sulfitobacter*, *Labrenzia*, *Rhodococcus*, *Kangiella,* and *Henticiella* were only isolated from NSW samples, which demonstrates a different microbial community structure.

Comparison of isolates with ASVs in Meta16S revealed a total of 124 ASVs were assigned to those sequences of isolates with 100% identity, including 51 dominant ASVs (>0.1% abundance) (**Supplementary Table 3**). In addition, 6 out of 14 key ASVs found in the Meta16S sequences were successfully isolated. ASV0013 and ASV0016 belonging to *Alteromonadaceae,* the relative abundance of these two key ASVs were 0.0090 and 0.0082, respectively. They showed a significant increase at the EA stage with the relative abundance of 0.046 and 0.037, assigning to 10 isolated strains from GL, EA, LA, and JN samples and identified as genus *Alteromonas*. One of them ASV0016 was absent at FE and then slightly increased in the gastrula stage. ASV0019, ASV0022, ASV0026, and ASV0030 belonging to *Rhodobacteriaceae,* the relative abundance of them were 0.0072, 0.0070, 0.0063, and 0.0060, respectively. They showed a significant increase at the PT stage with relative abundance of 0.033–0.040%, assigning to 13 isolated strains collected from JN and PT, which appeared from the early auricularia stage (**Supplementary Table 3**). Interestingly, these five key ASVs were not detected in the fertilized egg rearing seawater. In addition, although 75.8% sequences in Meta16S analysis were unassigned to known species or genera, the taxonomic level of 81 ASVs were improved to species or genus level after taxonomic analysis with isolated strains, in which one unassigned

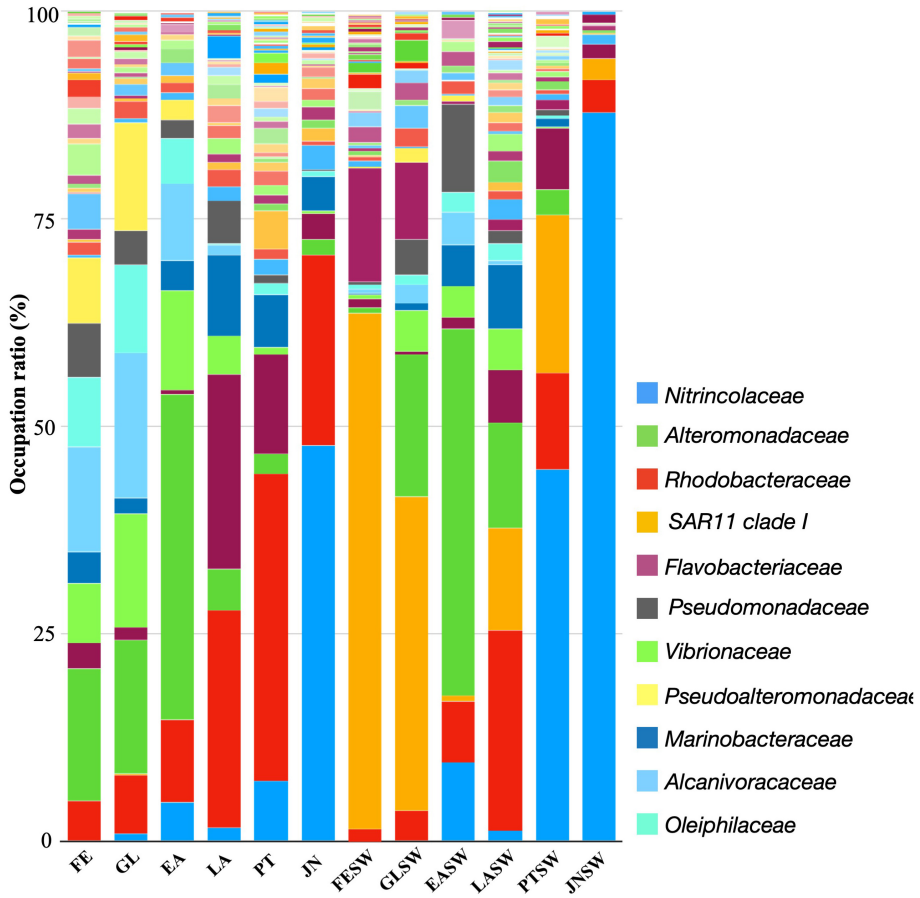

FIGURE 4 | Family-level taxonomic distribution among sea cucumber and seawater samples. Bars represent the relative percentage of each bacterial orders. FE, Fertilized Egg; GL, Gastrula; EA, Early Auricularia; LA, Late Auricularia; PT, Pentactula; JN, Juvenile; FESW, Kuma-shi seawater; GLSW, Gastrula rearing seawater; EASW, Early Auricularia rearing seawater; LASW, Late Auricularia rearing seawater; PTSW, Pentactula rearing seawater; JNSW, Juvenile rearing seawater.

ASV1407 was affiliated to strains NSW5 identified as *Kangiella geojedonens* and 10 uncultured ASVs were assigned to known species (**Supplementary Table 3**).

DISCUSSION

Microbiotas of sea cucumber *A. japonicus* has been intensively studied over recent decades aiming to discover beneficial bacteria that could contribute to sea cucumber aquaculture, disease control, and bioremediation (Chi et al., 2014; Yang et al., 2015b; Zhao et al., 2016; Chen et al., 2018; Ma et al., 2019). After the recent findings of potential physiological roles in gut microbiomes of the sea cucumber *A. japonicus* (Yamazaki et al., 2016), dynamics of the microbiome, in particular *How sea cucumbers shape their microbiomes?* has been a central question (Yamazaki et al., 2020). Among a series of studies on the sea cucumber microbiome, first colonizers on the animal guts called *Pioneer Microbes* are worth studying in sea cucumbers as they potentially contribute to improving aquaculture technology and accumulate biological knowledge on gut microbiome evolution compared to those in humans (Wopereis et al., 2014). Using a laboratory rearing system, we succeeded in conducting Meta16S sequencing to characterize microbiotas of fertilized eggs at major developmental stages up to juvenile and key bacteria were successfully isolated. To our knowledge, this is the first study to investigate the dynamics of microbiota during the larval development of sea cucumber and the possible relationship between early life microbiota and host biology in marine invertebrates.

New Insights Into the Dynamics of Microbiota Changes During Larval Development of Sea Cucumber

The pioneer microbiome in early life has been assessed mainly on human infant gut microbiome, and environmental factors, diets, developmental stage and genetics affecting microbiota shaping have been investigated (Nayak, 2010; Sullam et al., 2012; Mercier et al., 2015). *Bifidobacterium* and *Lactobacillus*, which are lactic acid utilizers, were prevalent in later infant microbiome (4–12 months) during breast feeding but decreased in adult-like microbiota when solid

FIGURE 5 | Dynamics of signature families at each stage. Scale represents relative abundance after *Z*-score calculation. FE, Fertilized egg; GL, Gastrula; EA, Early auricularia; LA, Late auricularia; PT, Pentactula; JN, Juvenile; FESW, Fertilized egg rearing seawater; GLSW, Gastrula rearing seawater; EASW, Early auricularia rearing seawater; LASW, Late auricularia rearing seawater; PTSW, Pentactula rearing seawater; JNSW, Juvenile rearing seawater.

food was introduced (Bäckhed et al., 2015; Hill et al., 2017). Similar to human infants, sea cucumber larvae's gut microbiota altered after feeding, which is probably linked to the changing of gut environment and its functional shifts. Many studies have also worked on pioneer microbiome using fish eggs and larvae to improve intensive aquaculture of larvae rearing (Vadstein et al., 2018), relationships to hatchability, and contribution to the formation of adult fish microbiota

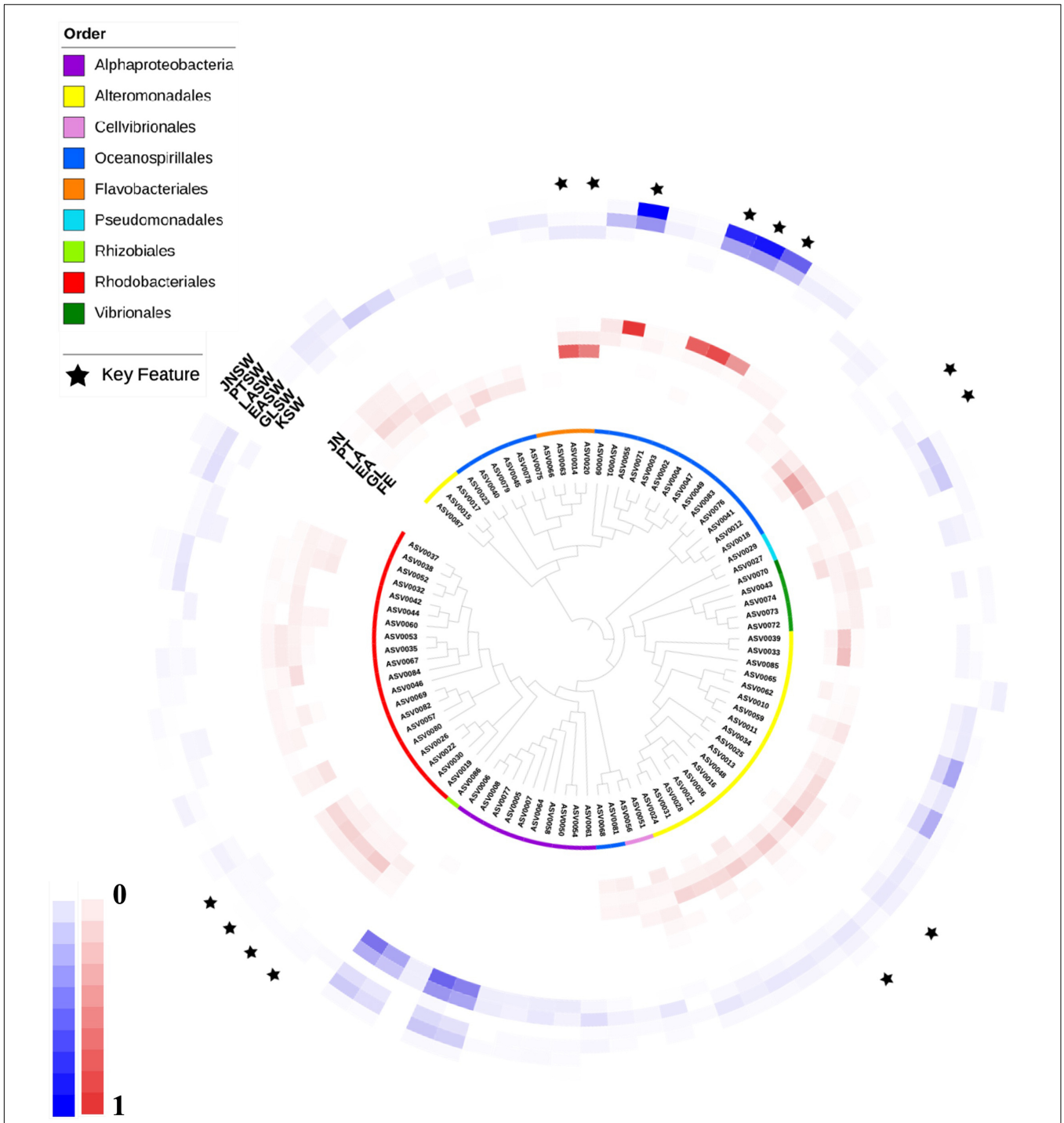

FIGURE 6 | A phylogenetic tree based on features having >500 reads with relative abundance. Inner circles represent taxonomic analysis at order lever. Red colored heatmap represent relative abundance of features in sea cucumber samples. Blue colored heatmap showed relative abundance of features in seawater samples. Key features were labeled with black stars. FE, Fertilized egg; GL, Gastrula; EA, Early auricularia; LA, Late auricularia; PT, Pentactula; JN, Juvenile; FESW, Fertilized egg rearing seawater; GLSW, Gastrula rearing seawater; EASW, Early auricularia rearing seawater; LASW, Late auricularia rearing seawater; PTSW, Pentactula rearing seawater; JNSW, Juvenile rearing seawater.

(Hansen and Olafsen, 1989, 1999; Califano et al., 2017). Whole-body microbiota of aquatic animals using fertilized eggs and post-hatch larvae of sea bream suggest that microbiotas of rearing water and diet affect the shaping of early life microbiota (Nikouli et al., 2019). The Meta16S analyses of sea cucumber during early developmental stages reveals several new findings

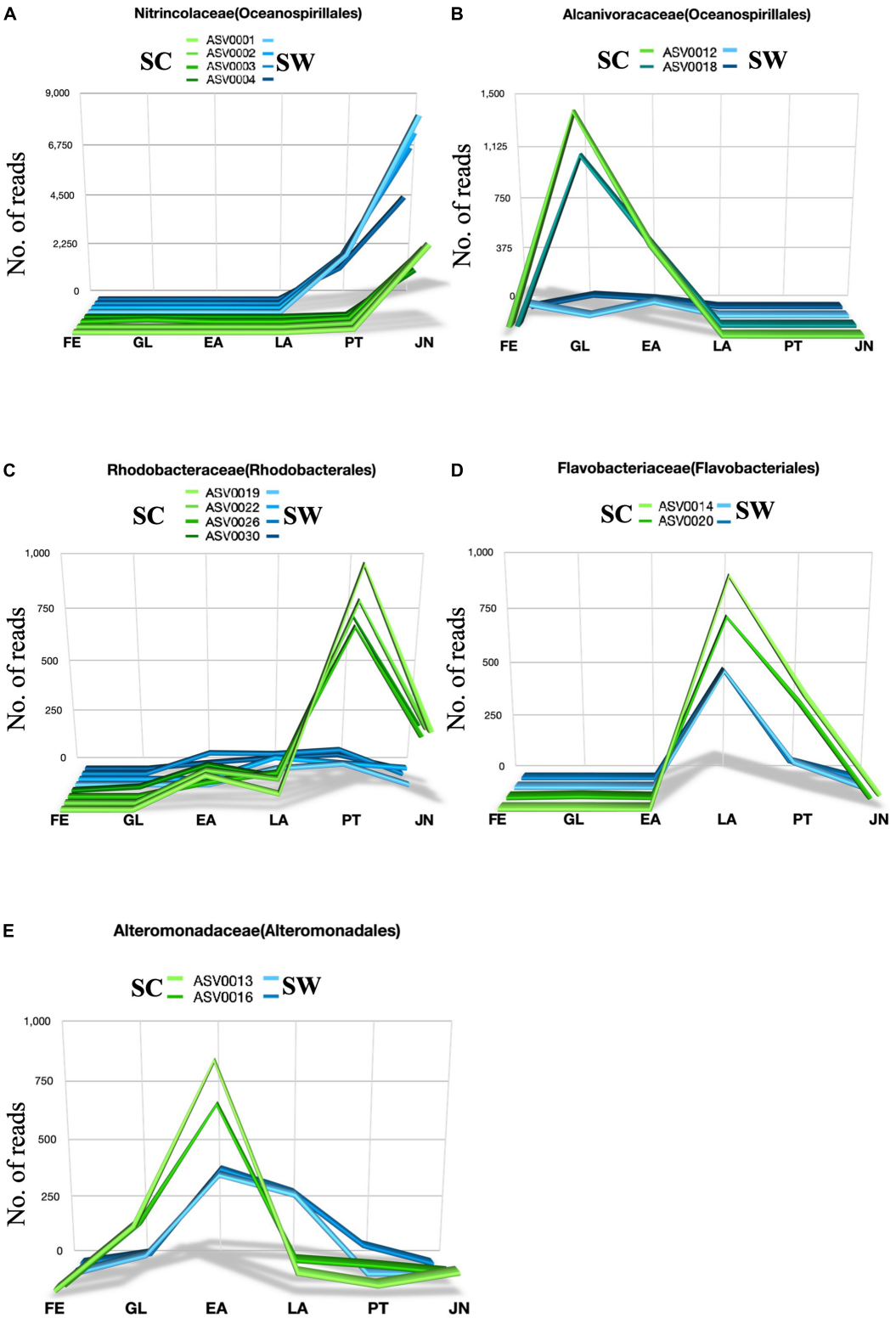

FIGURE 7 | Dynamics of relative abundance of key ASVs based on heatmap. 14 key ASVs were shown in panels **(A–E)**, respectively. X axis is developmental stage, and Y axis is number of reads. FE, Fertilized egg group; GL, Gastrula group; EA, Early auricularia group; LA, Late auricularia group; PT, Pentactula group; JN, Juvenile group.

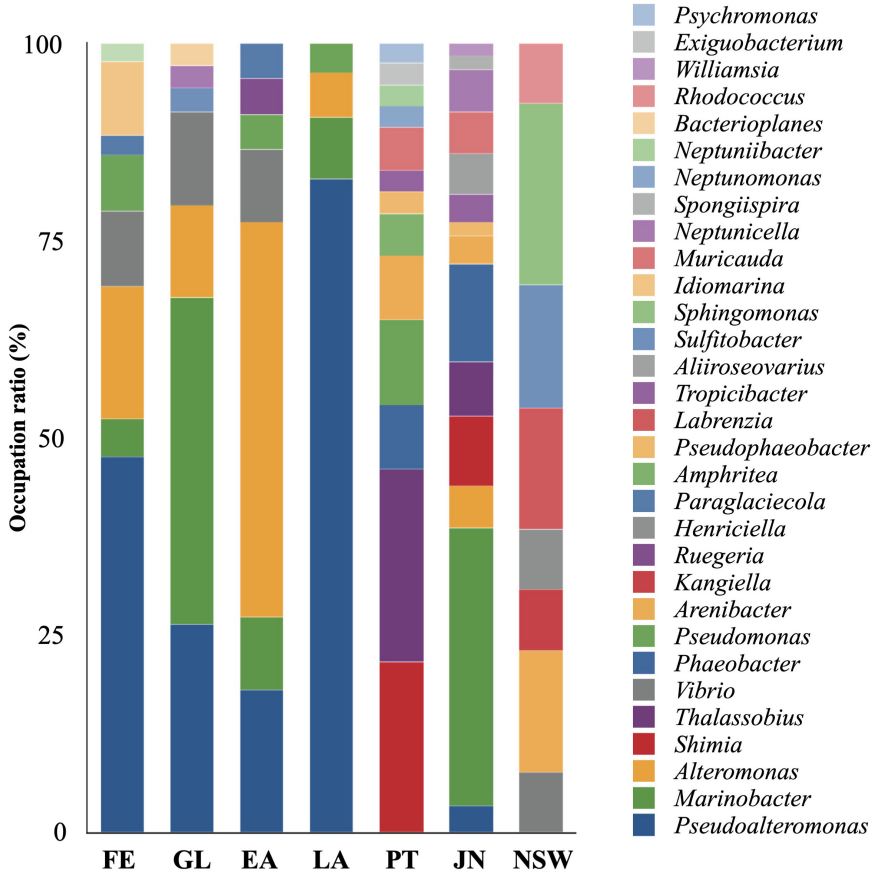

FIGURE 8 | Genus-level taxonomic distribution of isolates from sea cucumber and seawater. Bars represent proportion of isolates. FE, Fertilized egg; GL, Gastrula; EA, Early auricularia; LA, Late auricularia; PT, Pentactula; JN, Juvenile; NSW, Kumaishi nature seawater.

on microbiotas changes related to host development and organogenesis; (1) significant changes of microbiotas in the late auricularia stage, (2) possible first colonizers in gut assigned to *Rhodobacterales* and *Flavobacterales* appeared from auricularia stage, (3) significantly different microbiota in juveniles, and (4) significantly different microbiotas between host associated and environmental water.

Such microbiotas changes are likely to be related to organogenesis of the digestive system. Blastopore is formed in blastula and observed until the early auricularia stage. As the primitive gut-like structure in gastrula disappears, the buccal cavity, esophagus, intestine, cloaca and ciliary bands related to digestion, feeding and locomotion can be observed from the early auricularia stage and are well developed in the late auriculaira. Stomach, axohydrocoel and hyaline sphere are also clearly observed in the late auriculaira. After the pentacutula stage, sea cucumbers start a benthic lifestyle with more organogenesis of tentacles and ossicales formation, which are necessary for feeding and habitat settlement in adult individuals. Early life microbiome could be dynamically changed during the organogenesis, which allow microbes to colonize first on the tissue of host sea cucumber as they shape to a matured form.

Oceanospirillales, *Rhodobacteriales*, *Alteromonadales*, and *Flavobacterales* colonize sea cucumber's microbiota at different developmental stages and show significant changes during larval development. Many researchers have analyzed microbial communities and the impact of these individual bacteria on the host microbiome in sea cucumbers (Yang et al., 2015a; Yamazaki et al., 2016; Zhao et al., 2016; Kim et al., 2017; Li et al., 2018; Pagán-Jiménez et al., 2019; Ren et al., 2019; Yamazaki et al., 2019), but pioneer colonizers in fertilized eggs and larvae of sea cucumber have hardly been reported at all. *Rhodobacterales*, *Flavobacterales,* and *Alteromonadales* were also abundant in adult sea cucumber *A. japonicus* without similar microbial diversity (Yamazaki et al., 2016). Of particular interest in this study, *Rhodobacterales* was detected as the dominant bacterial order and key bacteria in microbiota in early life, which colonize in the early auricularia stage and significantly increase in the pentactula stage (**Figures 3**, **4**). *Rhodobacterales* is known as one of the primary surface colonizers and key players of biogeochemical cycling in marine environments, consisting of more than 350 species (Dang et al., 2008; Simon et al., 2017). Complicated taxonomic subgroups and worldwide distribution of *Rhodobacterales* helped increase research into the understanding the community

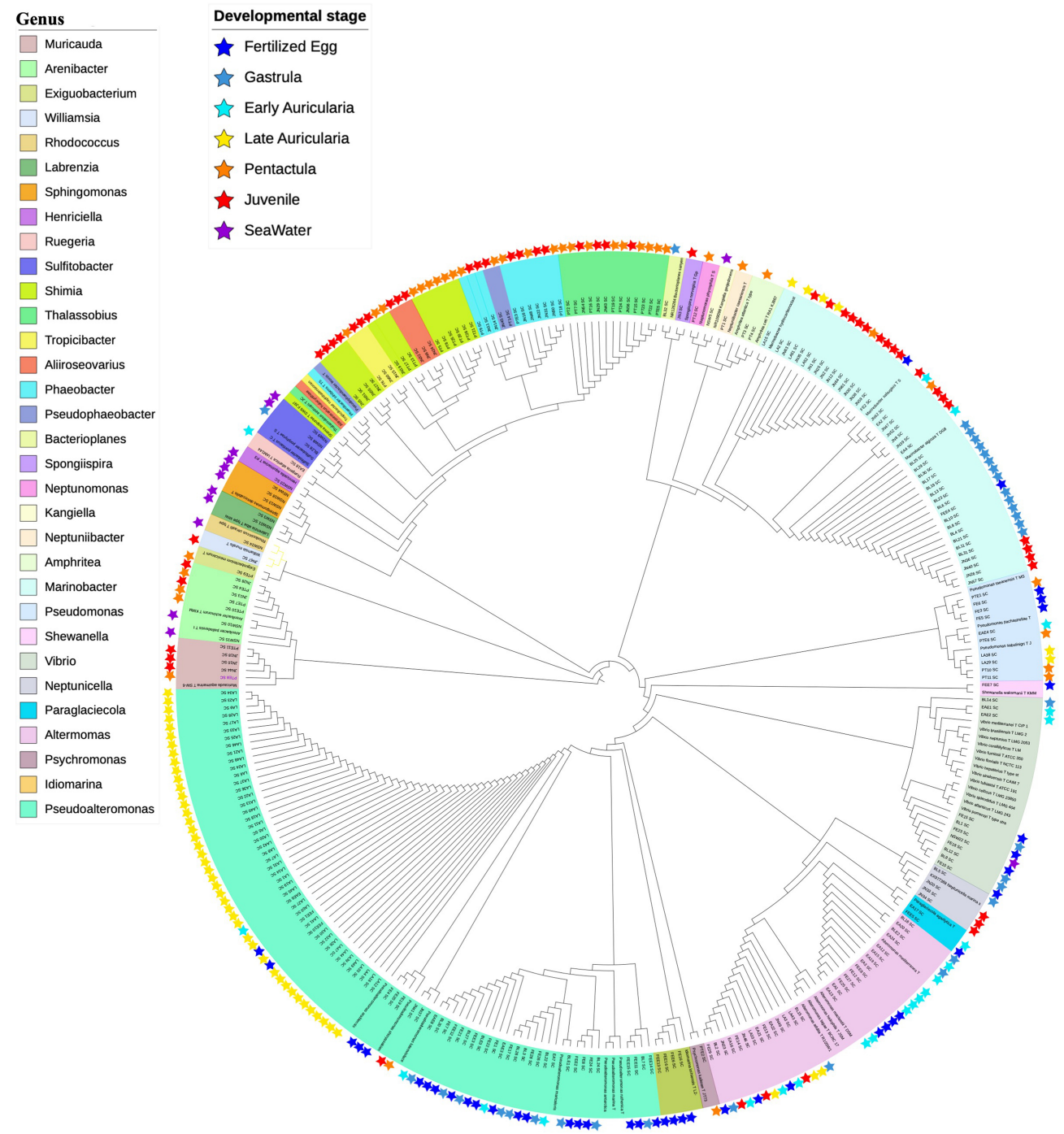

FIGURE 9 | Maximum-Likelihood phylogenetic tree based on partial 16S rRNA gene sequences of isolates. Isolates were shown with stars in specific color. Different genera were labeled with different color. 257 isolates were assigned to 32 genera and 45 species.

structures and molecular roles in *Rhodobacterales* (Fu et al., 2013; Rasmussen et al., 2018; Tavares et al., 2018). There are studies indicating *Rhodobacterales* could produce tropodithietic acid (TDA) against the pathogen *Vibrio* (Grotkjaer et al., 2016; Dittmann et al., 2020). In sea cucumber, Yamazaki et al. (2016) compared microbiota between larger and smaller individuals, in which *Rhodobacterales* was more abundant in larger sea cucumber, and it contains polyhydroxybutyrate (PHB) metabolism genes which could promote host growth (Yamazaki et al., 2016). Dietary β-glucan supplementation and dietary astragalus polysaccharide (APS) in sea cucumber *A. japonicus* have a positive impact on immune responses and enriched

relative abundance of *Rhodobacterales* in microbiota through the NF-κB signaling pathway (Yang et al., 2015b; Song et al., 2019). Currently, 51 isolates are assigned to *Rhodobacterales* and 13 of the 51 isolates are assigned to key ASVs detected in Meta16S analysis. The success of *Rhodobacterales* isolation opens up a way for us to perform bioassay, meta-transcriptomic analysis, molecular studies on host-microbiome association in the future.

Culture environments and supplied diets are always considered as major factors resulting in alteration of microbiota, many studies reveal the relationship of microbiotas between animal gut and environments. In some studies, microbiota of eggs shows highest similarity of 77% to that of rearing water (Nikouli et al., 2019). However, a study on sea bream discusses the potential effects of rearing water and diet on microbiota of different days post-hatch larvae, and they indicated that both rearing water and diet differ from host microbiota but somehow contribute to microbial community (Nikouli et al., 2019). According to Hansen and Olafsen (1989), there is a variation between microbiota in sea cucumber and rearing sea water. The microbiota of fertilized egg and gastrula in sea cucumber were significantly different from rearing water in our results. Only 6.3 and 15.5% ASVs in fertilized egg and gastrula were shared with rearing seawater and five key ASVs significantly changed along with developmental stages were not detected in early life rearing seawater. Nonetheless, with the development of sea cucumber larvae, the sharing ASVs increase to 42.5% at late auricularia stage but decreased to 8.3% at juvenile stage, indicating the majority of larvae microbiome were not affected by rearing seawater.

Factors Influencing Pioneer Colonizers of Sea Cucumber Gut Microbiome

Pioneer microbial colonization is related to birth process, gestational age, digestive system development and genetic factors (Benson et al., 2010; Karlsson et al., 2011; Gomez de Agüero et al., 2016; Borghi et al., 2019). In particular, genetic background of newborn individual influence gut microbial colonization, corresponding to functional development of digestive systems, were emerged in cod and chicken (Bakke et al., 2015; Schokker et al., 2015). Our results also show five key ASVs belonging to *Rhodobacterales* and *Flavobacteriales* are recognized as first colonizers in gut of sea cucumber's microbiota at different life stages. These bacteria were not detected in rearing seawater at corresponding life stages and before the feeding process. Based on these findings, it is assumed that the process of organ formation along with larval developments impacts first colonizers in gut as an influential factor. Interestingly, another variation of early life microbiota was also observed in juvenile individuals, which possess important organ tentacles. Therefore, we assume that organogenesis occurring in sea cucumber larvae is one of the likely factors resulting in the alteration of microbiota, but more insights into the role of individual pioneer colonizer are necessary for deeper understanding.

The auricularia stage is the longest period that primary organs of the digestive system are formed during larval development of sea cucumber, and is sustained for 8–12 days as reported in another study (Soliman et al., 2013). We also observed that the larvae in the auricularia stage have been continuously detected for more than 17 days, to a maximum 19 days after fertilization. We started feeding after the early auricularia stage, and buccal cavity, mouth, esophagus, stomach, intestine, and cloaca, these digestive organs in sea cucumber were clearly observed in late auricularia individuals. The late auricularia microbiota was different from the those of individuals before and after feeding, which indicates that organogenesis accompanied with feeding are major factors impacting on early life gut microbiota. As there are many studies on sea cucumber that indicate the supplied diet could regulate intestinal microbiota and immunity (Yang et al., 2015b; Chen et al., 2018; Song et al., 2019), dietary microalgae could be considered as one of the major factors causing alteration of microbiota observed in the auricularia stage, in fact microbiotas of seawater after diet microalgae spikes were different of those before the spikes. However, even in microbiotas changes of seawater between before and after diets spikes, we still observed differences between host-associated and environmental microbiotas, which means there is still a presence of host factors selecting colonizers and shaping microbiotas.

Culturing Pioneer Microbes

The combination of culture-independent and culture-dependent methods can improve the accuracy of taxonomic assignment in metagenomics. Also, the acquisition of reference genome sequences of isolates provides the possibility of meta-transcriptomes to understand the functions of host-associated bacteria. In this study, we were able to isolate 13 strains belonging to *Rhodobacterales* and they were also assigned 4 key ASVs detected by Meta16S analysis, emphasizing the importance of culture dependent method. *Rhodobacterales* has been reported as possible bacteria for promoting *A. japonicus* growth (Yamazaki et al., 2016). Our study opens the way to investigating the effects of individual bacteria on host physiology and emphasizes the importance of the culture dependent method.

In this study, we analyzed dynamic changes of microbiome for the first time alongside sea cucumber developmental stages and investigated potential factors impacting on the alteration of the microbiome during host development. Our results indicate early life microbiota in sea cucumber significant changes at the auricularia stage and is distinct from environmental seawater. We also isolated crucial bacteria candidates including pioneer microbes on fertilized egg and first colonizers in gut such as *Rhodobacterales* and *Alteromonadales* showing significant difference among larval developmental stages. Further study of individual host-associated bacteria could contribute to a deeper understanding of the interaction between bacteria and host biology.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found below: https://www.ddbj.nig.ac.jp/, DRR319863–DRR319874.

AUTHOR CONTRIBUTIONS

TS and JY designed the study. JY, TS, and YS performed experiments. JY and SM obtained and analyzed data. JY drafted the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.801344/full#supplementary-material

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