이학석사 학위논문

Changes in the Microbiome of the Fleshy Prawn *Fenneropenaeus chinensis* across the Early Developmental Stages

대하의 초기 유생 발달 시기에 따른 마이크로바이옴 변화 연구

2023 년 2 월

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이 논문을 이학석사 학위논문으로 제출함

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김 수 윤의 이학석사 학위논문을 인준함

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Abstract

The Fleshy Prawn *Fenneropenaeus chinensis* is a type of penaeid shrimp widely distributed around the coast of western Korea and eastern China. These days, however, repeated mass mortality due to pathogenic diseases and limited techniques for large-scale larvae production impose hardships in the active rearing of the shrimp, despite their economic importance in aquaculture. While the early developmental stage of penaeid shrimps is known to have significant relationships with its prokaryotic community due to frequent metamorphosis and feed input, the early-life *F. chinensis* remain unexplored. In this study, the changes in the microbiome of the larvae of *F. chinensis* across the early developmental stages were investigated. Eggs spawned from three wild-type maternal prawns were hatched and reared in individual tanks for 24 days. The prokaryotic community in larval shrimps at the egg, nauplius, zoea, mysis, and postlarval stages were analyzed. Moreover, influencing factors including the prokaryotic composition of the rearing water, feed, and environmental parameters were investigated. Results showed that the change in the developmental stage was the key factor that explained the differences between the prokaryotic communities, which grouped into egg and nauplius, zoea and mysis, and postlarvae stages. Egg and nauplius had a discrete community with relative enrichment of *Altermonadaceae* and *Pseudoalteromonadaceae*, implying vertical transmission from maternal prawns. Notably, the

zoea and mysis stages showed enrichment in the relative abundance of *Flavobacteriaceae*, and functional pathways related to glycan metabolism, suggesting an association with the feed input. The postlarval stages were enriched in *Rhodobacteraceae* and pathways related to the metabolism of amino acids and carbohydrates. Furthermore, assembly processes analysis, neutral model fitting results, and similarity and source tracking analyses showed that stochastic processes and the influence of the rearing water and feed were maximized at the zoea and mysis stage when feeding of the larvae was initiated. These results provide an understanding of the basal microbial community of *F. chinensis*, highlighting the importance of the early development stages in terms of aquaculture practices.

Keyword : *Fenneropenaeus chinensis*, Early Development, Microbiome, Marine invertebrate, Aquaculture

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Abbreviations

ASV	Amplicon Sequence Variant
BC	Bray–Curtis Distance
βMNTD	Beta–Mean Nearest Taxon Distance
βNTI	Beta–Mean Nearest Taxon Index
DO	Dissolved Oxygen
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG Ortholog
LDA	Linear Discriminant Analysis
LefSe	Linear Discriminant Analysis Effect Size
NMDS	Non–metric multidimensional scaling
PBS	Phosphate–Buffered Saline
PCoA	Principal coordinates analysis
PE	Paired–End
PICRUSt2	Phylogenetic Investigation of Communities by Reconstruction of Observed States version 2.5.0
RC_{bray}	Raup–Crick metric based on Bray–Curtis distance

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Chapter 1. Introduction

The Fleshy Prawn *Fenneropenaeus chinensis* is a penaeid shrimp that is widely found along the western coast of the Korean peninsula and the east coast of northern China (Jang *et al.*, 2009), and is one of the commercially important species in shrimp aquaculture, ranking the 4th most traded shrimp (Boyd & Jescovitch, 2020). In South Korea, aquaculture practices for *F. chinensis* started in 1963 (Kokkattunivarthil & Kim, 2020), and reached 3256 metric tonnes (mt) of production in 2001 (Jang *et al.*, 2011). However, due to the repeated mass mortality incidents in South Korea and China caused by White Spot Syndrome Virus infection, *F. chinensis* was replaced with the Pacific White Shrimp (*Litopenaeus vannamei*) (Jang *et al.*, 2009). The production of larvae in South Korea heavily depends on the capturing of wild maternal prawns during their spawning season, ranging from May to June along the west coast of South Korea (Jang *et al.*, 2009; Meng *et al.*, 2009). Hence, the history of repeated mass mortality and hardships of larval production impose a limitation in aquaculture practices of the *F. chinensis*.

Larval penaeid shrimps including fleshy prawns undergo several steps of metamorphosis during their early developmental stages, such as the nauplius, zoea, mysis, and postlarva stages. The early development stages are of critical importance in penaeid shrimp aquaculture practices. Live feed such as Artemia, *Chlorella*, and Rotifers are fed at nursery conditions, which acts as a possible

source of the carry-over of microbial agents. In addition, penaeid shrimps are highly susceptible to early mortality syndromes such as the zoea syndrome (Abdel-Latif *et al.*, 2022; Sathish Kumar *et al.*, 2017; Vandenberghe *et al.*, 1999) and translucent post-larva disease (Yu *et al.*, 2022; Zou *et al.*, 2020), primarily due to pathogenic microbial sources. Therefore, understanding the characteristics of microbial communities and their succession pattern along the developmental stages is a crucial step for successful aquaculture practices.

An approach that focuses on the microbial communities associated with the early developmental stages of penaeid shrimps has been conducted on the Pacific White Shrimp *L. vannamei* (Y. Wang *et al.*, 2020) and the Black Tiger Shrimp *Penaeus monodon* (Angthong *et al.*, 2020), respectively. Previous studies have both reported a succession pattern along larval development, showing high similarities of communities at each developmental stage. In particular, Wang *et al.* highlighted the importance of the mouth-opening stage starting from the zoea stage, indicating a strong correlation with the microbial community to the host' s morphological transition and behavior. However, previous studies had not considered the influence of feed and environmental parameters, suggesting the need of a more comprehensive understanding of the contributing factors that shape the microbial community of larval shrimps.

Hence, in this study, I aimed (1) to characterize the prokaryotic communities associated with the larvae of *F. chinensis* across its

early developmental stages, starting from the eggs until the postlarval stages. Moreover, I aimed (2) to assess the factors affecting the structure of the prokaryotic communities associated with the larvae including feed sources, the rearing water, and environmental factors.

Chapter 2. Materials & Methods

2.1. Experimental Design and Sampling Procedures

Maternal shrimps were captured in the coastal area of the Yellow Sea, South Korea and acclimated to rearing conditions in a cylindrical shaped tank (surface area = 12.56 m^2, height = 1.2 m) in an indoor shrimp mariculture experimental facility located in Dangjin, South Korea (36.925° N, 126.7781° E). Three cylindrical shaped tanks (surface area = 0.785 m^2, height = 1 m) named D2, D3, and D5 were selected for the hatching and rearing of larval shrimps. Seawater was pre-treated before introducing the maternal shrimp to the rearing tank. Each maternal shrimp was placed into individual rearing tanks to avoid the possible impact of the genetic divergence of the host. After the maternal shrimps spawned at the tank, days were counted starting from the hatching of the eggs. Environment factors including temperature, salinity, pH, and dissolved oxygen (DO) concentration of individual tanks were constantly measured in situ using water quality monitoring equipment 6600EDS (YSI, USA).

The selected sampling periods of larval shrimps and rearing water are described in Figure 1. Briefly, starting from the eggs across the nauplius, zoea, mysis, and postlarval stages, lasting for 24 days. The egg and larval shrimps were collected with a sterile 15 ml conical tube. The morphology and developmental stage of the larvae were verified manually by light microscopy and washed with sterile Phosphate-Buffered Saline (PBS) (Bioneer, South Korea) to avoid

the possible carry-over of rearing water (De Schryver *et al.*, 2014; Heyse *et al.*, 2021). The larval samples were subsequently transferred to cryovials to be preserved at −80 ℃ until the extraction of genomic DNA.

The rearing water was sampled at selected periods considering the full transition of the larval shrimp to the next developmental stage (Figure 1). Up to two liters of rearing water was sampled in a sterile LDPE water bottle and subsampled for macronutrient analysis, and subsequently pre-filtered with a sterile 100 μm pore-sized sieve. The pre-filtered rearing water was filtered with a 3.0 μm pore-sized PC membrane filter (MF-Millipore™, Germany) and subsequently filtered with a 0.2 μm pore-sized PC membrane filter (MF-Millipore™, Germany) to collect free-living microbial populations. The filters were stored at −80 ℃ before analysis. The macronutrient concentration of the subsampled rearing water including ammonium (NH_4), nitrite plus nitrate (NO_2+NO_3), phosphate(PO_4), and silicate(SiO_2) were measured in triplicates for each sample with QuAAtro AntoAnalyzer (SEAL Analytical, Germany).

The feed supplied for the larval shrimps, including Artemia cysts, *Chlorella*, Rotifers, and pellet feed were subsampled in sterile conical tubes and freeze-stored before analysis. A total of 105 larval shrimps, 20 rearing water filter samples, and 12 feed samples were used in the analysis.

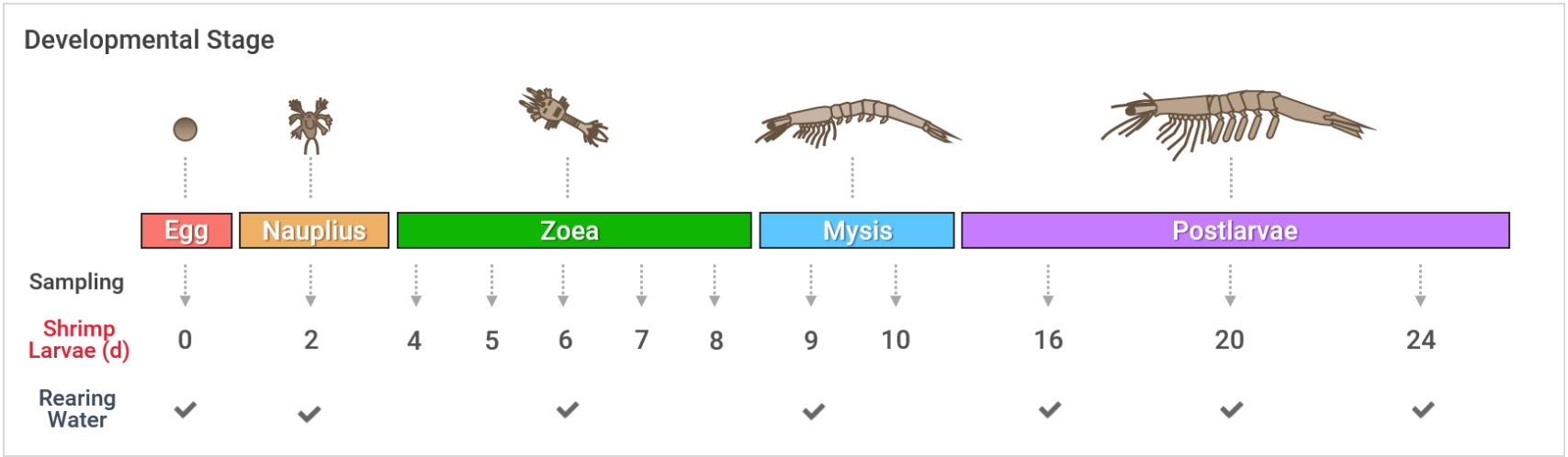

Figure 1 Sampling period of the larval shrimps and the rearing water followed by the developmental stage.

2.2. Genomic DNA Extraction, 16S rRNA Gene Amplification and Library Construction

The genomic DNA (gDNA) from the larval shrimps, filters of the rearing water, and feed were extracted using the DNeasy Power Soil Pro Kit (MoBio Laboratories, USA), according to the manufacturer's protocols. The concentration of gDNA was quantified with NanoDrop 2000/2000c spectrometers (Thermo Scientific, USA). A set of barcoded universal primers targeting the hypervariable V4 region (515F/806R) of the prokaryotic 16S rRNA gene (Y. Wang *et al.*, 2020) was used for the generation of PCR amplicons. 3 μl of the template gDNA was used with the forward and reverse primers (final concentration = 0.4 μM) at a total volume of 20 μl. The amplicons were generated by PCR at the following conditions: initial denaturation at 95 ℃ for 3 minutes, followed by denaturation at 95 ℃ for 30 seconds, annealing at 55 ℃ for 30 seconds, extension at 72 ℃ for 45 seconds repeated for 27 cycles, and followed by a final extension step at 72 ℃ for 10 minutes (Y. Wang *et al.*, 2020). The PCR products were purified and pooled to contain an equimolar amount of the PCR product. The library was constructed with the Nextera XT index Kit (Illumina, USA) and sequenced by Illumina MiSeq PE (paired-end) at LAS, South Korea.

The raw FASTQ data obtained were subject to quality control by FASTQC version 0.11.9 (Andrews, 2010), and adapter sequences were trimmed with Trimmomatic version 0.39 (Bolger *et al.*, 2014). Trimmed and paired sequences were demultiplexed by its set of

barcode sequences into independent samples with Cutadapt version 4.1 (Martin, 2011). The demultiplexed sequences were denoised with DADA2 (Callahan *et al.*, 2016), with Phred score 30 as the cutoff score at the QIIME2 environment (core 2022.8 distribution) (Bolyen *et al.*, 2019). The amplicon sequence variants (ASVs) were taxonomically assigned using the Naive Bayes classifiers trained on SILVA 138 99% OTUs from the 515F/806R region of sequences (Bokulich *et al.*, 2018). Chloroplast, Eukaryota, mitochondria, and unassigned sequences were excluded from the dataset.

2.3. Microbial Community Analysis

All statistical analyses were conducted in R version 4.2.1 (RCoreTeam, 2022). Larval shrimp samples were grouped into three groups, 'Egg and Nauplius', 'Zoea and Mysis', and 'Postlarvae' according to the developmental stage of the larval shrimps. Analysis of Similarities (ANOSIM) with 999 permutations was performed to determine if the prokaryotic communities from larval shrimps were differentiated by the developmental stage groups.

Alpha-diversity indices including Chao1, Shannon, and Inverse Simpson were calculated using the 'Phyloseq' R package version 1.40.0 (McMurdie & Holmes, 2013). Mean Nearest Taxon Distance (MNTD) was calculated using the 'mntdn' function implemented in the 'iCAMP' R package version 1.5.12 (Ning *et al.*, 2020). The prokaryotic taxa were agglomerated at the class and family level, respectively, to analyze the taxonomic composition of prokaryotic communities, and ten taxa with the highest relative abundance were plotted. Alpha diversity indices and the relative abundances of the ten bacterial families with the highest abundance at each developmental stage group were compared by the Kruskal-Wallis test and pairwise Wilcoxon tests with Bonferroni corrections for multiple comparisons were implemented for groups with significant differences.

Core bacterial ASVs present in at least 90 % of the larval shrimp samples were identified. The ASV sequences were BLAST searched against the latest updated version of the EzBioCloud database (July

2021) (Yoon *et al.*, 2017). The distinctive taxa appearing at each developmental stage group were identified using the Huttenhower Galaxy server version of linear discriminant analysis effect size (LEfSe) (Segata *et al.*, 2011) with linear discriminant analysis (LDA) score cutoff as 2.0, and all-against-all comparisons.

Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarities was applied to identify the clustering and succession pattern of prokaryotic communities from larval shrimps, rearing water, and feed.

The assembly mechanism of the larvae-associated prokaryotic community was assessed following the null model approach previously developed by Stegen *et al.*, 2013. Briefly, beta-mean nearest taxon distance (βMNTD) was used as a measure of phylogenetic distances between two communities. The null distribution of βMNTD was computed after randomizations (1000 permutations). The beta-mean nearest taxon index (βNTI) value, which is calculated as the deviation of the observed βMNTD values from the null βMNTD values was used to quantify the relative contribution of ecological processes governing prokaryotic community assembly. βNTI value greater than 2 indicates a higher level of phylogenetic divergence than expected from the null model, hence indicating heterogeneous selection. βNTI value less than −2 indicates a lower level of phylogenetic divergence than expected, hence indicating homogeneous selection by the host or the environment. On the contrary, $|\beta NTI| < 2$ indicates a dominance of

stochastic process. The Raup-Crick metric based on Bray-Curtis distances (RC_{bray}) was employed to quantify the different stochastic processes for Samples with $|\beta NTI| < 2$. $RC_{bray} > 0.95$ indicates a higher level of taxonomic divergence, hence the dominance of dispersal limitation. $RC_{bray} < -0.95$ indicates a lower level of taxonomic divergence, hence the dominance of homogenizing dispersal. $|RC_{bray}| < 0.95$ refers to the undominated processes (i.e. ecological drift). The assembly processes were quantified within larval samples of different sampling days and different developmental groups and between larval samples against the rearing water and feed. The function 'qpen()' in the 'iCAMP' R package was used.

The Sloan neutral model for prokaryotes was employed to identify the ecological processes affecting the assembly of prokaryotic communities associated with larval shrimps (Burns *et al.*, 2016; Sloan *et al.*, 2006). The larval metacommunity, which refers to the entire pool of prokaryotes associated with larval shrimps was considered the source for individual samples. The observed frequency of occurrence among the metacommunity of each ASV was plotted against its log-transformed mean relative abundance and fitted by the neutral model by the estimated migration rate (m). The estimated migration rate refers to the chance that an ASV will be replaced by the metacommunity through neutral processes. Hence, a lower 'm' value indicates a higher limitation on dispersion. R^2 metrics of the neutral model fit were utilized as the 'goodness of fit', where R^2 values close to 1 were considered as a good fit, indicating the

dominance of a neutral process (i.e. dispersal). ASVs with frequencies above or below the 95 % confidence level of the predicted frequencies were considered the ASVs that were selected by deterministic processes. The neutral model fitting was performed with the 'snm.comm()' function implemented in the R package 'iCAMP' version 1.5.12, considering each developmental stage group and reared days as different 'treatments', respectively.

To assess the effect of rearing water and feed on the larvae-associated community composition, βMNTD, Bray-Curtis distances (BC), βNTI, and RC_{bray} values calculated between larval shrimps and the rearing water from the same day and tank calculated from above were compared against each developmental group. The four metrics from above were also calculated between larval shrimps and four types of feed (*Artemia*, *Chlorella*, Pellet Feed, and Rotifer) and compared against each developmental group. SourceTracker2 (Knights *et al.*, 2011) was employed to calculate the relative contribution of prokaryotic communities associated with the rearing water and feed on the larval shrimps. Larval shrimps were set as 'sinks', and the rearing water and feed were set as 'sources' for source tracking analyses.

The correlation between the relative abundance of ten major bacterial families and environmental factors including temperature, salinity, pH, DO, and macronutrient profiles were assessed by calculating the Spearman correlation coefficients. The results were visualized with the 'corrplot' R package (Wei & Simko, 2021). All

other plots in this study were generated by using the ‘ggplot2’ R package (Wickham, 2016).

2.4. Functional Inference

PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Observed States version 2.5.0) was employed for the prediction of functional potentials of the prokaryotic communities associated with larval shrimps (Douglas *et al.*, 2020). The ASV table and corresponding sequences were set as input of the python script *picrust2_pipeline.py*. The Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs (KOs) resulting from the prediction were categorized into level 3 KEGG pathways in the KEGG BRITE hierarchy. The calculated abundance of KEGG pathways was standardized into relative abundance in each sample. Pathways showing differential abundance from each developmental group were identified by LefSe analyses with the same parameters as described above.

Chapter 3. Results

3.1. Diversity and Structure of prokaryotic communities associated with larval shrimps

3.1.1. Rearing and Sequencing Results

The rearing results of larval shrimps are described in Table 1. Briefly, all the larval fleshy shrimps were at their egg stage on day 0, nauplius stage on day 2, and zoea stage on day 4. However, the day larval shrimps reached their mysis stage was varied: larval shrimps at tanks D3 and D5 reached their mysis stage on day 9, while shrimps from tank D2 reached their mysis stage on day 10. The shrimps were fully verified as postlarval shrimps on day 16 and until the end of the experiment (day 24).

A total of 1,508,477 high-quality sequences were used in the study. A total of 2580 distinct ASVs were identified among all samples. The average number of high-quality sequences and ASVs per sample are described in Table 1 and Table 2. The average number of ASVs from larval shrimps, rearing water, and feed were 192±64, 107±22, and 111±35 ASVs, respectively.

Table 1 Rearing results of larval fleshy shrimps and the average number of high-quality sequences and number of ASVs used in the study.

Larval Shrimp							
Sample Name	**Sampling Date**	**Tank**	**Reared Days**	**Developmental Stage**	**Feed Type**	**Average Number of High-Quality Sequences**	**Average Number of ASVs**
D2_0d	2022-05-11	D2	0	Egg	None	14822 ± 4064	168 ± 24
D3_0d	2022-05-05	D3	0	Egg	None	21047 ± 2354	295 ± 33
D5_0d	2022-05-12	D5	0	Egg	None	8043 ± 8778	151 ± 99
D2_2d	2022-05-13	D2	2	Nauplius	None	10457 ± 3479	141 ± 11
D3_2d	2022-05-07	D3	2	Nauplius	None	9886 ± 3686	143 ± 36
D5_2d	2022-05-14	D5	2	Nauplius	None	10607 ± 1284	144 ± 12
D2_4d	2022-05-15	D2	4	Zoea	Chlorella	13230 ± 1693	229 ± 11
D5_4d	2022-05-16	D5	4	Zoea	Chlorella	10967 ± 5721	183 ± 35
D2_5d	2022-05-16	D2	5	Zoea	Chlorella	5917 ± 262	157 ± 5
D3_5d	2022-05-10	D3	5	Zoea	Chlorella	10110 ± 2597	178 ± 29
D5_5d	2022-05-17	D5	5	Zoea	Chlorella	7825 ± 3870	173 ± 43
D2_6d	2022-05-17	D2	6	Zoea	Chlorella	7840 ± 1523	207 ± 27
D3_6d	2022-05-11	D3	6	Zoea	Chlorella	8718 ± 1125	164 ± 12
D5_6d	2022-05-18	D5	6	Zoea	Chlorella	4658 ± 743	142 ± 19
D2_7d	2022-05-18	D2	7	Zoea	Chlorella	8652 ± 458	140 ± 5
D3_7d	2022-05-12	D3	7	Zoea	Chlorella	9490 ± 3739	187 ± 38
D5_7d	2022-05-19	D5	7	Zoea	Chlorella	10880 ± 2825	199 ± 28
D2_8d	2022-05-19	D2	8	Zoea	Chlorella	18437 ± 3816	246 ± 23
D3_8d	2022-05-13	D3	8	Zoea	Chlorella	5059 ± 1330	142 ± 26
D5_8d	2022-05-20	D5	8	Zoea	Chlorella+Rotifer+Artemia	7279 ± 2130	146 ± 13
D2_9d	2022-05-20	D2	9	Zoea	Chlorella+Rotifer+Artemia	16981 ± 10451	259 ± 74
D3_9d	2022-05-14	D3	9	Mysis	Chlorella	10795 ± 3300	189 ± 26
D5_9d	2022-05-21	D5	9	Mysis	Artemia	12528 ± 2883	273 ± 65
D2_10d	2022-05-21	D2	10	Mysis	Artemia	9637 ± 4515	250 ± 56
D3_10d	2022-05-15	D3	10	Mysis	Chlorella	7462 ± 2308	165 ± 16
D5_10d	2022-05-22	D5	10	Mysis	Artemia	8675 ± 2121	181 ± 26
D2_16d	2022-05-27	D2	16	Postlarvae	Chlorella+Artemia	43898 ± 55721	295 ± 155
D3_16d	2022-05-21	D3	16	Postlarvae	Chlorella+Artemia	17198 ± 7093	239 ± 45
D5_16d	2022-05-28	D5	16	Postlarvae	Chlorella+Artemia	6904 ± 2624	138 ± 37
D2_20d	2022-05-31	D2	20	Postlarvae	Chlorella+Artemia	27423 ± 10855	280 ± 118
D3_20d	2022-05-25	D3	20	Postlarvae	Chlorella+Artemia	12196 ± 4767	196 ± 38
D5_20d	2022-06-01	D5	20	Postlarvae	Artemia	6660 ± 2211	116 ± 11
D2_24d	2022-06-04	D2	24	Postlarvae	Artemia	8948 ± 2686	160 ± 26
D3_24d	2022-05-29	D3	24	Postlarvae	Chlorella+Artemia	27218 ± 6478	242 ± 52
D5_24d	2022-06-05	D5	24	Postlarvae	Chlorella+PelletFeed	15865 ± 12017	199 ± 47

Table 2 Sample information of the rearing water and feed, and the average number of high-quality sequences and number of ASVs used in the study.

Rearing Water							
Sample Name	**Sampling Date**	**Tank**	**Reared Days**	**Developmental Stage of Larval Shrimps**	**Feed Type**	**Average Number of High-Quality Sequences**	**Average Number of ASVs**
FL_D2_0d	2022-05-11	D2	0	Egg	None	11390	92
FL_D5_0d	2022-05-12	D5	0	Egg	None	8594	111
FL_D2_2d	2022-05-13	D2	2	Nauplius	None	12908	123
FL_D3_2d	2022-05-07	D3	2	Nauplius	None	4298	113
FL_D5_2d	2022-05-14	D5	2	Nauplius	None	13537	149
FL_D2_6d	2022-05-17	D2	6	Zoea	Chlorella	6558	137
FL_D3_6d	2022-05-11	D3	6	Zoea	Chlorella	3381	100
FL_D5_6d	2022-05-18	D5	6	Zoea	Chlorella	4435	120
FL_D2_9d	2022-05-20	D2	9	Zoea	Chlorella+Rotifer+Artemia	14241	134
FL_D3_9d	2022-05-14	D3	9	Mysis	Chlorella	4380	101
FL_D5_9d	2022-05-21	D5	9	Mysis	Artemia	5595	121
FL_D2_16d	2022-05-27	D2	16	Postlarvae	Chlorella+Artemia	1177	55
FL_D3_16d	2022-05-21	D3	16	Postlarvae	Chlorella+Artemia	2661	86
FL_D5_16d	2022-05-28	D5	16	Postlarvae	Chlorella+Artemia	2771	91
FL_D2_20d	2022-05-31	D2	20	Postlarvae	Chlorella+Artemia	5915	84
FL_D3_20d	2022-05-25	D3	20	Postlarvae	Chlorella+Artemia	5729	85
FL_D5_20d	2022-06-01	D5	20	Postlarvae	Artemia	7501	99
FL_D2_24d	2022-06-04	D2	24	Postlarvae	Artemia	11088	107
FL_D3_24d	2022-05-29	D3	24	Postlarvae	Chlorella+Artemia	5648	124
FL_D5_24d	2022-06-05	D5	24	Postlarvae	Chlorella+PelletFeed	5108	117
Feed							
ART	2022.05.23.			Artemia		5463 ± 4208	82 ± 31
CHL	2022.05.31			Chlorella		3081 ± 556	93 ± 10
ROT	2022.05.31			Rotifer		7308 ± 4320	119 ± 35
FD	2022.05.07			Pellet Feed		5024 ± 878	151 ± 16

3.1.2. Prokaryotic communities differentiated by groups of developmental stages

Non-metric multidimensional scaling (NMDS) analysis and ANOSIM analysis results for prokaryotic communities associated with larval shrimps (stress=0.088, R=0.8097, p=0.001) based on Bray-Curtis dissimilarities showed that the prokaryotic composition was best differentiated by the developmental stage group of larval shrimps (Figure 2). The developmental stage groups were 'egg and nauplius', 'zoea and mysis', and 'postlarvae'. In addition, the ANOSIM analyses showed that the composition of prokaryotic communities associated with larval shrimps at egg and nauplius stages was not differentiated at a statistically significant level. The community composition between the zoea and mysis stages was also not differentiated at a statistically significant level.

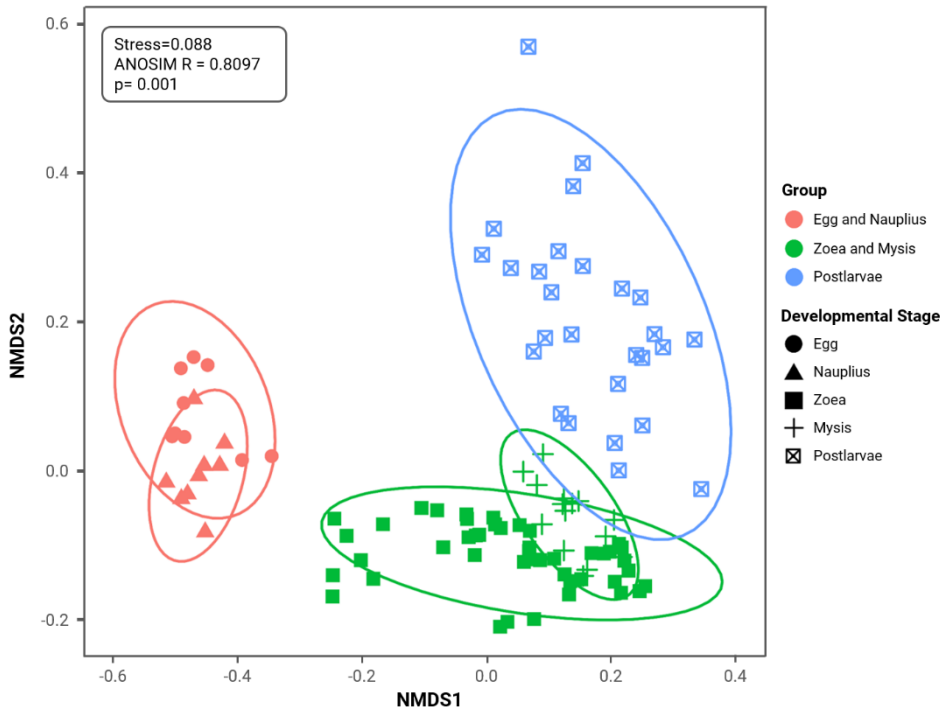

Figure 2 Non-metric multidimensional scaling (NMDS) analysis plot for prokaryotic communities associated with larval shrimps (stress = 0.088) based on Bray-Curtis dissimilarities based on the ASVs rarefied to an even depth (sample size = 2000). (R= 0.8097, p=0.001)

3.1.3. Alpha Diversity

Alpha diversity indices including Chao1, MNTD, Shannon, and Inverse Simpson were measured for the prokaryotic communities associated with larval shrimps and the rearing water (Table 3). Analyses showed that Chao1, MNTD, Shannon, and Inverse Simpson indices ranged from 85−473, 0.02−0.15, 3.09−4.93, and 4.71−65.76 for the larval shrimps respectively, and 55−149, 0.03−0.11, 2.59−4.21, and 4.39−48.06 for the rearing water.

Alpha diversity indices compared by each developmental stage group showed that species richness (Chao1) and phylogenetic diversity (MNTD) did not change significantly along larval development (Figure 3). However, species evenness (Shannon, Inverse Simpson) was significantly increased at the zoea and mysis stages ($p<0.001$ and $p<0.05$ respectively), compared to the earlier and later stages, indicating a higher level of biodiversity at the stage.

In addition, species richness (Chao1), phylogenetic diversity (MNTD) and species evenness (Shannon) were significantly higher in larval shrimps than in the rearing water community. Shannon indices were higher in larval shrimps than in the rearing water community, but Inverse Simpson indices were not significantly different.

Table 3 Summary of the diversity indices (Average±SD) of each sample.

Sample Name	Chao1	MNTD	Shannon	Inverse Simpson
D2_0d	168 ± 24	0.04 ± 0.01	3.59 ± 0.02	11.77 ± 0.93
D3_0d	295 ± 33	0.03 ± 0.00	4.29 ± 0.20	28.48 ± 6.63
D5_0d	151 ± 99	0.07 ± 0.03	4.14 ± 0.27	34.45 ± 2.52
D2_2d	141 ± 11	0.06 ± 0.01	3.81 ± 0.10	21.66 ± 2.88
D3_2d	143 ± 36	0.04 ± 0.01	3.66 ± 0.10	17.53 ± 1.19
D5_2d	144 ± 12	0.08 ± 0.02	3.64 ± 0.31	17.66 ± 6.74
D2_4d	229 ± 11	0.06 ± 0.01	4.46 ± 0.28	47.16 ± 18.46
D5_4d	183 ± 35	0.07 ± 0.01	3.9 ± 0.19	13.98 ± 4.31
D2_5d	157 ± 5	0.07 ± 0.01	4.2 ± 0.10	29.08 ± 6.63
D3_5d	178 ± 29	0.06 ± 0.01	4.13 ± 0.14	28.66 ± 11.75
D5_5d	173 ± 43	0.07 ± 0.01	4.31 ± 0.05	39.48 ± 8.43
D2_6d	207 ± 27	0.07 ± 0.01	4.62 ± 0.10	57.67 ± 7.20
D3_6d	164 ± 12	0.05 ± 0.00	4.15 ± 0.05	32.21 ± 2.63
D5_6d	142 ± 19	0.10 ± 0.00	4.27 ± 0.15	40.60 ± 6.92
D2_7d	140 ± 5	0.15 ± 0.01	3.25 ± 0.03	6.20 ± 0.12
D3_7d	187 ± 38	0.07 ± 0.02	4.49 ± 0.20	54.30 ± 10.96
D5_7d	199 ± 28	0.07 ± 0.01	4.42 ± 0.09	45.55 ± 8.53
D2_8d	246 ± 23	0.08 ± 0.01	4.27 ± 0.01	25.19 ± 1.17
D3_8d	142 ± 26	0.10 ± 0.02	4.27 ± 0.16	43.84 ± 7.50
D5_8d	146 ± 13	0.13 ± 0.02	3.64 ± 0.32	12.97 ± 8.22
D2_9d	259 ± 74	0.05 ± 0.01	4.23 ± 0.23	19.3 ± 3.99
D3_9d	189 ± 26	0.11 ± 0.00	4.07 ± 0.06	21.62 ± 5.46
D5_9d	273 ± 65	0.06 ± 0.00	4.58 ± 0.31	47.61 ± 13.28
D2_10d	250 ± 56	0.08 ± 0.01	4.71 ± 0.14	53.26 ± 2.13
D3_10d	165 ± 16	0.08 ± 0.01	4.31 ± 0.01	45.64 ± 2.69
D5_10d	181 ± 26	0.10 ± 0.01	3.88 ± 0.07	17.29 ± 1.00
D2_16d	295 ± 155	0.06 ± 0.03	3.77 ± 0.37	16.08 ± 5.62
D3_16d	239 ± 45	0.06 ± 0.01	4.44 ± 0.08	43.01 ± 1.00
D5_16d	138 ± 37	0.10 ± 0.02	3.69 ± 0.16	16.08 ± 2.21
D2_20d	280 ± 118	0.05 ± 0.01	3.65 ± 0.48	11.77 ± 6.10
D3_20d	196 ± 38	0.06 ± 0.01	4.24 ± 0.18	33.75 ± 6.30
D5_20d	116 ± 11	0.05 ± 0.01	3.27 ± 0.10	9.96 ± 1.29
D2_24d	160 ± 26	0.09 ± 0.01	3.94 ± 0.16	25.13 ± 4.36
D3_24d	242 ± 52	0.06 ± 0.01	3.45 ± 0.31	8.83 ± 2.06
D5_24d	199 ± 47	0.12 ± 0.02	3.54 ± 0.39	9.83 ± 4.76
FL_D2_0d	92	0.05	2.59	5.56
FL_D5_0d	111	0.04	3.82	25.18
FL_D2_2d	123	0.04	3.47	15.62
FL_D3_2d	113	0.08	4.21	48.06
FL_D5_2d	149	0.03	3.60	14.48
FL_D2_6d	137	0.06	4.12	32.57
FL_D3_6d	100	0.07	4.09	43.85
FL_D5_6d	120	0.07	4.08	31.28
FL_D2_9d	134	0.03	3.27	9.40
FL_D3_9d	101	0.06	3.87	24.22
FL_D5_9d	121	0.07	3.74	12.24
FL_D2_16d	55	0.10	3.69	32.34
FL_D3_16d	86	0.09	3.75	21.09
FL_D5_16d	91	0.09	3.92	31.17
FL_D2_20d	84	0.04	2.93	7.87
FL_D3_20d	85	0.04	3.07	8.77
FL_D5_20d	99	0.11	2.61	4.38
FL_D2_24d	107	0.03	2.86	7.42
FL_D3_24d	124	0.07	4.18	41.33
FL_D5_24d	117	0.07	3.94	22.85

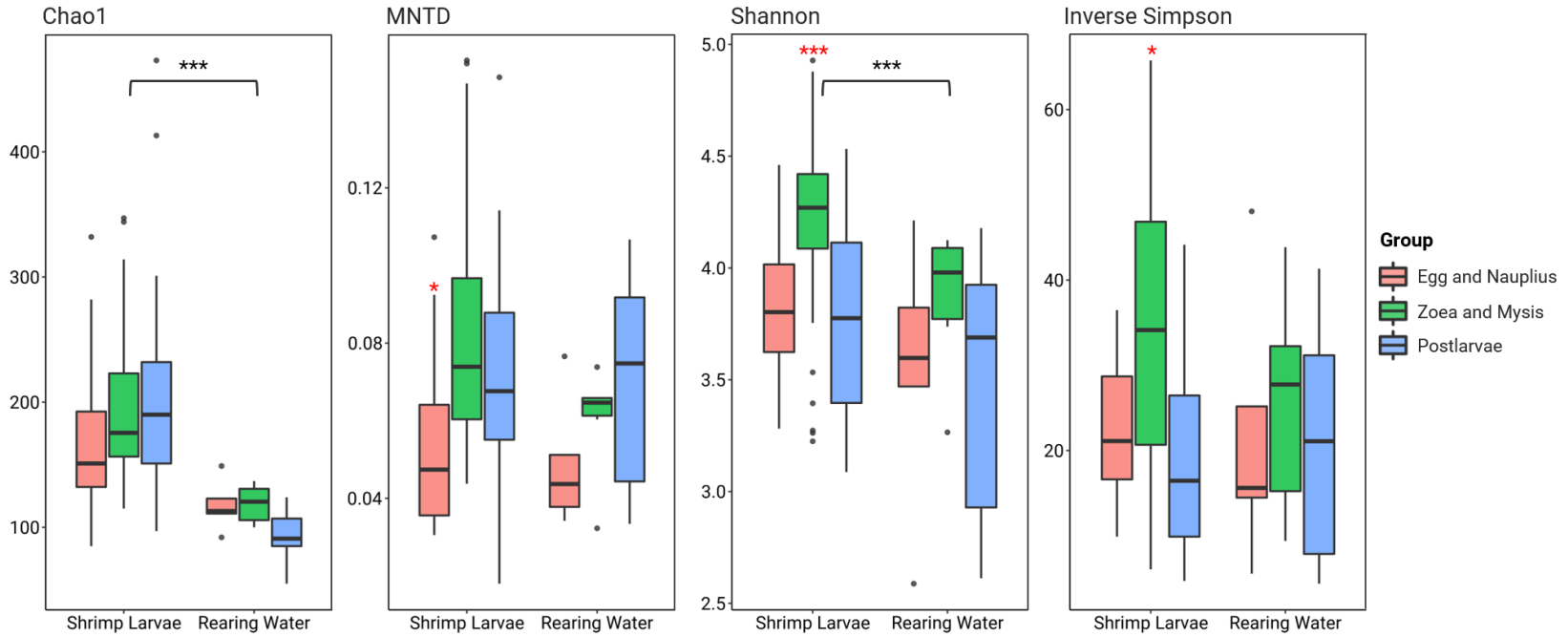

Figure 3 Alpha diversity indices (Chao1, MNTD, Shannon, and Inverse Simpson) of the prokaryotic communities associated with larval shrimps and the rearing water. Statistical significance was tested by pairwise Wilcoxon tests with p-values adjusted with the "Bonferroni" method in R (***p<0.001, *p<0.05, respectively).

3.1.4. Taxonomic Composition and Dynamics of Major Taxa from larval shrimps

The taxonomic composition analysis of the prokaryotic communities associated with larval shrimps agglomerated at the bacterial class level revealed that Gammaproteobacteria (31.46 ± 14.14 %), Bacteroidia (28.74 ± 8.38 %), Alphaproteobacteria (23.75 ± 10.66 %), Oligoflexia (6.7 ± 9.52 %), Verrucomicrobiae (2.75 ± 3.74 %), Polyangia (0.85 ± 1.48 %), Phycisphaerae (0.81 ± 0.79 %), Desulfuromonadia (0.73 ± 0.62 %), Fusobacteriia (0.47 ± 1.99 %), Planctomycetes (0.46 ± 0.64 %) were the major classes composing the prokaryotic communities (Figure 4).

The taxonomic composition of the prokaryotic communities associated with larval shrimps demonstrated that bacterial families: *Alteromonadaceae*, *Cellvibrionaceae*, *Pseudoalteromondaceae*, *Vibrionaceae* (Gammaproteobacteria), and an unknown family in the Gammaproteobacteria, *Rhodobacteraceae* (Alphaproteobacteria), *Crocinitomicaceae*, *Flavobacteriaceae* (Bacteroidota), *Oligoflexales* (Oligoflexia), and *Rubritaleaceae* (Verrucomicrobiae) were the major families composing the prokaryotic communities (Figure 4). The comparison of the relative abundance among each developmental stage group demonstrated that the relative abundance of *Rhodobacteraceae* and *Rubritalaceae* increased along development ($p<0.001$), while *Alteromondaceae*, *Cellvibrionaceae,* *Crocinitocaceae* and *Pseudoalteromonadaceae* showed a higher abundance at the egg and nauplius stage ($p<0.001$). The relative

abundance of *Flavobacteriaceae* was increased during the zoea and mysis stages and decreased afterward ($p<0.05$) (Figure 5).

Core ASVs that were present in more than 90 % of the larval shrimps were identified by BLAST searches against the EzBioCloud database (Table 4). The ASVs were mainly affiliated with *Flavobacteriaceae* and *Rhodobacteraceae*. Moreover, the relative abundance of some core ASVs showed a change of relative abundance along host development. Notably, ASV 1019 (*Alteromonas* sp.), ASV 856 (*Aestuariicella* sp.) and ASV 1583 (*Salinirepens* sp.) showed higher relative abundances at egg and nauplius stages, while ASV 965(*Pseudobacteriovorax* sp.) were enriched at zoea and mysis stages. ASV 331 (*Rubritalea* sp.) was enriched at the postlarval stage. LefSe results further indicated that certain taxa were distinctively appearing at each developmental stage group, noticeably *Aestuariicella*, *Aquimarina*, *Winogradskyella* at the egg and nauplius stages, *Oligoflexia*, *Pseudobacteriovorax*, *Marinicella*, *Tenacibaculum*, *Maribacter*, *Sulfitobacter*, *Jannaschia*, *Cryomorpha* at zoea and mysis stages, and *Rubritalea*, *Arenibacter* at postlarval stages (Figure 7).

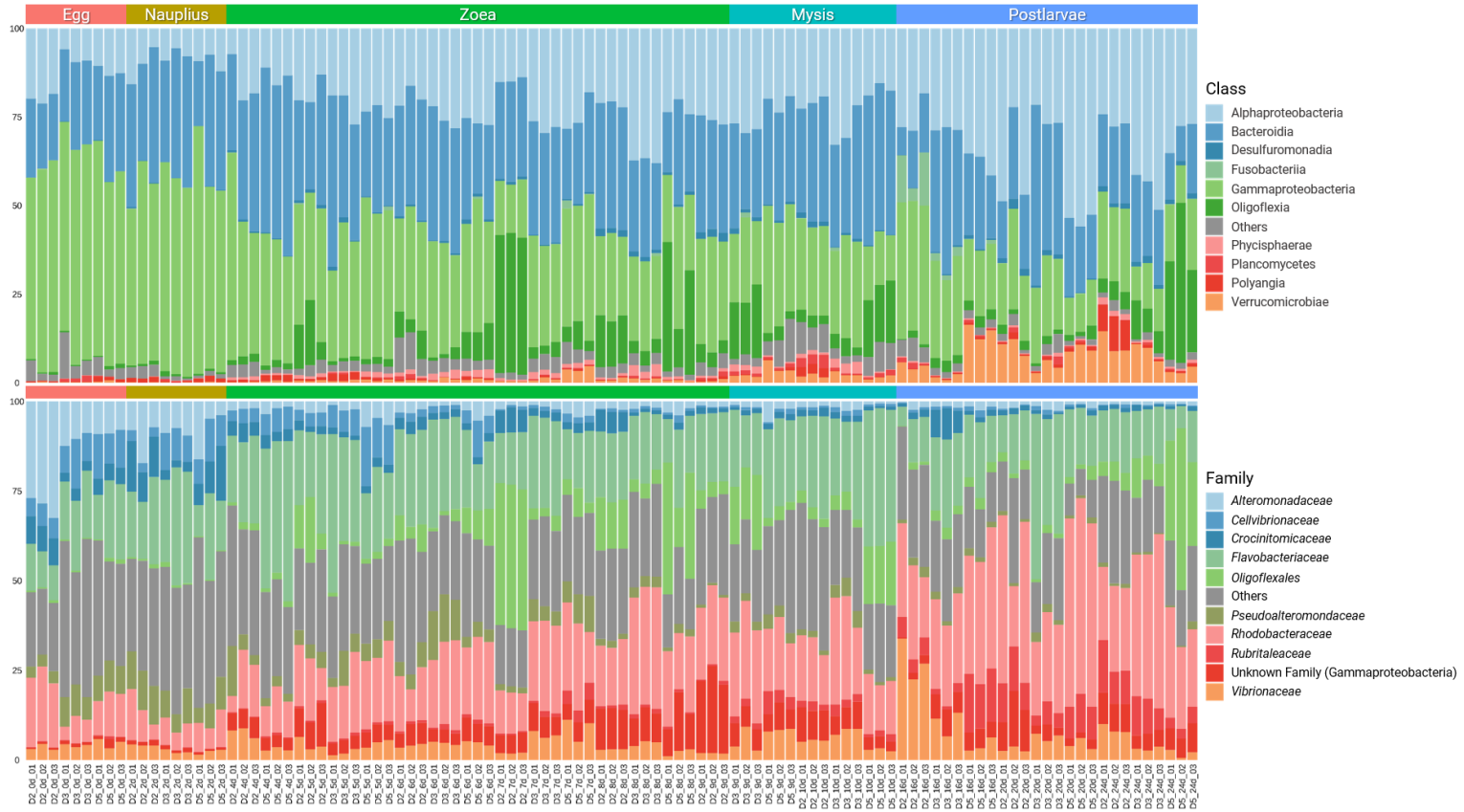

Figure 4 Taxonomic composition of the prokaryotic communities associated with larval shrimps grouped in each developmental stage, at the class (top) and family (bottom) level. Ten taxa with the highest abundances are plotted and the other taxa are grouped as "Others".

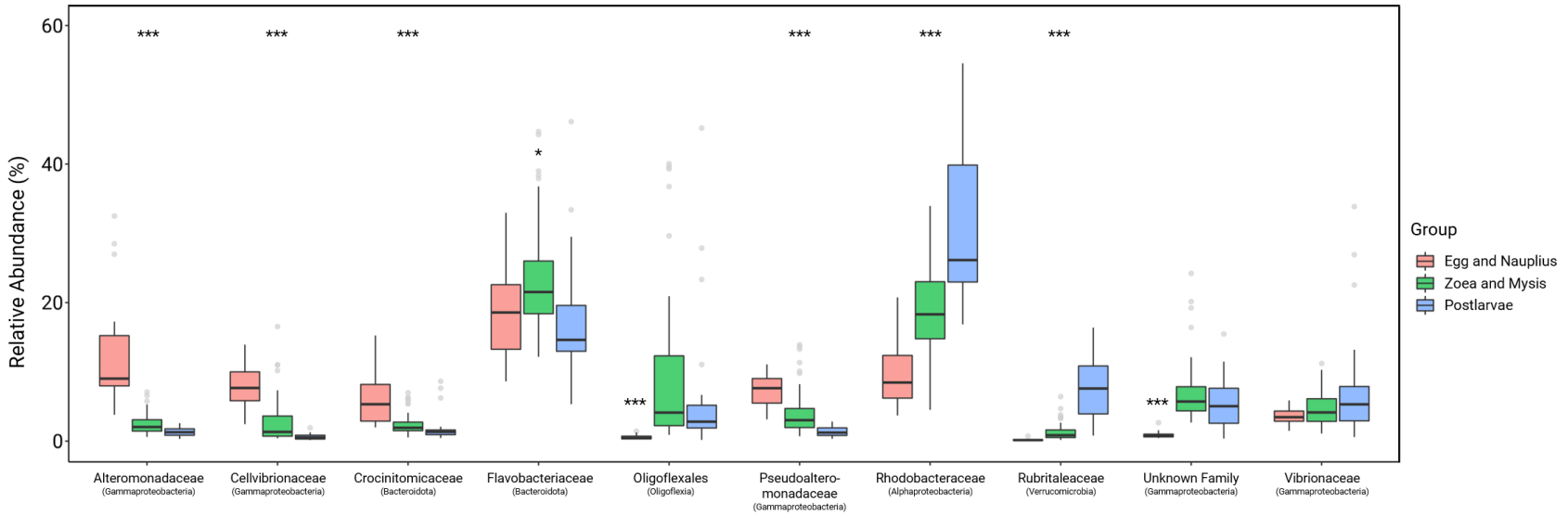

Figure 5 Comparison of the relative abundance of major bacterial families in larval shrimps grouped by developmental stages. (***p<0.001, **p<0.01, *p<0.05)

Table 4 Core bacterial ASVs present in at least 90% of larval shrimp samples. Results were obtained by running BLAST searches against the latest updated version of the EzBioCloud database (July 2021).

ASVs	Closest BLAST matches*	Average Relative Abundance at Each Developmental Stage (%)			Presence among larval shrimp samples (%)	Accession Number	Sequence Similarity (%)
		Egg and Nauplius	Zoea and Mysis	Postlarvae			
ASV628	*Marinicella sediminis*	1.73 ± 1.07	14.36 ± 8.16	13.81 ± 7.56	100	MVBD01000022	100
ASV1019	*Alteromonas macleodii*	27.89 ± 15.0	3.39 ±2.92	3.26 ± 2.45	100	CP003841	100
ASV1385	*Planktosalinus lacus*	0.73 ± 0.52	7.32 ± 3.12	6.25 ± 4.59	100	KJ782427	98.82
ASV1583	*Salinirepens amamiensis*	13.4 ± 10.49	1.84 ± 2.06	1.13 ± 1.06	100	AB517714	96.06
ASV696	*Pseudoalteromonas espejiana*	9.64 ± 5.04	6.49 ± 5.27	2.31 ± 1.56	99.05	CP011028	100
ASV1351	*Aquimarina macrocephali*	9.30 ± 10.06	6.05 ± 11.37	1.23 ± 1.07	99.05	JACA01000084	98.43
ASV1431	*Tenacibaculum mesophilum*	2.24 ± 1.50	7.86 ± 5.65	4.03 ± 3.09	99.05	jgi.1107970	100
ASV2529	*Sulfitobacter geojensis*	1.05 ± 0.84	5.95 ± 2.85	5.38 ± 2.36	99.05	JASE01000005	100
ASV856	*Aestuariicella hydrocarbonica*	21.64 ± 12.44	3.52 ± 4.13	1.54 ± 0.99	98.10	KF982858	99.61
ASV1468	*Winogradskyella haliclonae*	0.51 ± 0.43	3.47 ± 2.24	7.21 ± 6.32	98.10	KX640900	98.43
ASV2016	*Pseudobacteriovorax antillogorgiicola*	1.31 ± 1.11	16.16 ± 16.24	14.42 ± 16.58	98.10	FWZT01000055	98.03
ASV1421	*Tenacibaculum litopenaei*	0.97 ± 1.22	2.36 ± 2.45	0.54 ± 0.42	96.19	DQ822567	98.82
ASV331	*Rubritalea marina*	0.47 ± 0.34	2.14 ± 2.73	22.83 ± 14.13	95.24	DQ302104	96.06
ASV1798	*Phaeodactylibacter luteus*	0.24 ± 0.29	1.77 ± 1.68	0.73 ± 0.44	95.24	KM235292	92.52
ASV2076	*Haliangium ochraceum*	1.16 ± 0.90	0.74 ± 1.25	0.27 ± 0.19	95.24	CP001804	93.31
ASV1740	*Lishizhenia tianjinensis*	0.1 ± 0.10	1.72 ± 0.96	2.02 ± 1.90	94.29	jgi.1076210	88.98
ASV965	*Pseudidiomarina planktonica*	0.50 ± 0.54	2.32 ± 1.82	0.83 ± 0.77	93.33	FXWH01000005	98.82
ASV2451	*Jannaschia cystaugens*	0.22 ± 0.24	3.44 ± 1.88	2.41 ± 1.15	93.33	CYRX01000003	100
ASV722	*Vibrio pomeroyi*	3.08 ± 1.66	4.3 ± 2.67	0.73 ± 0.72	92.38	AJ491290	100
ASV2513	*Sagittula stellata*	0.48 ± 0.53	2.89 ± 1.44	2.66 ± 1.35	92.38	AAYA01000003	100
ASV1492	*Winogradskyella poriferorum*	2.99 ± 1.68	0.85 ± 0.85	3.29 ± 4.84	91.43	AY848823	99.61
ASV1524	*Arenibacter troitsensis*	0.34 ± 0.35	1.07 ± 1.07	3.13 ± 1.82	91.43	jgi.1048893	100

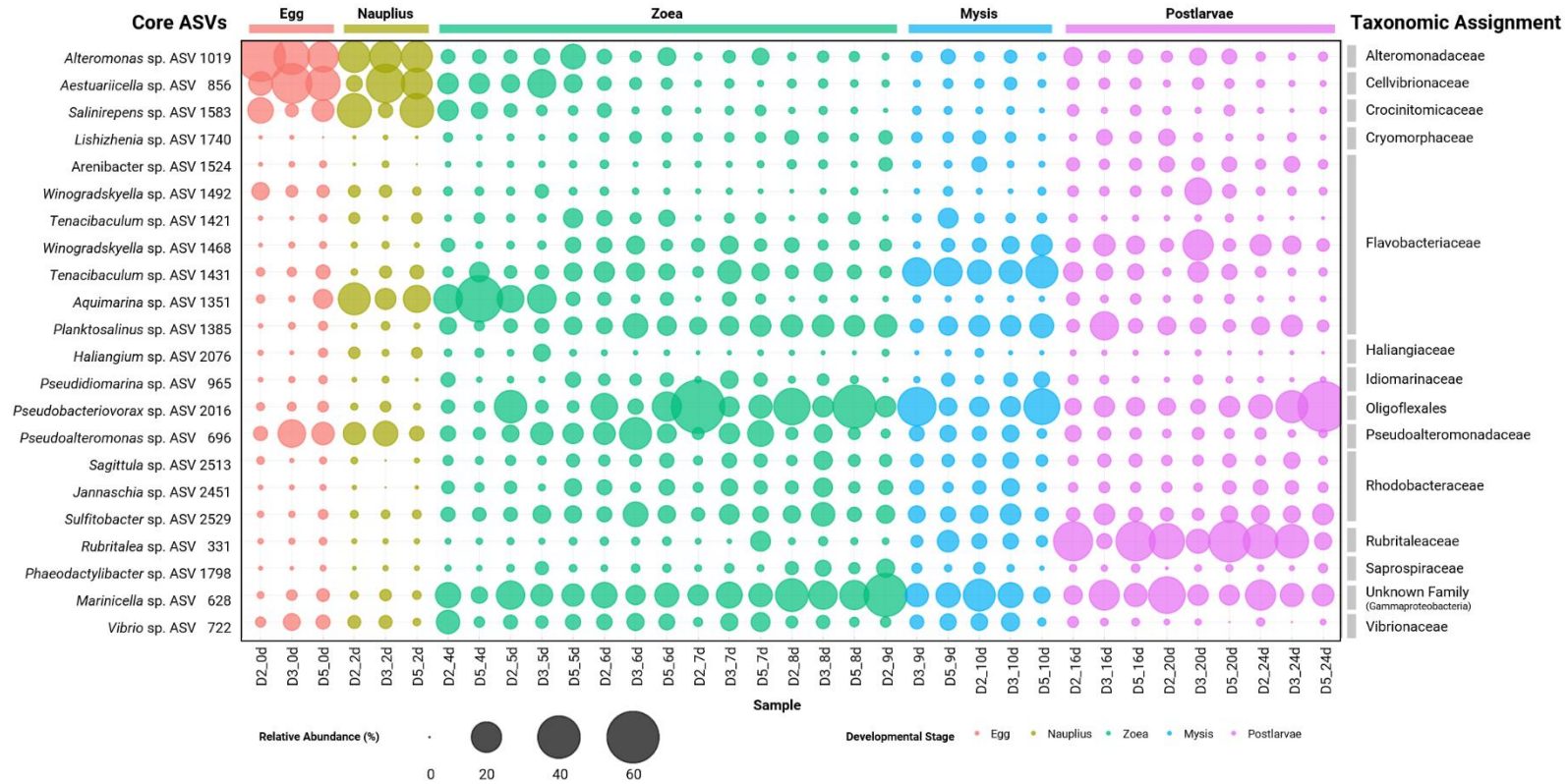

Figure 6 Abundance dynamics of core bacterial ASVs present in at least 90% of larval shrimp samples (n=3) and the corresponding taxonomic assignment at the family level. The size of the bubble represents the relative abundance (%) of each ASV and the color scale represents the developmental stage of the larval shrimp at each sampling period.

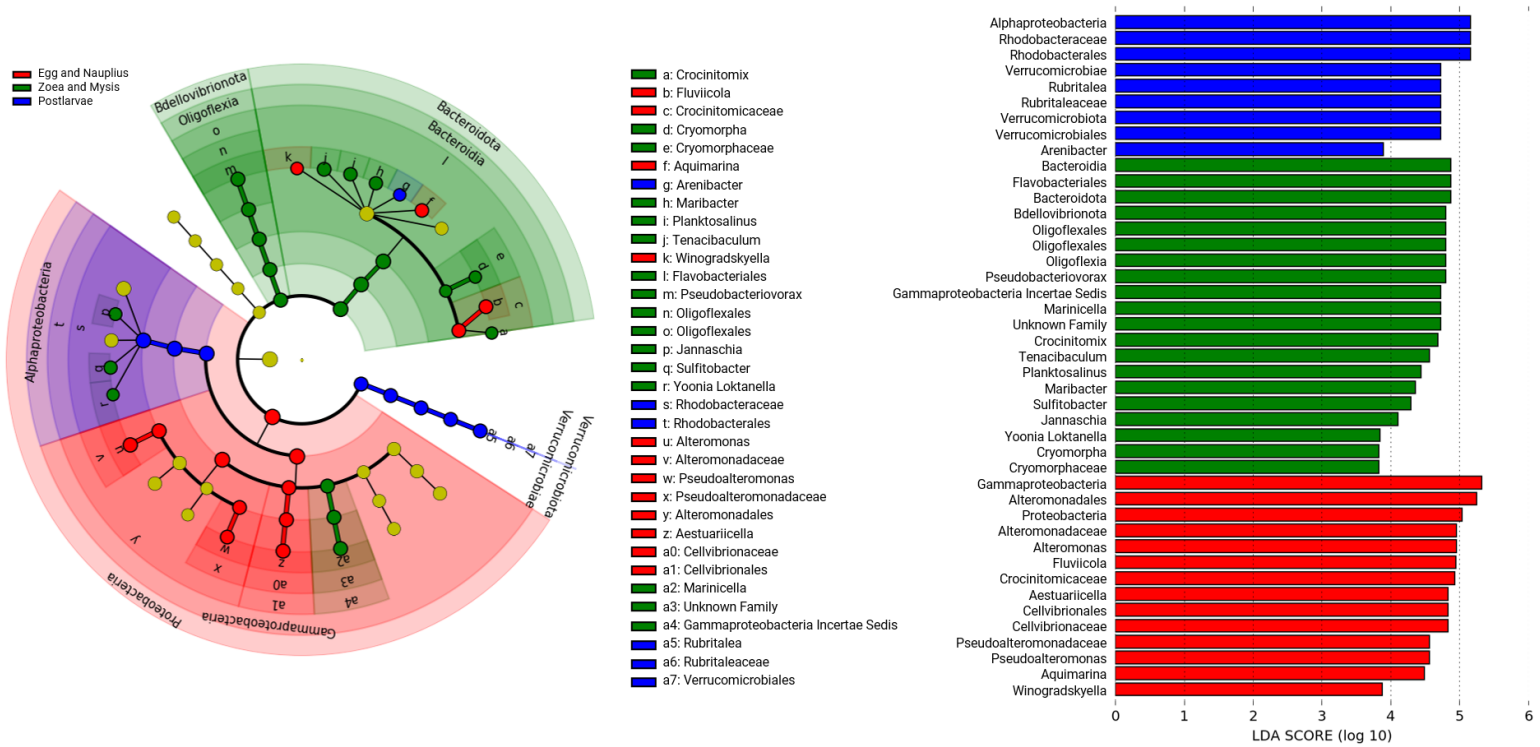

Figure 7 LefSe (Linear discriminant analysis effect size) analysis results. Cladogram (left) and LefSe analysis scores of each taxon grouped by developmental stage groups (right) (LDA score threshold= 2.0).

3.1.5. Prokaryotic Communities from the Rearing Water and Feed

The taxonomic composition of the prokaryotic communities associated with the rearing water demonstrated high dominance of Proteobacteria. The major bacterial families composing the prokaryotic communities were *Colwelliaceae*, *Idiomarinaceae*, *Pseudoalteromondaceae*, *Thiothrichaceae*, *Vibrionaceae* (Gammaproteobacteria), *Rhodobacteraceae* (Alphaproteobacteria), *Crocinitomicaceae*, *Flavobacteriaceae* (Bacteroidota), *Microbacteriaceae* (Actinomycetia) and *Rubritaleaceae* (Verrucomicrobiae) (Figure 8).

The prokaryotic communities associated with four types of feed: *Artemia*, *Chlorella*, *Pellet Feed*, and *Rotifer* were investigated. Notably, the major bacterial families composing the prokaryotic communities were *Halomonadaceae*, *Idiomarinaceae*, *Pseudoalteromondaceae*, *Vibrionaceae* (Gammaproteobacteria), an unknown family in the Gammaproteobacteria, *Bacillus* (Bacilli), *Oligoflexales* (Oligoflexia), *Flavobacteriaceae* (Bacteroidota), and *Saprospiraceae* (Saprospiria) (Figure 9).

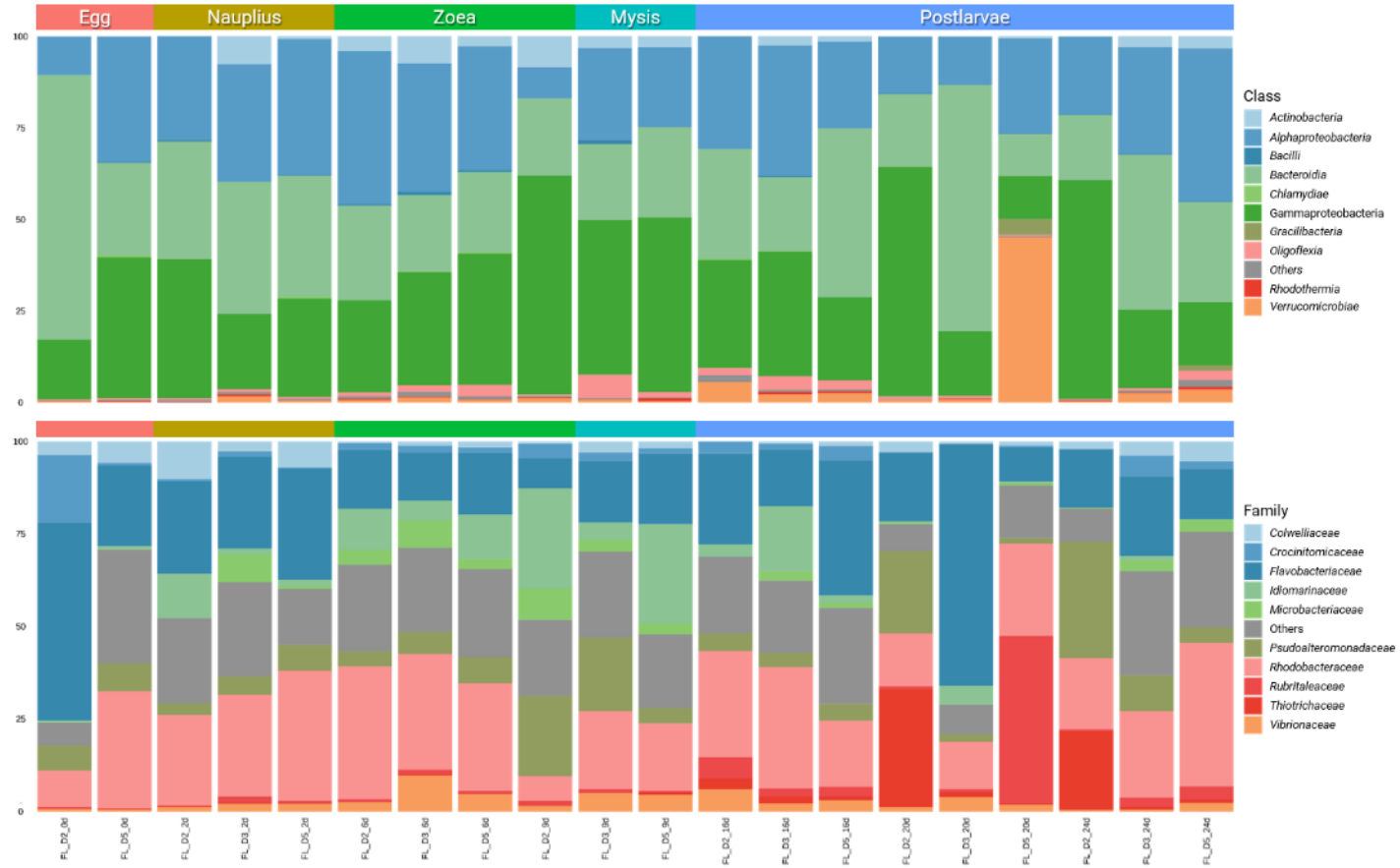

Figure 8 Taxonomic composition of the prokaryotic communities associated with the rearing water grouped in each developmental stage of the larval shrimp, at the class (top) and family (bottom) level. Ten taxa with the highest abundances are plotted and the other taxa are grouped as "Others".

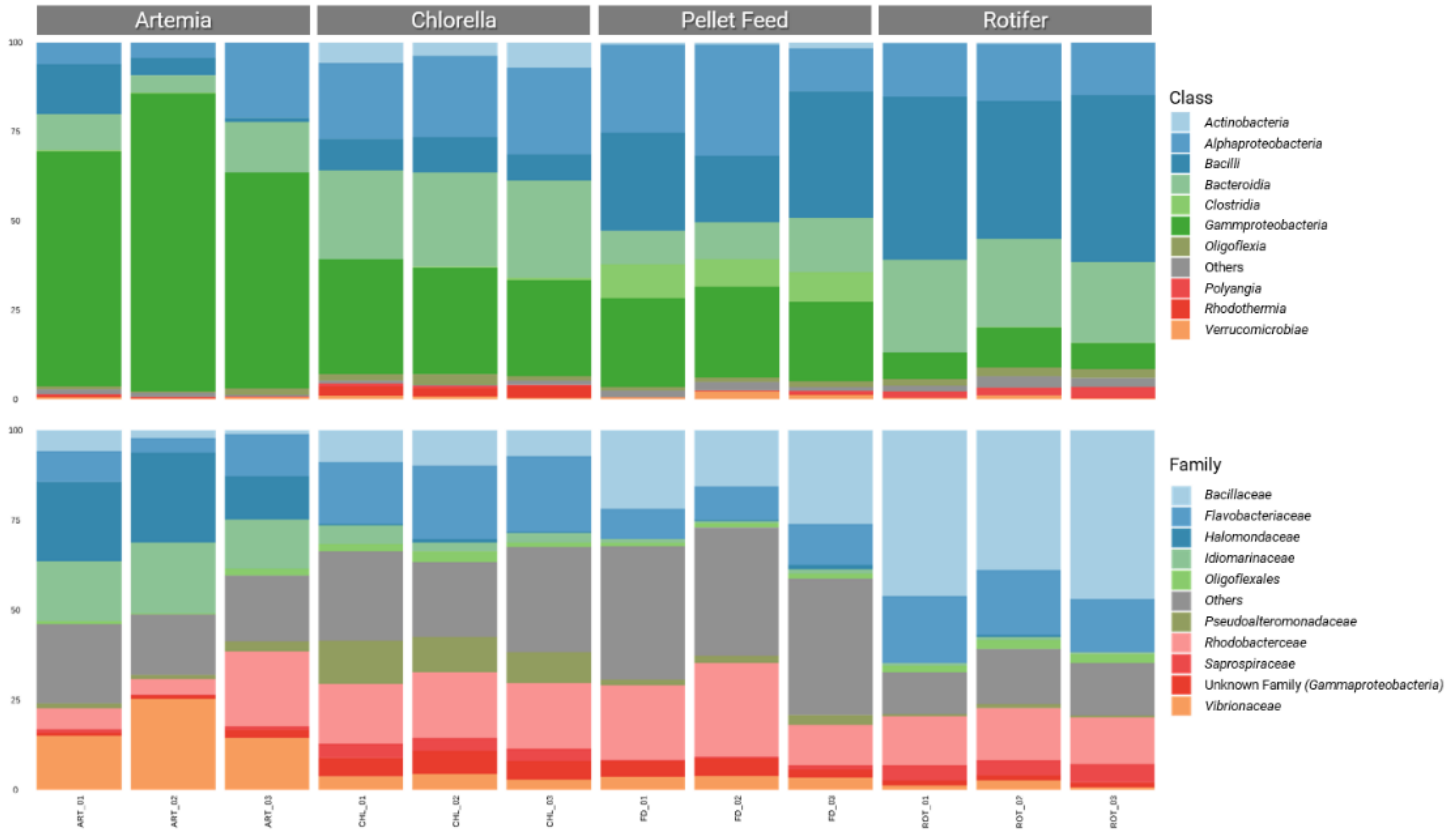

Figure 9 Taxonomic composition of the prokaryotic communities associated with feed, at the class (top) and family (bottom) level. Ten taxa with the highest abundances are plotted and the other taxa are grouped as "Others".

3.1.6. Functional Inference

KEGG pathways (level 3) inferred from the prokaryotic community composition by PICRUSt2 showed that the major metabolic pathways were related to the metabolism of amino acids (10.66 ± 0.31 %), membrane transport (10.03 ± 0.91 %), metabolism of carbohydrates (8.96 ± 0.25 %), replication and repair (7.96 ± 0.24 %), and energy metabolism (5.63 ± 0.16 %) among all larval shrimp samples (Table 4). Moreover, the relative abundance of KEGG pathways showed a shifting pattern along the development of larval shrimps (Figure 10). NMDS and ANOSIM analyses indicated a shift of the KEGG pathway abundance (stress=0.14, R=0.4595, p=0.001), indicating a shift of the function of the prokaryotic community associated with the larval shrimps across larval development.

The KEGG pathways that were significantly enriched in certain developmental stage groups were further assessed by LefSe analyses. The relative abundance of KEGG pathways related to cell motility and signal transduction significantly decreased following larval growth, while pathways related to the metabolism of amino acids and carbohydrates, along with the biosynthesis and metabolism of secondary metabolites showed an increase following larval growth. The relative abundance of the pathway related to the biosynthesis and metabolism of glycan was significantly higher at the zoea and mysis stages.

Table 5 Relative abundance (%) of KEGG Pathways (Level 3) of prokaryotic communities from larval shrimps and each developmental stage group.

KEGG Pathways (Level 3)	Average Relative Abundance (%)		
	Egg and Nauplius	Zoea and Mysis	Postlarvae
Amino Acid Metabolism	10.43 ± 0.23	10.71 ± 0.22	10.7 ± 0.45
Biosynthesis of Other Secondary Metabolites	0.78 ± 0.03	0.83 ± 0.03	0.84 ± 0.06
Cancers	0.23 ± 0.02	0.28 ± 0.02	0.31 ± 0.03
Carbohydrate Metabolism	8.66 ± 0.19	8.94 ± 0.12	9.23 ± 0.22
Cardiovascular Diseases	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
Cell Growth and Death	0.59 ± 0.02	0.66 ± 0.02	0.68 ± 0.05
Cell Motility	3.80 ± 0.31	3.62 ± 0.41	3.42 ± 0.54
Cellular Processes and Signaling	4.34 ± 0.16	3.93 ± 0.19	3.80 ± 0.29
Circulatory System	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.01
Digestive System	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
Endocrine System	0.34 ± 0.02	0.38 ± 0.04	0.36 ± 0.05
Energy Metabolism	5.37 ± 0.07	5.68 ± 0.11	5.67 ± 0.15
Environmental Adaptation	0.20 ± 0.01	0.19 ± 0.02	0.18 ± 0.03
Enzyme Families	2.08 ± 0.08	2.05 ± 0.05	1.94 ± 0.10
Excretory System	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
Folding, Sorting and Degradation	2.90 ± 0.11	2.85 ± 0.09	2.69 ± 0.14
Genetic Information Processing	2.83 ± 0.07	2.73 ± 0.08	2.65 ± 0.10
Glycan Biosynthesis and Metabolism	2.09 ± 0.09	2.25 ± 0.12	2.15 ± 0.14
Immune System	0.06 ± 0.00	0.06 ± 0.01	0.06 ± 0.01
Immune System Diseases	0.06 ± 0.00	0.05 ± 0.01	0.05 ± 0.01
Infectious Diseases	0.64 ± 0.02	0.62 ± 0.04	0.64 ± 0.09
Lipid Metabolism	3.53 ± 0.16	3.60 ± 0.12	3.67 ± 0.16
Membrane Transport	9.55 ± 0.64	9.75 ± 0.61	10.96 ± 0.97
Metabolic Diseases	0.09 ± 0.00	0.10 ± 0.01	0.09 ± 0.01
Metabolism	2.42 ± 0.05	2.25 ± 0.08	2.15 ± 0.06
Metabolism of Cofactors and Vitamins	4.17 ± 0.06	4.28 ± 0.08	4.26 ± 0.09
Metabolism of Other Amino Acids	1.92 ± 0.04	1.91 ± 0.06	1.89 ± 0.11
Metabolism of Terpenoids and Polyketides	1.59 ± 0.05	1.61 ± 0.04	1.61 ± 0.08
Nervous System	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
Neurodegenerative Diseases	0.51 ± 0.04	0.54 ± 0.06	0.61 ± 0.07
Nucleotide Metabolism	3.69 ± 0.05	3.77 ± 0.08	3.76 ± 0.11
Poorly Characterized	6.03 ± 0.14	5.51 ± 0.25	5.23 ± 0.20
Replication and Repair	8.00 ± 0.20	8.02 ± 0.20	7.79 ± 0.28
Sensory System	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Signal Transduction	2.39 ± 0.09	2.29 ± 0.17	2.18 ± 0.25
Signaling Molecules and Interaction	0.17 ± 0.01	0.18 ± 0.01	0.17 ± 0.03
Transcription	2.50 ± 0.04	2.34 ± 0.12	2.32 ± 0.12
Translation	5.10 ± 0.15	5.17 ± 0.15	4.98 ± 0.22
Transport and Catabolism	0.33 ± 0.02	0.35 ± 0.01	0.36 ± 0.02
Xenobiotics Biodegradation and Metabolism	2.31 ± 0.23	2.22 ± 0.11	2.28 ± 0.18

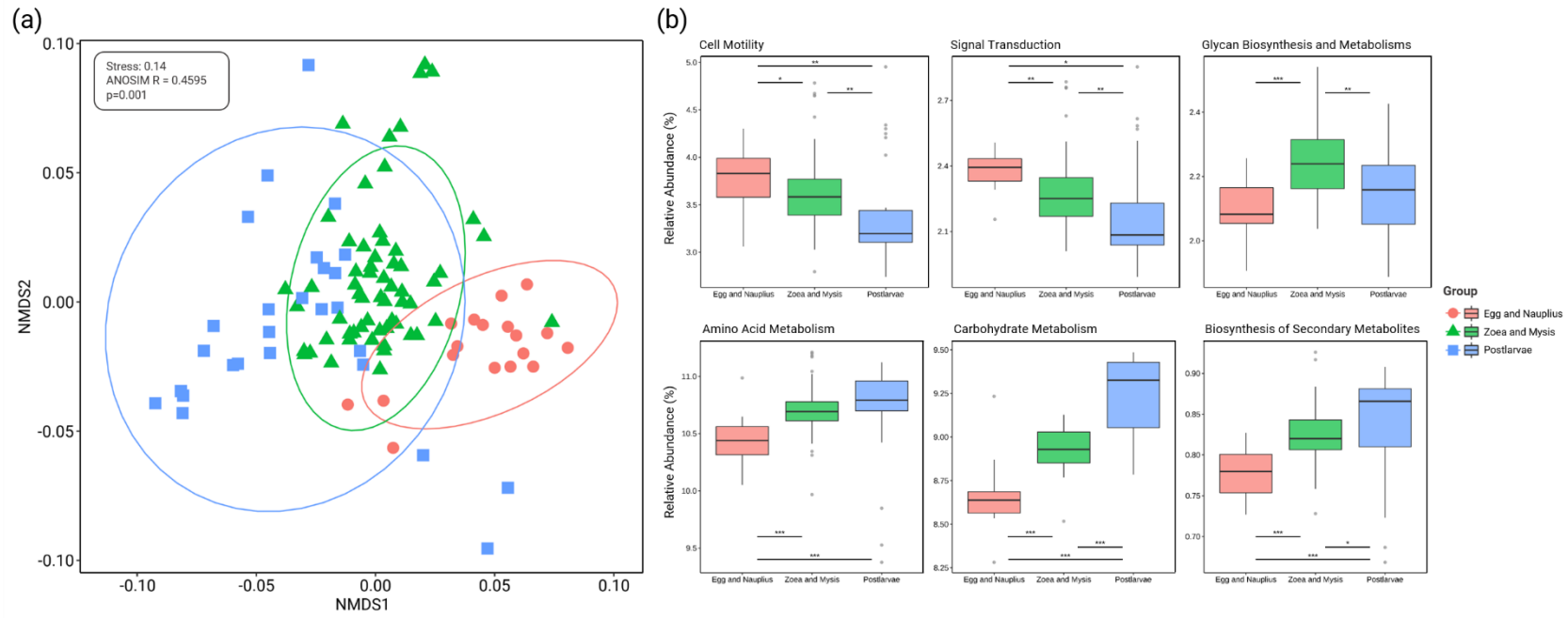

Figure 10 Non-metric multidimensional scaling (NMDS) plot of the relative abundance of the KEGG functional pathways (level 3) predicted from larval shrimp-associated prokaryotic communities by PICRUSt2 (a) and relative abundance (%) of functional pathways differing by developmental stage groups (b). Pathways with LDA score > 2.0 compared against each developmental stage group are shown. Statistical significance was tested by pairwise Wilcoxon tests with p-values adjusted with the "Bonferroni" method in R (***p<0.001, **p<0.01, *p<0.05, respectively).

3.2. Correlation between larval shrimps and influencing factors

3.2.1. PCoA Analysis of all samples

PCoA Analysis based on Bray-Curtis dissimilarities and among all samples showed a clear succession pattern along larval development (Figure 11). Pronounced clustering of microbial communities was observed from each developmental stage group, as demonstrated above (Figure 2). Prokaryotic communities associated with larval shrimps were distinguished from those from the rearing water and fed at egg and nauplius and postlarval stages. Association with feed was also observed, as the feed was introduced at specific stages of development.

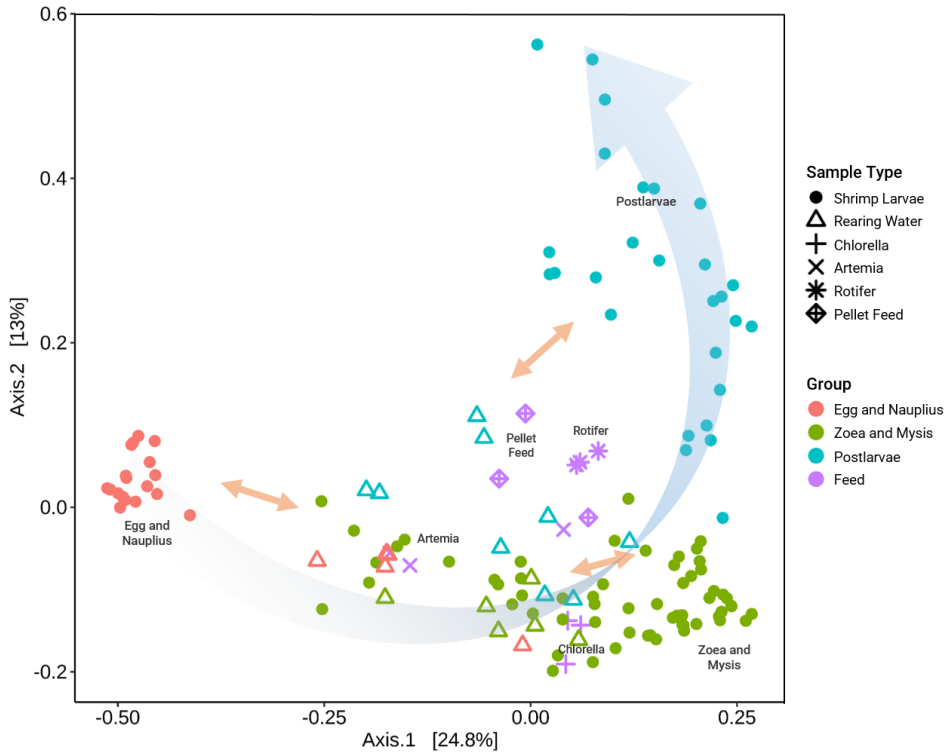

Figure 11 Principal Coordinated Analysis (PCoA) results for prokaryotic communities associated with all samples, including larval shrimps, rearing water, and feed, based on Bray–Curtis dissimilarities.

3.2.2. Succession patterns and assembly mechanism of larvae-associated prokaryotic communities

The succession of prokaryotic communities associated with larval shrimps was assessed with time decay analyses. The slopes of linear regression lines of βMNTD (0.0001, R^2=0.0783, p<0.001) and BC (0.0018, R^2=0.4276, p<0.001) calculated between different sampling days were both positive (Figure 12a and Figure 12b). This result indicates a sign of turnover of the microbial community along host development both in terms of phylogeny and taxonomy.

βNTI calculated between different sampling days showed that 70.51% of the βNTI values were higher than −2 and lower than 2, indicating the dominance of stochastic processes in community assembly (Figure 12c). Among the βNTI values representing stochastic assembly, 60.70 % of the RC_{bray} values were higher than 0.95, representing a dominance of dispersal limitation (Figure 12d). Overall, these results show that larval shrimps harbor a high taxonomic turnover and relatively intermediate levels of phylogenetic turnover of prokaryotic community composition along the development.

Stage-specific assembly mechanisms for larvae-associated prokaryotic communities were further identified using βNTI and RC_{bray} values calculated between samples within the same developmental stage groups. The median βNTI values were −2.58, −1.63, and −1.43 at the 'egg and nauplius', 'zoea and mysis', and 'postlarvae' stages, respectively (Figure 13a). Although not

statistically significant, the βNTI values were generally lower at the egg and nauplius stages. Accordingly, deterministic processes had a higher contribution at egg and nauplius stages than later stages (Figure 13b). Notably, the relative contribution of homogeneous selection was 71.11 % and decreased afterward, indicating a weakened effect of selection along host development. Meanwhile, the relative contribution of stochastic processes increased from 28.89 %, 67.39 %, and 70.37 %.

Neutral model fitting results further demonstrated the contribution of stochastic processes in larvae-associated prokaryotic communities. Generalized R^2 metrics from the Sloan neutral model fitting had a positive value throughout larval development, indicating that the prokaryotic ASVs associated with the larval shrimps were generally assembled by neutral (i.e. stochastic) processes rather than deterministic processes such as the selection or omission by the host larval shrimps (Figure 14a). However, neutral model fitting results further showed the varying contribution of ecological processes during each developmental stage group. Notably, the R^2 metrics were increased at the zoea and mysis stages and decreased afterward (Figure 14b). In addition, the estimated migration rate (m) decreased at the zoea and mysis stages, indicating a limitation of dispersal. This result aligns with the previously observed pattern of the increased relative contribution of dispersal limitation (Figure 13b).

Overall, these results demonstrate the importance of stochastic

processes in the assembly of the prokaryotic communities associated with larval shrimps. Moreover, the relative contribution of these ecological processes differed by the developmental stage groups, as primarily high influences of selection was decreased and influences of stochastic processes were increased at the zoea and mysis stages.

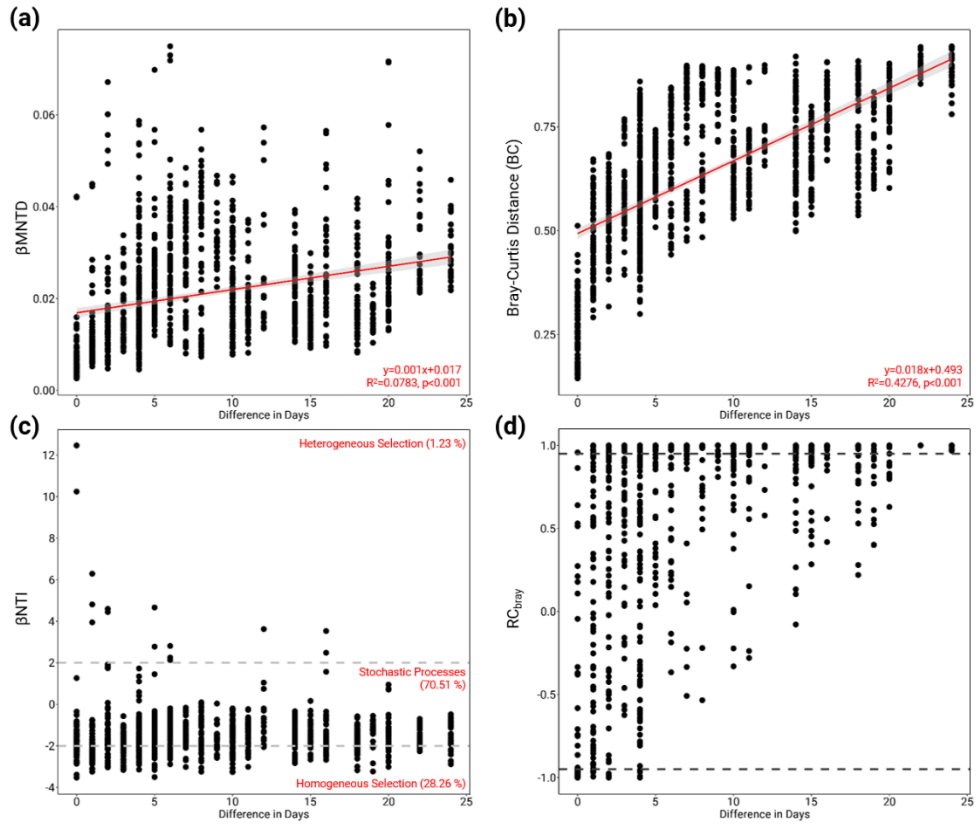

Figure 12 Pairwise distance calculation results of prokaryotic communities associated with larval shrimps from different sampling days. (a) Beta mean nearest taxon distance (βMNTD), (b) Bray–Curtis distance (BC), (c) beta mean nearest taxon index (βNTI), and (d) Raup–Crick indices based on Bray–Curtis distances (RC_{bray}) were used as dissimilarity metrics.

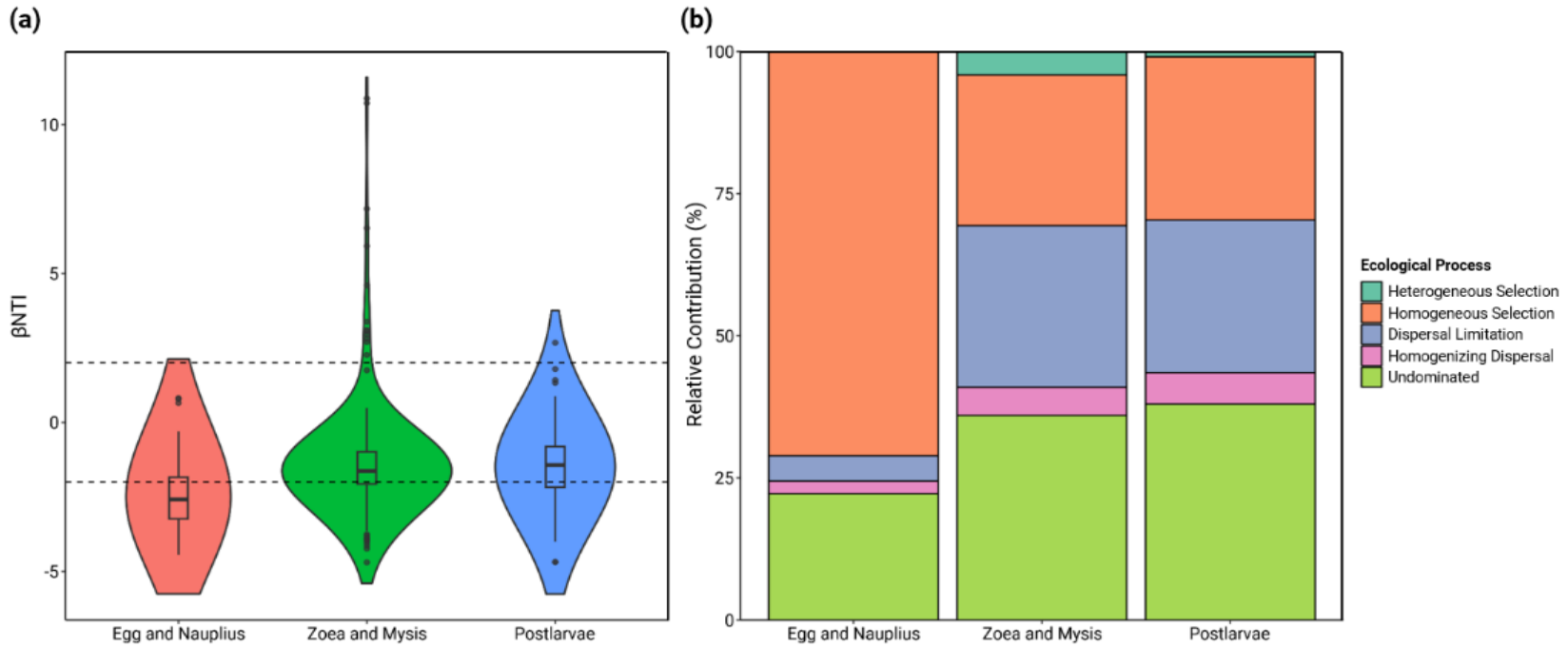

Figure 13 (a) βNTI values and (b) the relative contribution of ecological processes within each developmental group.

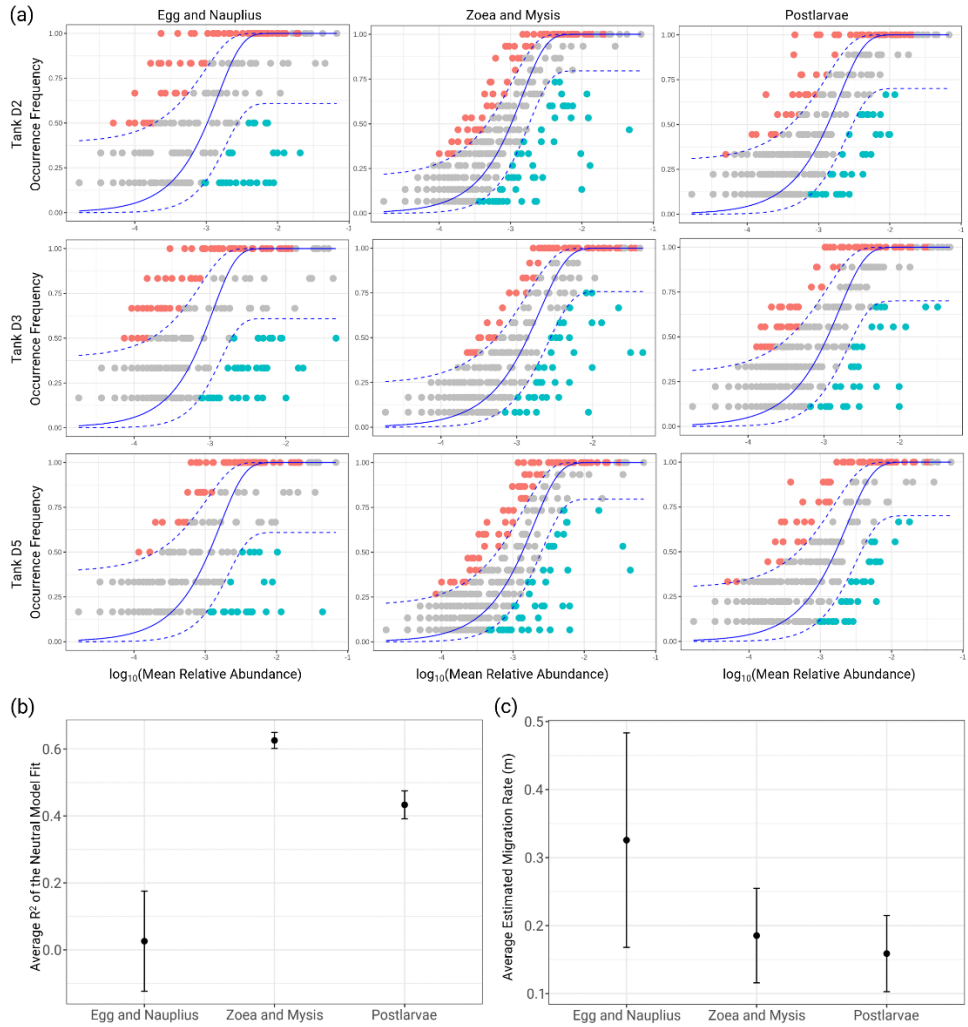

Figure 14 (a) Neutral model fitting results for larval bacterial communities by each developmental stage group and rearing tank. The ASVs that had frequencies above the predicted frequencies are colored in red, while those with frequencies below the prediction are shown in blue. ASVs considered neutrally distributed are colored in grey. Blue dashed lines represent 95% confidence intervals around the model prediction. (b) R^2 metrics of the neutral model fit and (c) Estimated Migration Rate (m) averaged by model fitting results from each tank at each developmental stage group.

3.2.3. Influence of the Rearing Water and Feed on the larvae-associated communities

Phylogenetic and taxonomic distances calculated between larval shrimps and the rearing water indicated a stage-specific dynamic of the larvae-associated communities (Figure 15). While no significant differences were detected among the phylogenetic distances, Bray-Curtis distance comparisons further revealed the changing community dissimilarity between the prokaryotic community in larval shrimps and postlarvae previously observed from the PCoA (Figure 11). Bray-Curtis distances between larval communities and the rearing water were significantly higher at the egg and nauplius stages ($p<0.001$) and decreased afterward.

Community distances compared between larval shrimps and feed showed differing patterns by feed type and developmental stage groups (Figure 16). Notably, prokaryotic communities associated with *Chlorella* were both phylogenetically and taxonomically significantly less distant to larvae-associated prokaryotes at the zoea and mysis stages ($p<0.001$ for both metrics). Other feeds such as *Artemia*, pellet feed, and rotifers generally showed higher similarities to larvae-associated shrimps at the zoea and mysis stages.

The estimated relative contribution of the ecological processes between the prokaryotic communities in the rearing water and those in the larvae showed that stochastic processes accounted for 60.00 %, 50.00 %, and 62.96 % of the total ecological processes at each

developmental stage group (Figure 17). The estimated relative contribution of the ecological processes between the prokaryotic communities in the feed and those in the larvae showed that stochastic processes account for 77.78 %, 85.12%, and 87.35 % of the total ecological processes at each developmental stage group.

Source tracking analysis results by SourceTracker2 demonstrated the relative microbial contribution of the rearing water and feed to the larval shrimps (Figure 16). The total relative contribution of both rearing water and the feed was highly increased at the zoea and mysis stages (61.39 %), compared to the previous (3.39 %) and latter developmental stages (28.27 %). The contribution of the rearing water and feed along development both reached the maximum at the zoea and mysis stage (38.30 % and 23.09 %, respectively) and declined afterward.

Overall, these results indicate stage-specific changes of influences of both the rearing water and feed-borne bacteria, especially showing higher influence starting from the zoea and mysis stages.

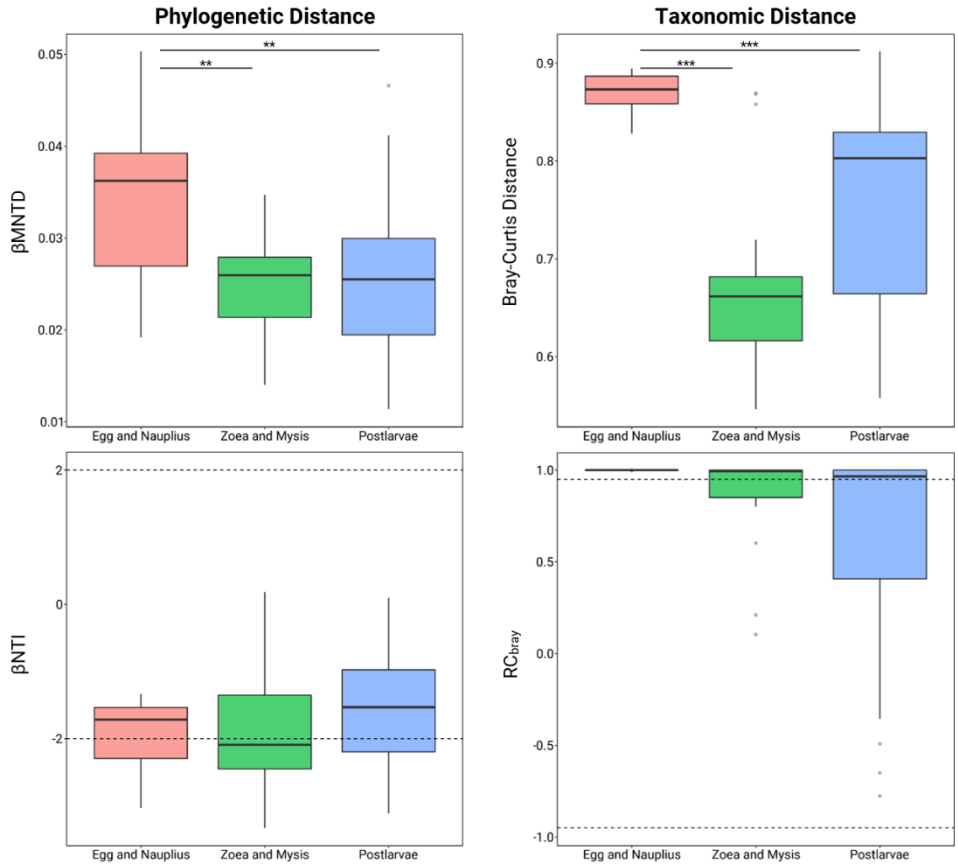

Figure 15 Box plots showing phylogenetic and taxonomic distances calculated between larval shrimps and the rearing water. Statistical significance was tested by pairwise Wilcoxon tests with p−values adjusted with the "Bonferroni" method in R (***p<0.001, **p<0.01).

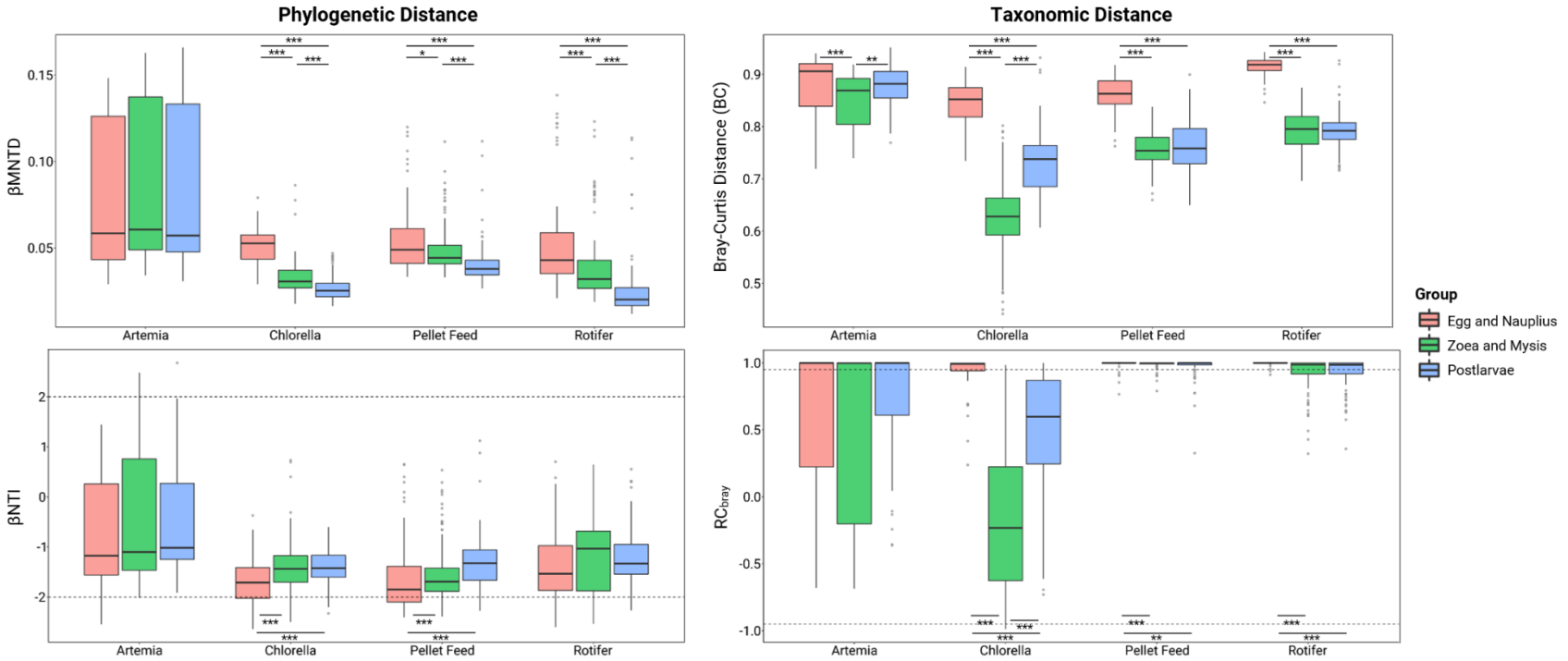

Figure 16 Box plots showing phylogenetic and taxonomic distances calculated between larval shrimps and each type of feed. Statistical significance was tested by pairwise Wilcoxon tests with p-values adjusted with the "Bonferroni" method in R (***p<0.001, **p<0.01, *p<0.05).

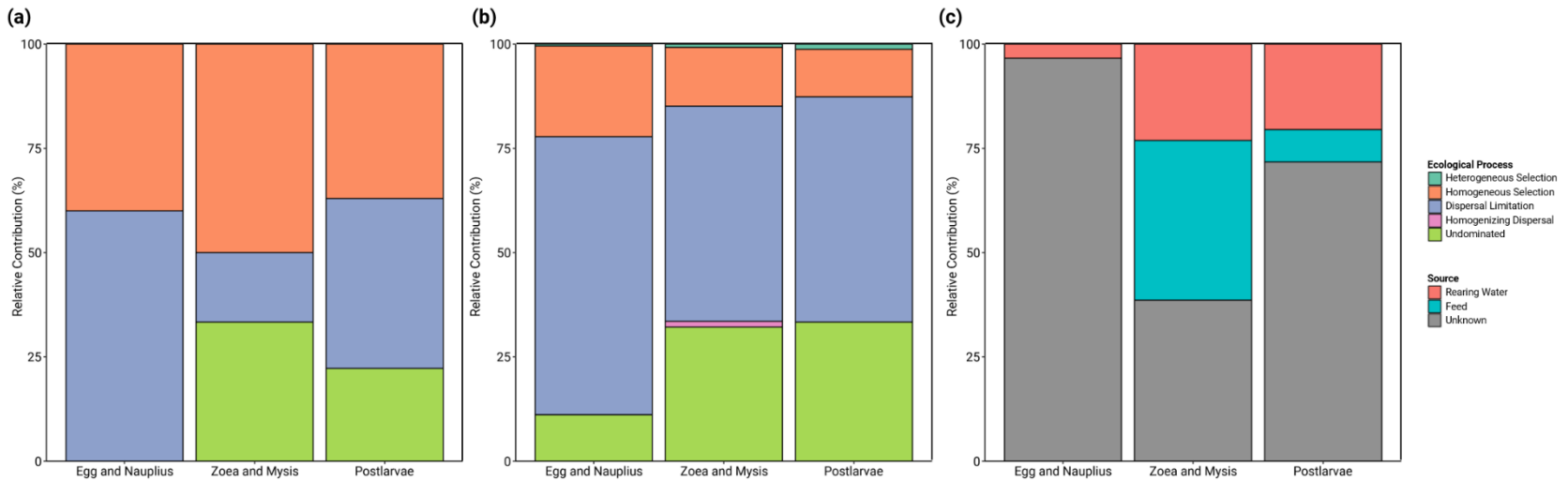

Figure 17 The relative contribution of ecological processes between larvae–associated prokaryotic communities and (a) the rearing water, and (b) the feed at each developmental stage group. (c) Source tracking analysis results by SourceTracker2. Larval shrimps were set as microbial 'sinks', and feed and the rearing water were set as microbial 'sources'.

3.2.4. Correlation with Environmental Factors

The Spearman correlation between environmental factors including temperature, DO, pH, salinity, and macronutrient concentrations including nitrate plus nitrite (NO_2+NO_3), ammonia (NH_4), phosphate (PO_4), and silicate (SiO_2) were calculated against the relative abundance of major bacterial families (Figure 16). Notably, the relative abundance of *Rubritaleaceae* showed a positive correlation to temperature and salinity, while those of *Cronitomicaceae* and *Pseudoalteromondaceae* showed a negative correlation to temperature and salinity. Moreover, the relative abundance of *Oligoflexales* showed a positive correlation with the concentration of macronutrients including ammonia, phosphate, and silicate. Overall, these results indicate that the relative abundance of major taxa correlates with the environmental factors of aquaculture conditions.

Figure 18 Spearman Correlation plot of environmental factors and dominant bacterial families. The color and size gradient denotes the Spearman correlation coefficients.

Chapter 4. Discussion

4.1. Host microbiome and the lack of study on Fleshy Prawn

The host microbiome of marine aquaculture organisms is known to play an essential role in the growth, physiological health, and nutrition of its host (Infante-Villamil *et al.*, 2021; Perry *et al.*, 2020; Rajeev *et al.*, 2021). Meanwhile, the microbiome is influenced by host-related factors such as host genetics and developmental stages, the health of the host, and environmental conditions such as diet, the surrounding water, and abiotic factors (e.g. water quality) (Chen *et al.*, 2022; Holt *et al.*, 2021; Yukgehnaish *et al.*, 2020). However, the interrelationships among these factors and their impact on the host remain largely unexplored.

Among the factors, the development stage of the host is known to be one of the critical influencing factors. Various kinds of aquaculture animals, including fish, mollusks, and shrimps undergo multiple steps of metamorphosis along the early developmental stages (Wilkes Walburn *et al.*, 2019; Yang *et al.*, 2020). Along with metamorphosis, the microbial communities that are associated with their hosts are known to undergo frequent reassembly, due to frequent changes in interactions with their hosts and other microbes (McFall-Ngai *et al.*, 2013; Vadstein *et al.*, 2018). The shifting prokaryotic community composition of along larval growth has previously been reported from penaeid shrimps such as *L. vannamei*

(Y. Wang *et al.*, 2020) and *P. monodon* (Angthong *et al.*, 2020), and the suggested developmental stage as the major factor that determines the differentiation of microbial communities. However, the prokaryotic communities associated with the larvae of *F. chinensis* remain largely unknown and the impacts of possible factors influencing the feed are not well-described.

4.2. Dominance of different taxa and functional groups by developmental stages

The results of this study demonstrate a clear differentiation among the prokaryotic community associated with larval shrimps by its developmental stage, shown by NMDS analyses (Figure 2) and PCoA (Figure 11). This reflects the shift in the taxonomic composition of the community across development, which was further described by the alteration of the relative abundance of major bacterial families (Figure 5) and ASVs (Table 4 and Figure 6). Notably, *Alteromonadaceae* and *Pseudoalteromonadaceae* showed their highest relative abundance at the egg and nauplius stage and a decline afterward. *Alteromonadaceae* and *Pseudoalteromondaceae* have recently been reported as the core bacterial taxa found both in the maternal and paternal reproductive organs and the eggs and nauplius of *L. stylirostris*, possibly indicating vertical transmission from the parents (Giraud *et al.*, 2022). In other words, the decrease of these taxa may be reflecting the fading influence of the vertically transmitted populations along host development. Moreover, the genus *Alteromonas* has also been reported as the most dominant genera in eggs and nauplii of the Indian white shrimp *Penaeus indicus* (Vinay *et al.,* 2022), further supporting the results of this study.

Moreover, *Flavobacteriaceae* and *Rhodobacteraceae* dominated the larvae-associated communities in terms of bacterial family abundance (Figure 4). In addition, the core ASVs associated with larval shrimps were mostly affiliated with these families (Figure 6).

The dominance of *Flavobactericaeae* and *Rhodobacteraceae* in aquaculture conditions had been widely reported (Moschos *et al.*, 2022; Roquigny *et al.*, 2021). However, each of the families showed stage-specific dynamics. The relative abundance of *Flavobacteriaceae* was increased at the zoea and mysis stages and declined afterward. This tendency of enrichment at the zoea stage has previously been reported in *L. vannamei* (Zheng *et al.*, 2017). *Flavobacteriaceae* are widely found in shrimp aquaculture environments (Wang *et al.*, 2020; Xue *et al.*, 2020), and are known to degrade organic matter derived from algal sources (McBride, 2014; Sato *et al.*, 2010). The larval shrimps were fed algal feed such as *Chlorella* spp., starting from the zoea stages. Accordingly, *Flavobacteriaceae* were also found in the *Chlorella* feed (Figure 9). Moreover, functional inference results showed that the metabolism of glycan was significantly enriched at the zoea stage (Figure 10). From these results, we speculate that *Flavobacteriaceae* have a strong contribution in terms of abundance and function at the zoea and mysis stages.

Rhodobacteraceae generally increased along larval development until the postlarval stage. *Rhodobacteraceae* are commonly found in marine environments and are associated with symbiosis with aquatic organisms (Simon *et al.*, 2017). They have also known to harbor a wide range of metabolic activity (Pujalte *et al.*, 2014). Moreover, *Rhodobacteraceae* are known to be related to the promotion of the growth of shrimp larvae (Cardona *et al.*, 2016; Liu *et al.*, 2019; Shen

et al., 2022). Functional inference results further show the increase of metabolisms of amino acids and carbohydrates, along with the metabolism of secondary metabolites across development (Table 5 and Figure 10).

The relative abundance of *Rubritaleaceae* in the class Verrucomicrobiae was significantly increased. In particular, the average relative abundance of the core ASV 331 (*Rubritalea* sp.) showed a high increase at the postlarval stages (22.83 ± 14.13 %) (Table 4). The genus *Rubritalea* comprises members that produce antioxidants and carotenoids, and hence confer a potential benefit to the shrimp host (Lv *et al.*, 2020; Rosenberg, 2014). Overall, the dynamics of the taxonomic composition and their function across the developmental stages demonstrate a developmental stage-specific change.

4.3. Assessment of influencing factors on the prokaryotic communities associated with larval shrimps

Another important aim of the study was to understand the influencing factors on larvae-associated prokaryotic communities. We hypothesized the prokaryotic community of the rearing water and feed as the main factors. Results showed that prokaryotic communities associated with shrimps were distinct from the surrounding communities and the feed (Figure 11).

Phylogenetic and taxonomic succession patterns revealed assembly processes along host development (Figure 12). Moreover, the stage-specific assembly mechanism of larvae-associated prokaryotic communities based on phylogenetic and taxonomic metrics further emphasizes the importance of zoea and mysis stages, since stochastic processes including dispersal limitation, homogenizing dispersal and undominated processes (i.e. ecological drift) were highly increased (69.37 %) compared to the previous period (28.89 %) (Figure 13).

This was further supported by the Sloan neutral model fitting results that emphasizes the importance of stochastic processes at the zoea and mysis stage (Figure 14). Neutral processes dominated the ecological regimes at the zoea and mysis stage, supported by the higher average R^2 values of the model fit. Generally, neutral processes are known to decrease along host development, due to the increase of deterministic processes by the host and the limitation of dispersal (Burns *et al.*, 2016). However, a pattern of increase at the

zoea stage and the subsequent decrease of the neutral process was observed (Figure 16b). The overall results strongly suggest the relative importance of the zoea and mysis stage, showing the high influence of the rearing water and feed. This may be reflecting the feeding behaviors of shrimp larvae as suggested previously, which emphasized the mouth-opening event between the nauplius and zoea stages (Y. Wang *et al.*, 2020).

Community distance comparisons and source tracking results further elucidate the relative contribution of rearing water and feed. Notably, the Bray-Curtis similarities and phylogenetic similarities compared against both the rearing water and the feed were significantly increased from the zoea and mysis stages (Figure 15 and Figure 16). Source tracking results showed a similar trend, demonstrating a higher relative contribution of rearing water and feed to the microbial community composition (Figure 17).

4.4. Implications on the aquaculture of Fleshy Prawn and penaeid shrimps

Larval shrimps spend their early developmental stages mostly in nursery ponds in aquaculture conditions, and move to bigger ponds at the end of the postlarval stage (Mishra *et al.*, 2008). This nursery period is a key step in successful aquaculture practices, considering the frequent mass mortality caused by microbial agents (Correia *et al.*, 2014). Therefore, a fundamental understanding of the microbial community that is already present among larval shrimps, and an understanding of dynamics in a constantly changing condition in terms of host development, feed, and rearing water is critical. Given the limited aquaculture practices of the *F. chinensis* in South Korea and east Asian countries, we conclude that the zoea and mysis stages span from day 4 to day 10 since hatching must be carefully treated in terms of pathogen management and probiotic treatment.

However, it is important to consider that many of the ASVs were taxonomically unassigned at specific levels of taxonomy, due to limitations in cultivability and characterization up to now. This indicates a need for both a metagenomics approach and a culture-based approach for aquaculture conditions and marine invertebrates in general. Nonetheless, this study provides an elementary knowledge of the prokaryotic communities among *F. chinensis*, which may confer substantial aid in the application of aquaculture practices of *F. chinensis* and larval penaeid shrimps.

Chapter 5. Conclusions

In this study, the structure and function of the prokaryotic community associated with larvae of the Fleshy Prawn (*F. chinensis*) across its early development stage were investigated. The results validate the hypothesis that the developmental stage is the key factor that explains the differences between the prokaryotic communities. Moreover, the results revealed a stronger influence of the rearing water and feed at the zoea and mysis stages than other stages. To our knowledge, this is the first study that investigates the microbial community of *F.chinensis* at its early developmental stage, spanning from the egg to the postlarvae stage. This study will provide an understanding of the baseline microbial community of *F.chinensis* and insights into the host–microbe interaction and microbial community assembly mechanism of marine invertebrate hosts.

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Abstract in Korean

대하(*Fenneropenaeus chinensis*)는 보리새우 과에 속하는 새우의 일종으로, 한국 서해안과 중국 동안에서 발견되는 종이며, 수산 분야에서 경제학적으로 중요한 종 중 하나이다. 그러나 대하는 반복되는 대량 폐사와 제한적인 종묘 생산으로 인해 현재 국내에서는 대체 종들에 비해 활발하게 양식되고 있지 않다. 한편, 새우는 초기 유생 발달 과정에서 유생에 동반되는 원핵생물 군집과 중요한 상관관계를 가진다고 알려져 있으며, 유생의 잦은 변태 과정과 변화하는 먹이 급여 등이 요인으로 알려져 있다. 반면, 대하의 초기 유생 단계에 동반되는 원핵생물 군집의 구조와 기능에 대한 연구는 현재까지 없는 실정이다. 본 연구에서는 초기 유생 발달 단계에 따른 대하의 마이크로바이옴 변화 특성을 최초로 규명하고자 하였다. 3개체의 야생 모하로부터 산란한 알들을 3개의 유수식 탱크에 각각 나누어 24일 동안 사육하였으며 수정란부터 노플리우스, 조에아, 미시스, 포스트라바 단계의 대하 시료 내 미생물 군집을 분석하였다. 또한 대하의 초기 유생 마이크로바이옴 형성에 영향을 줄 것으로 여겨지는 사육수와 먹이원의 원핵생물 조성 및 사육 환경 인자와의 관계를 함께 조사하였다. 연구 결과, 미생물 군집 간의 차이를 가장 잘 설명하는 변인은 발달 단계의 변화였으며, 각각 초기 단계인 수정란과 노플리우스, 중기 단계인 조에아와 미시스, 그리고 후기 단계인 포스트라바 단계별로 군집이 클러스터링됨을 확인하였다. 수정란과 노플리우스 단계에서는 모하로부터의 수직 전달을 시사하는 *Alteromonadaceae*와 *Pseudoalteromonadaceae* 등의 분류군이 특징적으로 나타났다. 특히, 조에아와 미시스 시기에는 주변

사육수와 먹이의 영향을 나타내는 *Flavobacteriaceae* 와 glycan 대사 경로의 증가 양상이 나타났다. 후기 단계인 포스트라바 시기에는 *Rhodobacteraceae* 등의 분류군과 아미노산 및 탄수화물 대사 경로의 증가가 관찰되었다. 더 나아가, 미생물 형성 과정 분석, Neutral Model Fitting 분석, 그리고 유사도 분석 및 Source Tracking 분석 결과, 미생물의 확률적 형성과정과 사육수와 먹이의 영향이 극대화되는 시기는 섭식이 일어나기 시작하는 조에아와 미시스 시기로 나타났다. 이러한 결과는 대하의 기저 미생물 군집에 대한 이해를 도울 것으로 예상되며, 더 나아가 양식 현장에서 대하의 초기 유생 발달 시기가 가지는 중요성을 시사한다.

주요어 : 대하, 초기 발달 단계, 마이크로바이옴, 해양 무척추 동물, 수산

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