Microbial Biomarker detection in shrimp larvae rearing water as putative bio-surveillance proxies in shrimp aquaculture

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Background: Aquacultured animals are reared in water hosting various microorganisms with which they are in close relationships as those microorganisms can be involved in the animal health, physiology and fitness during their whole lifecycle. In aquaculture hatcheries, understanding the interactions existing between the rearing water microbiota, the animal larval stage and health status, and the natural seawater may allow to establish microbial proxies to monitor the rearing ecosystems, to promote the conducive microbiota to larval development and ultimately help microbial management. **Methods:** In this light, we daily followed the composition of the active microbiota of the rearing water, with or without antibiotic addition, in a hatchery of the Pacific blue shrimp *Penaeus stylirostris*, where both healthy larvae with a high survival rate, and unhealthy larvae with a high mortality rate, occurred during the same rearing cycle. Using HiSeq sequencing of the V4 region of the 16S rRNA gene of the water microbiota, coupled to zootechnical parameters of the reared larvae and statistical analysis, we aimed to distinguish microbial taxa related to mortality rate at a given larval stage. **Results:** We highlighted that the active microbiota of the rearing water was highly dynamic whatever the larval survival rate. A clear distinction of the microbial composition was shown from D2 between the water harboring heathy larvae reared with antibiotic *versus* the unhealthy larvae reared without antibiotic.Yet it is hard to untangle the antibiotic effect from the larval death on the active microbiota of the rearing water. Our results indicated that various active taxa of the rearing water were specific of a given larval stage and their survival rate except for the Zoea with a good survival rate. When these communities were compared to the primary reservoir containing the lagoon seawater and to the storage water, it appeared that many taxa were originally detected in the natural seawater. This highlighted the great importance of the microbial composition of the lagoon on the rearing water microbiota. The biomarker investigation did not allow to identify proxies at the family level that were

specific of a good survival rate nor to the bad survival rate. However, taking together the larval stage and survival allowed to highlight that the *Flavobacteriaceae,* and *Cryomorphaceae* were proxies of the healthy Mysis. The NS11-12 marine group was the main biomarker of the unhealthy Zoea; the AEGEAN-169 marine group and an unknown family related to the *Gammaproteobacteria* were specific of the unhealthy Mysis. All these biomarkers specific of healthy or unhealthy larvae, could be used as a routine proxy of early warning in the natural seawater and then during the first days of the larval rearing to monitor and to estimate the evolution of the larval rearing. Ultimately these biomarkers might help manage the rearing water microbiota and select beneficial microorganisms for the larvae.

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Key words: shrimp larvae, microbial biomarker, rearing water microbiota, healthy larvae, unhealthy larvae, bio-surveillance proxies, lagoon microbiota

Abstract

Background: Aquacultured animals are reared in water hosting various microorganisms with which they are in close relationships as those microorganisms can be involved in the animal health, physiology and fitness during their whole lifecycle. In aquaculture hatcheries, understanding the interactions existing between the rearing water microbiota, the animal larval stage and health status, and the natural seawater may allow to establish microbial proxies to monitor the rearing ecosystems, to promote the conducive microbiota to larval development and ultimately help microbial management.

Methods: In this light, we daily followed the composition of the active microbiota of the rearing water, with or without antibiotic addition, in a hatchery of the Pacific blue shrimp *Penaeus stylirostris*, where both healthy larvae with a high survival rate, and unhealthy larvae with a high

mortality rate, occurred during the same rearing cycle. Using HiSeq sequencing of the V4 region of the 16S rRNA gene of the water microbiota, coupled to zootechnical parameters of the reared larvae and statistical analysis, we aimed to distinguish microbial taxa related to mortality rate at a given larval stage

Results: We highlighted that the active microbiota of the rearing water was highly dynamic whatever the larval survival rate. A clear distinction of the microbial composition was shown from D2 between the water harboring heathy larvae reared with antibiotic *versus* the unhealthy larvae reared without antibiotic. Yet, it is hard to untangle the antibiotic effect from the larval death on the active microbiota of the rearing water. Our results indicated that various active taxa of the rearing water were specific of a given larval stage and their survival rate except for the Zoea with a good survival rate. When these communities were compared to the primary reservoir containing the lagoon seawater and to the storage water, it appeared that many taxa were originally detected in the natural seawater. This highlighted the great importance of the microbial composition of the lagoon on the rearing water microbiota. The biomarker investigation did not allow to identify proxies at the family level that were specific of a good survival rate nor to the bad survival rate. However, taking together the larval stage and survival allowed to highlight that the *Flavobacteriaceae,* and *Cryomorphaceae* were proxies of the healthy Mysis. The NS11-12 marine group was the main biomarker of the unhealthy Zoea; the AEGEAN-169 marine group and an unknown family related to the *Gammaproteobacteria* were specific of the unhealthy Mysis. All these biomarkers specific of healthy or unhealthy larvae, could be used as a routine proxy of early warning in the natural seawater and then during the first days of the larval rearing to monitor and to estimate the evolution of the larval rearing. Ultimately, these biomarkers might help manage the rearing water microbiota and select beneficial microorganisms for the larvae.

Introduction

In New-Caledonia, hatcheries of the Pacific blue shrimp *Penaeus stylirostris* face high larval mortality rates (Beliaeff et al., 2009; Pham et al., 2012). The causes of such mortalities are not understood yet and multi-factorial reasons seem to trigger the larval death. For example, only 128 million post larvae were produced in 2019; while it was up to 167 million in 2005 (https://www.agence-rurale.nc/filieres/peche-et-aquaculture/crevettes/). These larval mortalities create many issues as not enough post-larvae are available to be spread among all the earthen

ponds of the 18 farms of the Territory, where they grow until they reach a certain weight to be sold. This induces commercial deficit and economical loss for both the farmers, the workers and the Territory. Several factors, such as water quality or bacterial infections, could play a role on the larval mortalities but these hypotheses have been wrecked respectively because during a same rearing cycle using the same water, various larval survival rates were observed and because no larval septicemia was noticed. However, a rearing water dysbiosis could hamper the larval survival. Indeed, aquacultured animals are reared in water hosting various microorganisms with which they are in close relationships as several microorganisms of the water might be involved in the animal health, physiology and fitness during their whole lifecycle (Goarant et al., 2006; Ganguly & Prasad, 2012; Carbone & Faggio, 2016; Zheng et al., 2017; Sun et al., 2019; Wei et al., 2020; Wang et al., 2020; Angthong et al., 2020). It has been shown that the microbiota of the rearing water could influence pre-feeding fish larvae (Bledsoe et al., 2016; Wilkes Walburn et al., 2019) and may contribute to larval health in cod larvae (Lauzon et al., 2010). Regarding shrimps, it has been proved that the rearing water microbiota can interact and some taxa can be transmitted to the host microbiota at various lifecycle stage (Huang et al., 2018; Giraud et al., 2021). Thus, in shrimp hatcheries, it seems necessary to uncover the interactions between the rearing water microbiota, the animal larval stage and health status, and the natural seawater, in order to establish microbial proxies to monitor the rearing ecosystem. To date, few studies have been done on both diseased and healthy *Penaeus vannamei* shrimps at various life stages: larvae and adults. They still managed to establish link between the microbial communities and the health status, and identified bacterial indicators of diseased shrimps (Xiong, Zhu & Zhang, 2014; Zheng et al., 2017). However rearing methods of *P. vannamei* larvae slightly differ from the way *P. stylirostris* are raised; as in the first case rearing is done using oceanic water using both probiotics and antibiotics while, in New-Caledonia, larval rearing is done using lagoon seawater and antibiotic is often added until post-larval stage (Pham et al., 2012; Zheng et al., 2017). Thus, in order to overcome the larval deficit in New-Caledonia, is it imperative to establish microbial proxies to monitor the rearing ecosystems, to promote the conducive microbiota to larval development and ultimately help microbial management.

In this aim, we have daily followed the microbial composition of the rearing water containing larvae raised with or without antibiotic, to investigate if any microbial families were associated

to a certain mortality rate at a given larval stage. We also looked if the antibiotic and the natural seawater microbiota had a role on the active microbial communities inhabiting the rearing water. In the context of this study, we identified several biomarkers specific of a given larval stage and health, that might be used as a routine proxy of early warning in the natural seawater and then during the first days of the larval rearing. These biomarkers could help to manage the rearing water microbiota and select beneficial microorganisms for the larvae.

Materials & Methods

Study design, water collection and storage

The study was done in the experimental shrimp hatchery hosted in a shrimp farming research facility at the Station Aquacole de Saint Vincent (Boulouparis, New-Caledonia), in February 2019, where seawater from the water storages, larval rearing tanks and control tank were collected during a larval rearing cycle. Tanks in the hatchery were filled with natural seawater collected in the Saint Vincent Bay. The natural seawater was pumped through a 1cm pore size device into a primary reservoir (ResI); and was then filtered through a 10µm filter prior to storage into a $2m^3$ storage container with intensive bubbling, located in the hatchery (ResII). In New Caledonia, the reproduction of *Penaeus stylirostris* is done, as described by Pham et al. (2012), by inseminating artificially mature females. The same day, the hatchery tanks were filled with storage seawater in which 5 $g.m^{-3}$ of EDTA (ethylenediaminetetraacetic acid) were added and an intensive bubbling was implemented. Each tested condition was carried out in triplicates except for the control, per say: three larval tanks with erythromycin added at 2 ppm few hours after the EDTA (day 0) and then on days 3, 5, 7 and 9; three tanks with larvae without antibiotic and one tank Control (Control) without larvae, antibiotic or food. The larval feeding protocol was as follow: from Zoea 1 to Zoea 2, microparticles were added five times per day and frozen *Tetraselmis* sp. once a day; from Zoea 3 to Post-larvae, microparticles were added twice a day and living *Artemia* sp. nauplii (between 20 to 40 nauplii/larvae/day) twice a day. No water exchange was applied during the first 10 days of the larval rearing.

Natural seawater from the primary reservoir was sampled before the insemination, seawater from the storage container was sampled the insemination day; while samples from the rearing tanks were daily collected during 9 days, before the first feeding of the day. For each sample, 1L of

water was filtered on 0.2 µm sterile membrane filters (S-Pak, Millipore). All filters were stored at -80°C until RNA extractions.

Daily determination of the zootechnical parameters

Daily larval survival rates were estimated by counting the number of larvae in three samples of 100 mL while the larval stages were determined by the observation of 30 larvae using a binocular magnifying glass. This allowed to calculate the Larval Stage Index (LSI), using the modified equation of Maddox and Manzi (Maddox & Manzi, 1976) :

LSI = (0xNii+1xZ1+2xZ2+3xZ3+4xM1+5xM2+6xM3+7XPL) / N

where Nii is the number of larvae observed in the Nauplius stage, Z1 in the Zoea 1 stage, Z2 in Zoea 2, Z3 in Zoea 3, M1 in Mysis 1, M2 in Mysis M2, M3 Mysis M3, PL in Post-larvae 1; and N corresponds to the total number of observed larvae. The Larval Survival Rate (LSR) was defined by averaging 3 direct counts of the living and dead larvae in 1L of rearing water per tank and per day. The LSR was determined as follow LSR: 100*counted living larvae / initial number of nauplii.

RNA extractions, reverse transcriptions, sequencing and sequence processing

RNAs extractions were performed using the RNAeasy Powerwater kit (Qiagen) following the manufacturer's information. Then, the total RNAs were reverse transcripted into complementary DNA (cDNAs) using 10 µL of RNA (200 ng/µl), 1 µl of the reverse transcriptase M-MLV at 200 u/µl (PROMEGA), 2 µl random hexamers 50 µM, 4 µl of Buffer 5X, 2 µl of a mix of dNTP a 10 mM each and 1 µl of Rnase/Dnase free water. All the reverse-transcriptions were carried out in a Veriti™ instrument (Applied Biosystems), using the program: 10 min at 25°C, 2 h at 42°C and 5 min at 85°C. The cDNAs were stored at -80°C until shipping to MrDNA (Molecular Research LP, Shallowater, Texas, United States) where the PCR, barcode indexing and sequencing of the V4 hypervariable region of the 16S rRNA molecule were conducted using the universal primer combination 515f-806R (Caporaso et al., 2011; Hugerth et al., 2014). The Illumina HiSeq sequencing was done using MrDNA protocols with a 2x300 bp paired-end run and an average sequencing depth of 20k raw reads per sample. As described in Giraud et al, 2021 (Giraud et al., 2021), the raw data were first demultiplexed using the fastqSplitter available on the MrDNA website (https://www. Mrdnalab.com/mrdnafreesoftware/fastq-splitter.html). Then, the reads

were treated using the DADA2 (Callahan et al., 2016) package available in the Rstudio software, where the sequences were filtered using a maximum excepted error (maxEE) set at 2 and the taxonomy was assigned using the Silva 138 SSU Ref NR99 database (Quast et al., 2013). Once the ASV table was obtained, sequences with no affiliation or affiliated to the eukaryotas, mitochondrias and chloroplasts were removed before further analysis. All the 16S rRNA data are available in the NCBI SRA repository (Submission ID XXXX, BioProject ID PRJNA736535, xxx and: SRP14825206 for the ResI sample).

Downstream microbial analysis

The alpha diversity was calculated using the ACE, Chao 1, Shannon and Inverse Simpson (InvSimpson) indexes using the phyloseq package in RStudio (McMurdie & Holmes, 2013). Before other microbial analysis, the ASV table was normalized with the Count Per Million (CPM) method using the edgeR package under the RStudio software. The beta diversity was determined by the construction of a dendrogram and PCoAs, all based on a Bray-Curtis dissimilarity matrix and the Ward method, using the Vegan package (Jari Oksanen, 2022) for the dendrogram and the phyloseq package (McMurdie & Holmes, 2013) for the PCoAs in Rstudio. Prior to Venn diagram constructions, we made 5 groups of rearing water samples based on the larval stage: nauplii, zoea or mysis; and larval survival rate: good or bad. We considered larvae with a good survival rate when the daily counting was above, equal or slightly below the reference (less than 5%) (the reference is an average of survival rate calculated for each day using data of 10 years of successful rearing; Ifremer data, Pham comm. pers.). The 5 final defined groups were the nauplii, the zoea with a good survival rate (later named Zoea Good), the zoea with a bad survival rate (Zoea Bad), the mysis with a good survival rate (Mysis Good) and the mysis with a bad survival rate (Mysis Bad). Venn diagrams were then made using the Jvenn web application (Bardou et al., 2014) (http://bioinfo.genotoul.fr/jvenn/example.html). Linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) were performed with a threshold set at 4 using the microbiome Marker package (Cao) in RStudio to identify microbial biomarkers. The correlogram between the larval stage, the survival rate and the families detected with the LEfSe, was constructed using the microeco package in RStudio (Liu et al., 2021).

Results

Zootechnical parameters

Contrasted survival rates were observed between the two treatments (**Fig 1).** Larvae reared with antibiotic gave the best survival rates on D9 with more than 70% of surviving larvae, similar to the reference, that was the usual survival rate obtained for a specific day calculated for each day using data of 10 years of successful rearing (Ifremer data, Pham comm. Pers.). Inversely, larvae raised without antibiotic did not manage to reach the 9th day of rearing. The mortality started to occur on D4 except in the tank C, with only 75% of survival in the tanks A and B, against 85% for the reference. On D5 the survival of larvae in the three replicates without antibiotic were dramatically below the reference value. On day 9, only larvae from the tanks with antibiotic were still alive with a survival rate above the reference (70%) with an average of 73% of survival. Regarding the larval stage, none of the tanks, with or without antibiotic addition, reached the post larvae stage on D9 (Table 1**)**. All larvae metamorphosed in mysis on D7, one day later to the reference. Except the larvae of the tanks B and C reared without antibiotic (Without-B and Without-C in Table 1); all rearing stayed 2 days in the zoea 3 stage (D3 et D4) while it usually takes 1 day. Globally compared to the reference, whichever the rearing condition, larvae had a delay in their metamorphosis. Indeed, at D6 all larvae should be in Mysis 1 stage while they were still in Zoea 3 stage.

Time series of the active microbiota in the water

After eukaryotas, mitochondria, chloroplasts and unassigned sequences were removed, a total of 21,749,911 reads, spread into 6707 ASVs were obtained from the HiSeq Illumina sequencing of all samples. The smallest library was made of 207,221 reads and corresponded to the Control D3 sample (water without larvae, antibiotic or food); while the largest was composed by 650,143 for the sample Control D6.

Overall, the alpha diversity indexes were generally higher in the rearing water without antibiotic than in the rearing water with antibiotic and the control water. Also, the alpha diversity indexes of the control water were greater than the rearing water with antibiotic. The storage water samples (primary reservoir and container storage) were those exhibiting the higher alpha diversity indexes. Kruskal-Wallis tests were performed on the alpha diversity indexes (Table SI) between the different kinds of water samples: storage water, control, rearing water with antibiotic and rearing water without antibiotic. The tests showed that, for the Chao1 and ACE,

the rearing water with antibiotic was significantly different from the rearing water without antibiotic (p value = 0.000006). For the same indexes, the test exhibited that the control was significantly different from the rearing water without antibiotic (p value = 0.0013); and the storage water was significantly different from the rearing water with antibiotic (p value = 0.005). The same trend was observed for the ACE index, with significant differences between the rearing with and without antibiotic (p value = 0.000004), between the rearing water without antibiotic and the control (p value = 0.003); and between the storage water and the rearing water with antibiotic (p value = 0.005). Considering the Shannon and Inverse Simpson indexes, significant differences were highlighted only between the rearing water with antibiotic and rearing water without antibiotic, with respective p values at 0.000008 and 0.003. Comparisons were done with the samples collected on D1 in the rearing water with and without antibiotic. The p values were calculated at 0.513 for the ACE and Chao1, for rearing water samples collected at D1 with or without antibiotic; whereas significant differences were showed for the Shannon and inverse Simpson indexes with p values at 0.0495 for both proxies.

Using the whole microbial diversity, we visualized how the samples gathered together (Fig 2). The ascending hierarchical clustering with a threshold set at 1.5 exhibited 7 clusters. The three first cluster grouped the rearing water with antibiotic: first one included water samples collected from D7 to D9, the second cluster encompassed those collected from D4 to D6; while cluster 3 was composed by samples collected on D2 and D3. Thus, except for the rearing water with antibiotic samples collected at D1, all the rearing water with antibiotic samples belonged to a unique larger cluster. All the rearing water sampled on D1, plus the control water sampled on D0 and the storage container sample, were all gathered in cluster 4. All the control waters, except for the sample collected on D0, composed the cluster 5. Cluster 6 encompassed the rearing water without antibiotic collected on D7 and D8 and the primary reservoir sample. Rearing water without antibiotic collected from D2 to D6 formed the cluster 7. Thus, clusters 6, 7 containing solely samples collected in the rearing water without antibiotic, as well as cluster 4 that contained all the D1 samples were gathered together in a larger cluster.

In order to deeper investigate these results, 2 PCoAs were built, one for each water treatment (antibiotic or not). The ordinations of the samples collected in the rearing water without antibiotic using the Bray-Curtis matrix, exhibited a first cluster grouping the water samples from

the storage container ResII and the samples collected at D1 (Fig 3A), likewise the ordination made with the samples collected in the rearing water without antibiotic (Fig 3B). In both ordinations, a second cluster gathered the samples collected from D3 to D5, corresponding to the rearing water with larvae at the zoea stage. A third cluster was also observed in the 2 PCoAs, and encompassed samples collected from D7 to D9 for the rearing made with antibiotic and samples from D7 and D8 for the rearing made without antibiotic. These rearing days corresponded to the larvae at the mysis stage (Table 1). Thus, in both ordinations, the water collected in the primary reservoir (ResI) and those collected on D6 did not belong to any cluster.

The active microbiota of the primary reservoir (ResI) that contained lagoon seawater, was highly different from all the other samples, as most of its lineages belonged to families that were not represented by more than 1% of the relative abundance in the other samples (Fig 4**)**. The microbial composition of the control water, that contained no larvae, antibiotic or food, on D0 was identical to that of the storage container ResII and the rearing water with or without antibiotic collected on D1. The A*lteromonadaceae* and *Rhodobacteraceae* were dominant in all these samples. Their abundances remained high during the other nine days of rearing with, however, an increased proportion of *Pseudohongiellacaea* and *Bdellovibrionaceae* (Fig 4). The active microbial compositions of the 3 replicates samples for each condition displayed homogenous profiles through the whole rearing excepted on D8 for the samples collected in the rearing water without antibiotic (Fig 4). The active microbiota of the water samples exhibited different compositions and dynamics according to the rearing day as microbial shifts daily occurred, and according to the addition or not of antibiotic. However, from D2 and during the entire rearing in the presence or not of antibiotic, the *Rhodobacteraceae* had high abundances (Fig 4). The active microbial composition of the rearing water without antibiotic encompassed mostly members of the *Rhodobacteraceae*, *Alteromonadaceae*, *Saprospiracaea* and *Litoricolaceae* on D1and D2 (Fig 4). On D3, a shift occurred with the diminution of the proportion of the *Alteromonacaeae* accompanied with an increase of the *Saprospiraceae*; and until D5, the most abundant families were *Rhodobacteraceae*, *Alteromonadaceae* and *Saprospiracaeae*. From D6 to D8, *Alteromonadaceae* and *Saprospiraceae* decreased drastically. On D7 the main families were the *Rhodobacteraceae* and an unknown family related to the ASV19 affiliated to the *Gammaproteobacteria*. On D8, the dominant families varied among the

3 tanks, where in addition to the *Rhodobacteraceae*, the proportion of *Flavobacteriaceae*, *Vibrionaceae* and/or the *Pseudoalteromonadaceae* raised. In rearing water with antibiotic, on D1 the taxa affiliated to the *Rhodobacteraceae*, *Alteromonadaceae* and *Litoricolaceae* were mostly abundant; while on D2, the main families were the *Rhodobacteraceae, Alteromonadaceae* and the NS11-12 marine group (Fig 4**)**. On D3, a shift occurred with an increase of the proportion of the *Pseudoalteromonadaceae* and *Cryomorphaceae*, associated with a drop of the *Alteromonadaceae*. On D4, *Pseudoalteromonadaceae* decrease in favor to increase of the unknown family UBA12409; whereas on D5, the microbiota was dominated by members of the *Rhodobacteraceae* and *Sapropsiraceae*. On D6, *Rhodobacteraceae* and *Cryomorphaceae* greatly composed the microbiota. A prevalence of members of the *Rhodobacteraceae* and *Flavobacteraceae* was noticed on D7; while from D8 to D9, the main families of the rearing water microbiota with antibiotic were the *Rhodobacteraceae*, *Cryomorphaceae* and *Flavobacteraceae*.

Specific, shared and core microbiotas among the water samples and larval stages and health status

In order to determine ASVs specific to a given rearing condition, per say specific to a larval stage and survival rate, and common microbiota, several Venn diagrams were constructed.

A first Venn diagram was built to compare the rearing water hosting the nauplii, the zoea with a good survival rate (Zoea Good), the zoea with a bad survival rate (Zoea Bad), the mysis with a good survival rate (Mysis Good) and the mysis with a bad survival rate (Mysis Bad) (Fig 5A). As the larval mortalities occurred mainly in the rearing water without antibiotic after D4, the group nauplii was composed by nauplii from the 2 rearing. In the same way, the group Zoea Good encompassed all the samples collected in the rearing water with antibiotic from D2 to D6 and samples collected at D2 and D3 in the rearing water without antibiotic. Thus, the microbiome specific to the nauplii contained 658 ASVs. The diagram showed that no ASV was specific to the Zoea Good, while the Zoea Bad gathered 95 ASVs. The Mysis Good condition exhibited 79 common ASVs and the Mysis Bad had 141 specific ASVs. The diagram also displayed a core microbiota composed by 137 ASVs. These comparisons allowed to point out specific microbiotas of a given condition and a core microbiome. Natural water, per say the lagoon seawater stocked in the primary reservoir and then in storage container, was used for the rearing,

comparisons were done between these two water storages and the specific microbiotas and core microbiome. Thus, the specific microbiota of the rearing water hosting the nauplii had 85 unique ASVs, while 573 ASVs were already present in the natural seawater (Fig 5B). The comparison between the specific microbiota of the Zoea Bad and the natural seawater highlighted that 58 ASVs were not previously in the natural seawater whereas 37 ASVs from the natural seawater were only detected in this condition (Fig 5C). The Mysis Good condition had 48 specific ASVs and shared 31 ASVs with the natural seawater (Fig 5D). The Mysis Bad condition exhibited 79 specific ASVs and co-owned 62 ASVs with the natural seawater (Fig 5E). The comparison between the rearing water core microbiome and the natural seawater displayed that only 1 ASV was specific of the rearing water core microbiome while the 136 other ASVs were shared with the natural seawater (Fig 5F). Together, these comparisons exhibited the great role of the natural seawater on the rearing water microbiota, as several ASVs seemed to be able to re-activate during the rearing according to the larval stage and health status. As we observed a microbial clusterization (Figs 3 and 4) and specific microbiota associated to a given condition of larval stage and survival in the rearing water (Fig 5), we constructed a LEfSe to investigate how the larval stage, health or both interacted with the active rearing water microbiota at the family level.

Biomarkers at the family level according to the larval stages and health status.

A LEfSe analysis was done to investigate the families differentially abundant in the rearing water according to larval stage and health status (Fig 6A). When the rearing water hosted the nauplii with a good survival rate, 7 biomarkers were enriched and were the *Alteromonadaceae*, the *Litoricolaceae*, the *Alcalinovoraceae*, the *Hyphomonadaceae*, the NS9 marine group, the SAR11 Clade I and the *Rhizobiaceae.* The *Pseudoalteromonadaceae*, UBA12409, the *Idiomarinaceae* and *Legionellaceae* were statistically more enriched in the rearing water hosting the zoea with a good survival rate. The Saprospiraceae and NS11-12 marine group were statistically more enriched in the rearing water hosting the zoea with a bad survival rate. The biomarkers specific to the mysis with a good survival rate were the *Cryomorphaceae* and the *Flavobacteriaceae*; while those of the water hosting the mysis with a bad survival were the *Rhodobacteracaea*, the *Vibrionaceae*, an unknown family of the *Gammaproteobacteria* related to the ASV19, an unknown family related to the NRL2-ASV44 and the AEGEAN-169 marine group (Fig 6A). In order to untangle which of the larval stage, the larval health status or both taking together, were

the main factors to enrich specific families with the LEfSe, we did a Pearson correlation (Fig 6B). The correlogram highlighted that the good survival rate parameter was slightly positively correlated with the *Alcalinovoraceae* family that was greatly positively related to the nauplii. The bad survival parameter exhibited the greatest coefficient correlation with an unknown family related to the NRL2-ASV44 and also several significant positive correlations with an unknown family of the *Gammaproteobacteria* related to the ASV19 and with the AEGEAN-169 marine group. However, according to the Pearson coefficient correlation, none of these biomarkers can directly be linked with the bad survival as these 3 families exhibited a higher Pearson coefficient score with the Mysis with a bad survival rate. The nauplii were greatly positively correlated with the *Rhizobiaceae* and the NS9 marine group, and significantly to the *Litoricolaceae*, *Alteromonadaceae* and SAR11 Clade I; and in less extend to the *Hyhomonadaceae* (according to the color of the red gradient of the Pearson correlation). The *Pseudoalteromonadaceae*, *Legionellaceae* and *Idiomarinaceae* were lightly correlated with the Zoea parameters; yet, these 3 families exhibited a greater Pearson coefficient correlation with the Zoea with a high survival rate parameter (Zoea high in the correlogram, Fig 6B). Thus, we can suggest that the *Legionellaceae* and *Idiomarinaceae* with the bigger but still light Pearson coefficient, are likely linked with the presence of the zoea with a good survival rate in the rearing water. The Zoea Bad parameter was highly positively correlated with the NS11-12 marine group. The Mysis parameter exhibited a great correlation with the *Rhodobacteraceae*. The Mysis with a good survival rate had a high Pearson coefficient score with the *Flavobacteriaceae* and *Cryomorphaceae* (Fig 6B). All together the correlogram data displayed that no biomarker was highlighted to be specific of a good survival rate nor to the bad survival rate. It also seemed that the *Idiomarinaceae* and *Legionellaceae* were biomarkers specific to the Zoea with a high survival rate; and the *Flavobacteriaceae* were the main biomarker specific of the healthy Mysis, along with the Cryomophacaea and Rhodobacteraceae but in a lesser extent. Concerning the unhealthy larvae, the NS11-12 marine group was the biomarker specific of the Zoea Bad parameter; while the AEGEAN-169 marine group, the unknown family related to the *Gammaproteobacteria* ASV19 and an unknown family related to the NRL2-ASV44 were biomarkers specific to the Mysis Bad parameter (Fig 6B).

Discussion

*** Main families of rearing water microbiota**

A noticeable evolution of the active rearing water microbiota was pointed out either in the presence of antibiotic or not. Despite a clear distinction of the rearing water microbiota between the water with antibiotic harboring heathy larvae and the water without antibiotic having unhealthy larvae, several main families were common between the 2 rearing conditions. The dominant families were the *Rhodobacteraceae* (*Alphaproteobacteria*), *Alteromonadaceae* (*Gammaproteobacteria*), *Saprospiraceae* (*Bacteroidia*) and in less extent *Litoricolaceae* (*Gammaproteobacteria*), *Cryomorphaceae* (*Bacteroidia*) and *Flavobacteriaceae* (*Bacteroidia*) (Fig 4). Overall, during the rearing, the proportion of the *Bacteroidia* increased while the part of the *Alpha*- and *Gammapropteobacteria* decreased, which was similarly highlighted in the rearing water of *Penaeus vannamei* (Zheng et al., 2017) and *Penaeus monodon* (Angthong et al., 2020). The *Rhodobacteraceae* are often detected in the rearing water of marine shrimp larvae such as *P. vannamei* (Zheng et al., 2017; Heyse et al., 2021), and *P. stylirostris* (Giraud et al., 2021, 2022). The *Alteromonadaceae* have been previously detected in the rearing water of the eggs and the nauplii of *P. stylirostris* (Giraud et al., 2022) and in the rearing water of *P. vannamei* larvae, until stage Mysis 1 (Zheng et al., 2017). Interestingly the *Alteromonadaceae* abundance was unevenly distributed during the 2 rearing; and their proportions were higher in the samples without antibiotic, similarly to the *Litoricolaceae* trend. This could be linked to the absence or presence of antibiotic, and/or to the larval health. It is also quite usual to detect the *Litoricolaceae* mostly in the beginning of the rearing as members of this family are known to grow on oligotrophic medium (Rosenberg et al., 2013); explaining their high abundance during the early larval stages as more organic matter accumulates throughout the rearing due to no water exchange. In the opposite, the *Cryomorphaceae* became more and more abundant at the end of the rearing, especially in the rearing water with antibiotic (Fig 4); which is in accordance with their features as they are often spotted in organic rich oceanic water (Bowman & McMeekin, 2015). Indeed, the rearing water became more and more eutrophic throughout the rearing with the accumulation of organic matter (feed, larval feces, exoskeleton, dead larvae) and with the absence of water renewal during those days. Interestingly the *Saprospiraceae*, have been detected in the rearing water of *P. vannamei* larvae at the mysis stage (Zheng et al., 2017), in a lower proportion than in our study. Also contrary to Zheng (Zheng et al., 2017), in this study, *Saprospiraceae* were

detected in high abundance from D1 to D6 in the rearing water without antibiotic and until the end of the rearing but in a lower proportion; and on D1 and D2 in the rearing water with antibiotic, meaning that the *Saprospiraceae* were mostly in the rearing water hosting the nauplii and the zoea. The *Flavobacteriaceae* have been found in the rearing water of adult *P. vannamei* shrimps (Md Zoqratt et al., 2018), in high abundance in the rearing water of *P. vannamei* larvae at the zoea stage (Zheng et al., 2017) and in the rearing water hosting the eggs and the nauplii of *P. stylirostris* (Giraud et al., 2022). In our study the *Flavobacteriaceae* were mostly detected in the rearing water with antibiotic and at the end of the rearing, when the water hosted the larvae at the mysis stage. Members of this family are known to be able to degrade various organic macromolecules such as fucoidan, microalgae or macroalgae (Bernardet & Nakagawa, 2006). Considering the cited metabolic abilities, that might be the reason explaining their prevalence at the end of the rearing as the rearing water became eutrophic, that could have accumulated in the water. Thus, the dynamic of the main families, and the others, in the rearing water appeared to be related to the rearing water quality and to the addition of antibiotic. However, a clear discrepancy was observed between the rearing water microbiota reared with or without antibiotic and the larval health.

*** Antibiotic and larval health as drivers of the rearing water microbiota?**

Despite the dominance of several main families, 2 large clusters were distinguished based on the presence or absence of antibiotic; one cluster encompassed samples with antibiotic and the other the samples without antibiotic, except for D1 where the conditions were gathered together. Thus, in our study, it is hard to untangle the effect of the antibiotic or the larval death rate on the rearing water microbiota. The use of antibiotic is a worldwide habit in shrimp hatcheries either to avoid larval mortalities caused by pathogenic *Vibrio* species (Holmström et al., 2003; Aftabuddin et al., 2009) or used for prophylactic reasons under veterinary instructions. The observed discrepancy between the microbial communities in our study could be due to the antibiotic action, to the larvae mortalities or both. Analysis of the alpha diversity indexes highlighted significant difference between the microbiota of the rearing water with antibiotic and those of the water without antibiotic; with higher diversity in the rearing water without antibiotic but with high larval mortalities. As all the massive larval mortalities occurred in the rearing water without antibiotic, one can assume that the alpha diversity indexes in this condition are shaped by the

mortality in combination or not with the antibiotic. However, since D1, it seemed that the antibiotic mostly affected the evenness, with significant differences for Shannon and inverse Simpson indexes (Kruskal-Wallis tests); while inversely, it appeared that the total richness (ACE and Chao1) was not impacted by the antibiotic. In addition, both samples collected on D1 with or without antibiotic clustered together; while after this rearing day the 2 rearing took 2 different paths in terms of water microbiota (Fig 2). The effect of the antibiotic on the rearing water of aquacultured animal is poorly documented, while several studies have investigated its effect on animal's heath, physiology or microbiota (Kim et al., 2019; Zeng et al., 2019; Holt et al., 2020; Yukgehnaish et al., 2020). To this day, only one study has dealt with antibiotic effect on the microbiota of the freshwater shrimp *Macrobrachium rosenbergii* at zoea stage (Ma et al., 2020); where it has been showed that the alpha diversity was not significantly different between the water control and the water where the zoea had been reared with antibiotic. These data differed from our findings as we observed significant differences on D1 when all the larvae were healthy and during the whole rearing where 2 factors at least could have affected the alpha diversity: the presence or not of antibiotic and the larval health which differed between the 2 rearing conditions. On the contrary, antibiotic treatment caused significant decrease of the microbial diversity in the cod fish larvae (Lauzon et al., 2010). Likewise, the same conclusions were made with the gut microbiota of adult olive flounder (Kim et al., 2019). Together, our data implied that the use of antibiotic had a significant role on the rearing water microbiota and this explains, at least partially, why all the alpha diversity indexes were higher in the samples without antibiotic. The observation of the 2 large clusters, distinguishing the rearing water samples without antibiotic from those with antibiotic (Fig 2), was also congruent with previous studies that have proved that animal death (or death of any living organism) implied change in their microbiota (Preiswerk, Walser & Ebert, 2018; Benbow et al., 2019). Indeed, the microbiome associated with the living host will change to let place to the necrobiome from both the host and the closest environment, that will decompose the dead tissues and the host-derived organic matter (Cobaugh, Schaeffer & DeBruyn, 2015; Benbow et al., 2019). That will also involve change in the microbiota of the dead organism's surrounding ecosystem (Cobaugh, Schaeffer & DeBruyn, 2015; Finley et al., 2016; Lobb et al., 2020). Thus, we can easily assume that dead larvae could release their necrobiome that could then be present and detected in the rearing water and potentially become part of the highlighted specific microbiota (Fig 5). In addition, as the dead

larvae accumulate in the tanks with high mortalities and as the necrobiome decomposes the host-derived organic matter, we can also hypothesize that such change in the rearing condition could eventually involve change in the water composition. As we pointed out in the figure 2, such changes in the rearing water composition, could also potentially be related to the re-activation of specific lineages originating from the storage water and from the lagoon as they meet good conditions to develop. The change in the microbiota structure between the 2 rearing conditions occurred directly after the addition of antibiotic and 1 to 2 days before the beginning of the mortalities in the rearing without antibiotic. This implies that the use or not of antibiotic as well as the larval health (mortality and necrobiome) were drivers of the rearing water microbiota. It also means that, in our study, the use of antibiotic seemed to prevent the larval mortality. This could, ultimately, help us to unveil specific biomarkers to further help the microbial management of the rearing water by either suggesting new probiotic populations or beneficial taxa to enhance the water quality or the larval health; or to design new tools for an early surveillance of the rearing.

*** Interaction between the natural seawater, rearing water and larvae stage and health**

In the light of our data, we established that various taxa of the rearing water were specific of a larval stage and health; except for the zoea with a good survival rate for which no specific ASVs were found (Fig 5). This also exhibited a microbiota partitioning as also shown in the rearing water of *P. vannamei* larvae (Zheng et al., 2017); and revealed the great importance of larval stage and health on the rearing water microbiota. In addition, Giraud et al. (Giraud et al., 2021, 2022) have shown that a horizontal transmission occurs between the shrimp larvae and their surrounding rearing water, suggesting a putative dynamic between the larval microbiota and the rearing water microbiota. As explained above, the necrobiome inherent of the dead larvae may also interact with the rearing water microbiota and even become part of it, especially in the water hosting larvae with high mortality rates. In addition, we also exhibited that according to the specific microbiota of a given larval stage and survival rate in the rearing water, many of these specific ASVs were previously detected in the natural seawater suggesting a possible re-activation of taxa originally present in the natural seawater. Several studies have also showed that several lineages were shared between the early stage of the shrimps and the water reservoirs (Zheng et al., 2017; Wang et al., 2020; Giraud et al., 2021, 2022). This suggests the high

497 importance of the lagoon and the storage waters on the rearing water microbiota; as well as the
498 importance to look up in the natural seawater for specific and significant biomarkers before the
499 beginning of the larval rearing.
500
501 **Proxy uncovering: biomarker identification specific to a given rearing condition**
502 Our main objective was to unveil biomarkers at the family level specific of a larval stage and
503 health, to later use them to monitor larval health and manage the rearing water. For that, both
504 rearing water microbiota with and without antibiotic, were pooled together according to their
505 larval stage and health. The enriched families detected with the LEfSe, were then related to
506 rearing parameters (larval stage, health or both together) using Pearson correlations. The
507 correlogram (Fig 6) displayed that no strong relation was found between the families and the
508 good survival rate nor between the families and the bad survival rate. This meant that the
509 survival rate alone did not drive the enriched families within the rearing water microbiota.
510 Interestingly, no biomarker specific of the zoea stage was detected; while the *Rhodobacteraceae*
511 were the proxy for the mysis. That differed from Zheng et al, who detected, in the rearing water
512 of *P. vannamei* larvae, that the *Flavobacteriaceae* were specifically enriched in the rearing water
513 hosting the zoea and the BD1-5 clade were more abundant in the water with the mysis (Zheng et
514 al., 2017).
515 With the Pearson correlation, we evidenced several links between the families and the larval
516 stage and health. The *idiomarinaceae* and *Legionellaceae* families were slightly correlated with
517 the zoea with a good survival rate. It was then difficult to use them as real proxies of the zoea
518 with a good survival rate in the rearing water.
519 Our data displayed that the NS11-12 marine group was the specific proxy of the zoea with a high
520 mortality rate in the rearing water. This group was found in various sites and ecosystems, from
521 metal contaminated sites in an urbanized areas (Coclet et al., 2019), in rivers-estuaries sites
522 (Morency et al., 2022), in the Igoumenitsa Gulf (Meziti et al., 2015) and in mangrove areas
523 (Becker et al., 2020), to the rearing water of *P. vannamei* (Zheng et al., 2017). Unfortunately, the
524 ecological role and metabolic activities of the NS11-12 marine group are still unknown.
525 The *Flavobacteriacea,e* and the *Cryomophaceae* in a less extent, were the identified biomarker
526 of the mysis with a high survival rate. The *Flavobacteriaceae* was detected in the rearing water
527 of *P. vannamei* larvae, with higher abundance in the rearing water hosting the zoea (Zheng et al.,

2017); and also in various rearing water samples of adult *P. vannamei* (Md Zoqratt et al., 2018; Kim et al., 2021; Hu et al., 2022). The *Flavobacteriaceae* have been detected in the gut of *P. vannamei* post larvae and juveniles (Huang et al., 2016) as well as in the gut of adult shrimps of this species (Li et al., 2018; Md Zoqratt et al., 2018; Schleder et al., 2020; Rezende et al., 2022). Interestingly, in the gut of healthy *P. vannamei* adults, members of the *Flavobacteriaceaea* have been found in negative interaction with 2 *Vibrio* lineages; while in the gut of diseased shrimps the same *Flavobacteriaceae* taxa were in positive correlation with the 2 *Vibrio* (Dai et al., 2018). The authors argued that in the gut, *Flavobacteriaceae* members occupy specific ecological niches and therefore prevent the pathogens development (Dai et al., 2018). Thus, we can envisage that *Flavobacteriaceae* have a beneficial role on the mysis health and avert opportunistic microorganisms to negatively outcome the rearing water microbiota.

The *Cryomorphaceae* family is present from polar to tropical ecosystems and is often detected in organic-rich locations (Bowman, 2014, 2020). This was similar to our study, indeed, as no water renewal was applied, the rearing water had accumulated lot of organic matter from larval feed, feces, exoskeleton. *Cryomorphaceae* have also been found in the water of a biofloc technology system with *P. stylirostris* juveniles (Cardona et al., 2016), in the rearing water of *P. vannamei* (Md Zoqratt et al., 2018), in the rearing water of *P. vannamei* at the zoea stage (Zheng et al., 2017); as well as associated with *P. stylirsotris* eggs and nauplii (Giraud et al., 2022). Thus, it seemed that *Cryomorphaceae* could have important ecological role in the healthy larvae or could be able to overcome r-strategist microorganisms that could unbalance the rearing water microbiota. The LEfSe and the correlogram allowed to spot out 2 potential proxies in the rearing water containing mysis with a high mortality rate: the AEGEAN-169 marine group and an unknown family related to the *Gammaproteobacteria*. The NRL2 family was rule out as this family was also highly correlated with the Bad survival condition, so we could not untangle the mysis bad survival rate from the bad survival rate effect itself on this biomarker. The AEGEAN-169 marine group has been detected in various marine habitats such as coral reef seawater (Rosales et al., 2020), mangrove (Becker et al., 2020), ocean water column (Cram et al., 2015), in the rearing water of a polyculture aquaculture system with shrimps (*Penaeus chinensis*) jellyfish and clams (Guan et al., 2020); in the rearing water, the eggs and the nauplii of *P. stylirostris* (Giraud et al., 2021) as well as in phytoplankton blooms (Yang et al., 2015). However, this group lacks ecological and metabolic activity information. Considering the

559 unknown family related to the *Gammaproteobacteria*, it is tricky to envisage an ecological role
560 as we couldn't go deeper than the class in the phylogeny for this lineage nor relate it to any
561 function or activity. However, in a way or another these 2 lineages were involved in the rearing
562 water hosting unhealthy mysis either by opportunistic strategy, by being an actor of the larval
563 dysbiosis or by being part of the necrobiome.
564 Together, the LEfSe and the Pearson correlation permitted to uncover specific microbial proxies
565 of a given larval stage and health. In addition, as displayed above, several taxa were previously
566 detected in the natural seawater and could have re-activated according to the rearing conditions.
567 Thus, our data regarding the evidenced biomarkers and the role of the natural seawater will allow
568 us to further forecast the fate of the larval rearing; even before the beginning of the rearing, by
569 designing specific qPCR primers, to look at these proxies in the natural seawater: lagoon and
570 storage waters.
571

572 ## Conclusions

573 Our findings exhibited that the larvae rearing water is a complex and dynamic ecosystem, driven
574 by several parameters: the original natural seawater, the presence of antibiotic or not, the larval
575 stage, the larval health and the necrobiome. We also highlighted that it is hard to untangle the
576 antibiotic effect from the mortality effect on the rearing water microbiota, especially in the case
577 of mass mortalities occurring in the rearing water without antibiotic in comparison with great
578 survivals in the rearing condition with antibiotic. In addition, our results revealed that, given a
579 larval stage and survival rate, several active taxa were spotted out to be specific of this condition
580 (except for the zoea with a good survival rate); and that among these lineages, many of them
581 were originally detected in the natural seawater. That outcome disclosed the great importance of
582 the natural seawater microbiota on the rearing water microbiota. We also showed that the
583 necrobiome associated with dead larvae could also impact the structure of the rearing water
584 microbiota. The biomarker investigation allowed to highlight that *Flavobacteraceae* and
585 *Cryomorphaceae* were proxies in the rearing water of the mysis with a high survival rate; the
586 NS11-12 marine group was specific of the zoea with a bad survival rate; while, the AEGEAN-
587 169 marine group and an unknown family related to the *Gammaproteobacteria,* were the proxies
588 in the rearing water of the unhealthy mysis. To further understand the role of these specific
589 families in the rearing water or on the larvae, several studies are needed, in particular to uncover

their activities and ecological role. Despite the unknown role of these specific families during the rearing, these biomarkers could be used to design specific qPCR primers to be used as a routine proxy to forecast the larval health. They could also be used as an early warning before, per say in the natural seawater, and during the first days of the rearing by detecting and quantifying all the highlighted proxies. Ultimately, the same proxies could also help to monitor and to estimate the evolution of the larval rearing; and to further manage the rearing water microbiota and select beneficial microorganisms for the larvae.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Nolwenn Callac conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Carolane Giraud analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Viviane Boulo, Nelly Wabete and Dominique Pham conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

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Figure

Figure 1: Evolution of the larval survival during the experiment.
Evolution of the larval survival during the experiment compared to the reference in turquoise (e.g.: usual survival rate obtained for a specific day, calculated for each day using data of 10 years of successful rearing; Ifremer data, Pham comm. pers.).Without-A, Without-B, Without-C, correspond to the rearing water without antibiotic in the tanks A, B and C; and With-A, With-B, With-C, stand for the rearing water with antibiotic in the tanks A, B and C. D0 to D9 correspond to the day of the rearing.

Figure 2: Water sample clusterization. Hierarchical clustering diagram based on microbial community dissimilarity, made at the ASV level using the Bray-Curtis matrix. In order to define the 7 clusters, a threshold at 1.5 was set (red dotted line).

Figure 3: Ordination of the water samples based on the PCoA method and a Bray-Curtis dissimilarity matrix. A) PCoA of the rearing water samples without antibiotic according to the sampling day and the 2 water storage samples. **B**) PCoA of the rearing water samples with antibiotic regarding the sampling day and the 2 water storage samples. For each sampling day the larval stage and health were displayed with good for good survival rates and bad for low survival rates, each color correspond to a sampling day in the figure and the sampling day corresponding is noted in the same color in the caption on the right of each PCoA.

Figure 4: Microbial composition of the water samples.
Relative abundance of the main prokaryotic families. The relative abundance is represented in terms of percentage of the total prokaryotic sequences per sample. Only families representing more than 1% of the overall abundance in at least 3 samples are displayed on the barplot. ResI stands for the primary reservoir sample, ResII for the secondary reservoir sample, Control stands for the control water without larvae, antibiotic and food; Without antibiotic for the rearing water without antibiotic, With antibiotic for the rearing water supplemented with antibiotic. D0 to D8

correspond to the sampling day. Sample day are followed by A, B or C which correspond to the replicate tanks for the rearing water with or without antibiotic.

Figure 5: Microbial communities associated with the specific and core microbiomes of the whole rearing experiment in the rearing water and the water storages.

A) Venn diagram of shared ASVs among all the rearing water samples. The light red ellipse corresponds to the ASVs common to all the rearing water hosting the nauplii. The light khaki ellipse corresponds to the ASVs common to all the rearing water hosting the zoea with a good survival rate (Zoea Good). The light green ellipse represents the ASVs common to the rearing water hosting the zoea with a bad survival rate (Zoea Bad). The light blue ellipse corresponds to the ASVs common to the rearing water hosting the mysis with a good survival rate (Mysis Good). The light purple ellipse represents the ASVs common to the rearing water hosting the mysis with a bad survival rate (Mysis Bad). The overlapping area between all the ellipses, corresponds to the core microbiome composed by the 137 ASVs common to all the samples. The numbers inside the ellipses and in the overlapping zones correspond to the total number of ASVs present in the condition. B) Venn diagram of shared ASVs between the specific ASVs of the nauplii in the light red ellipse and the ASVs of the water storages: in light yellow the primary reservoir and in light maroon the secondary reservoir. C) Venn diagram of shared ASVs between the specific ASVs of the Zoea Bad in the light green ellipse and the ASVs of the water storages: in light yellow the primary reservoir and in light maroon the secondary reservoir. D) Venn diagram of shared ASVs between the specific ASVs of the Mysis Good in the light blue ellipse and the ASVs of the water storages: in light yellow the primary reservoir and in light maroon the secondary reservoir. E) Venn diagram of shared ASVs between the specific ASVs of the Mysis Bad in the light purple ellipse and the ASVs of the water storages: in light yellow the primary reservoir and in light maroon the secondary reservoir. F) Venn diagram of the core microbiota of the rearing water and the ASVs of the water storages: in light yellow the primary reservoir and in beige the secondary reservoir.

Figure 6: Differentially abundant families according to the larval growth and survival status; and Pearson correlations between the biomarkers and larval growth and survival status

900 A) LEfSe, linear discriminant analysis (LDA) effect size, exhibiting the families significantly
901 more abundant in the rearing water according to the larval growth and survival status; B)
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903 growth, survival status and both the larval stage and survival status, with high for high survival
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906

Figure 1

Evolution of the larval survival during the experiment

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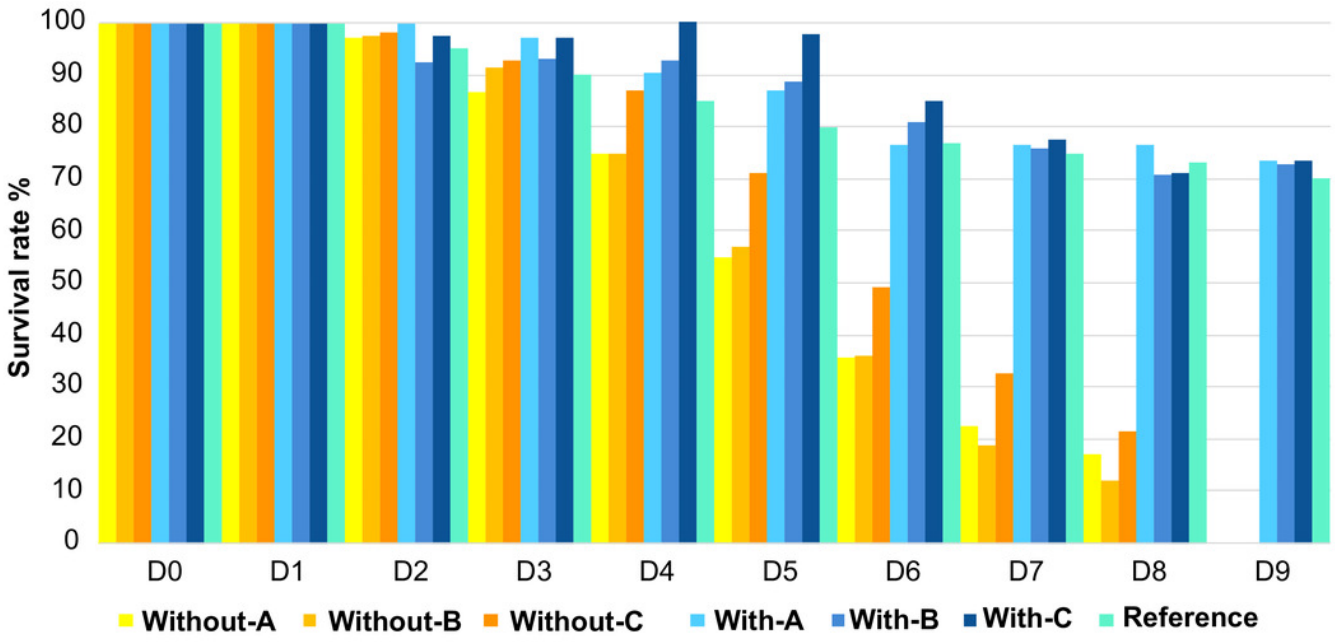
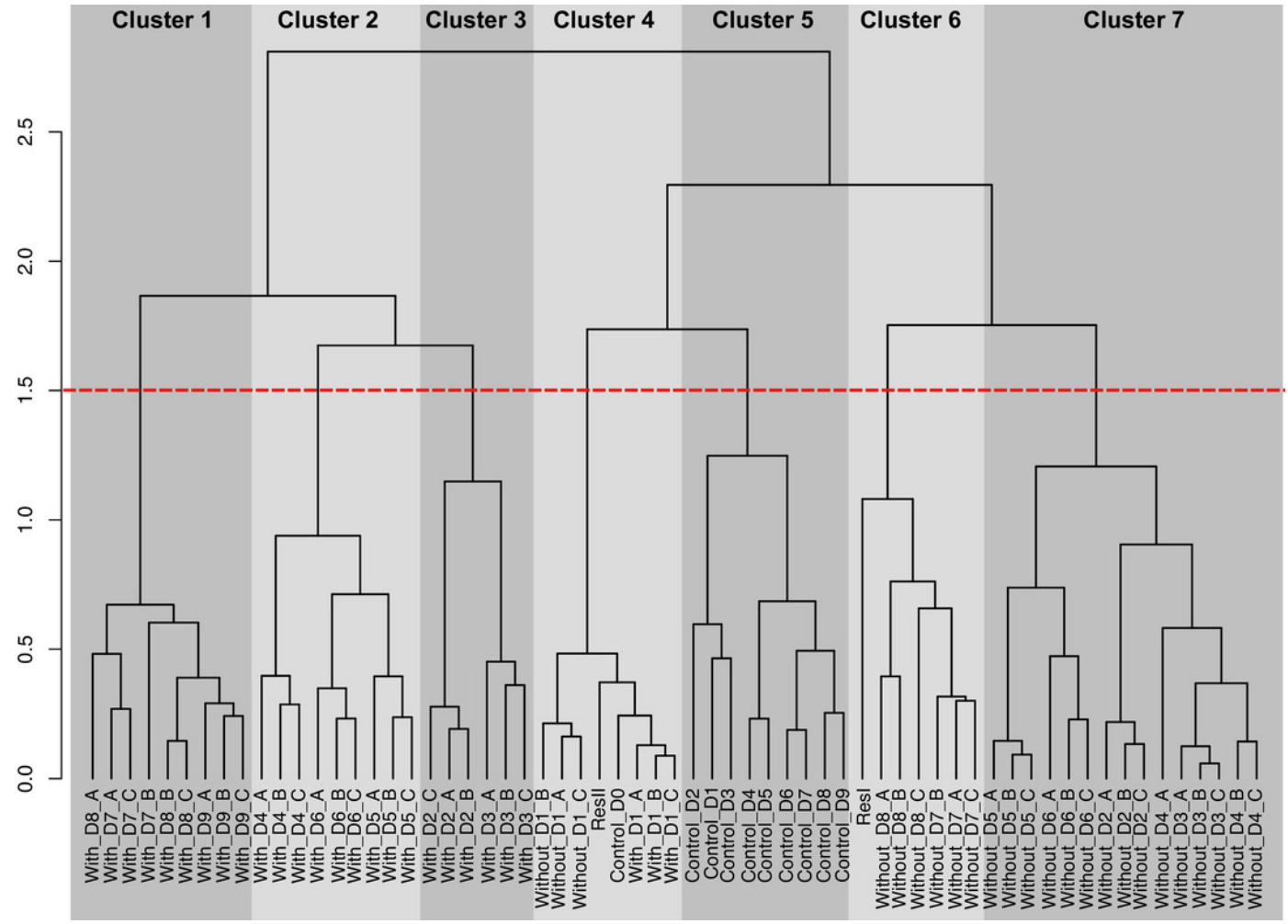


Figure 2

Water sample clusterization

Hierarchical clustering diagram based on microbial community dissimilarity, made at the ASV level using the Bray-Curtis matrix. In order to define the 7 clusters, a threshold at 1.5 was set (red dotted line).



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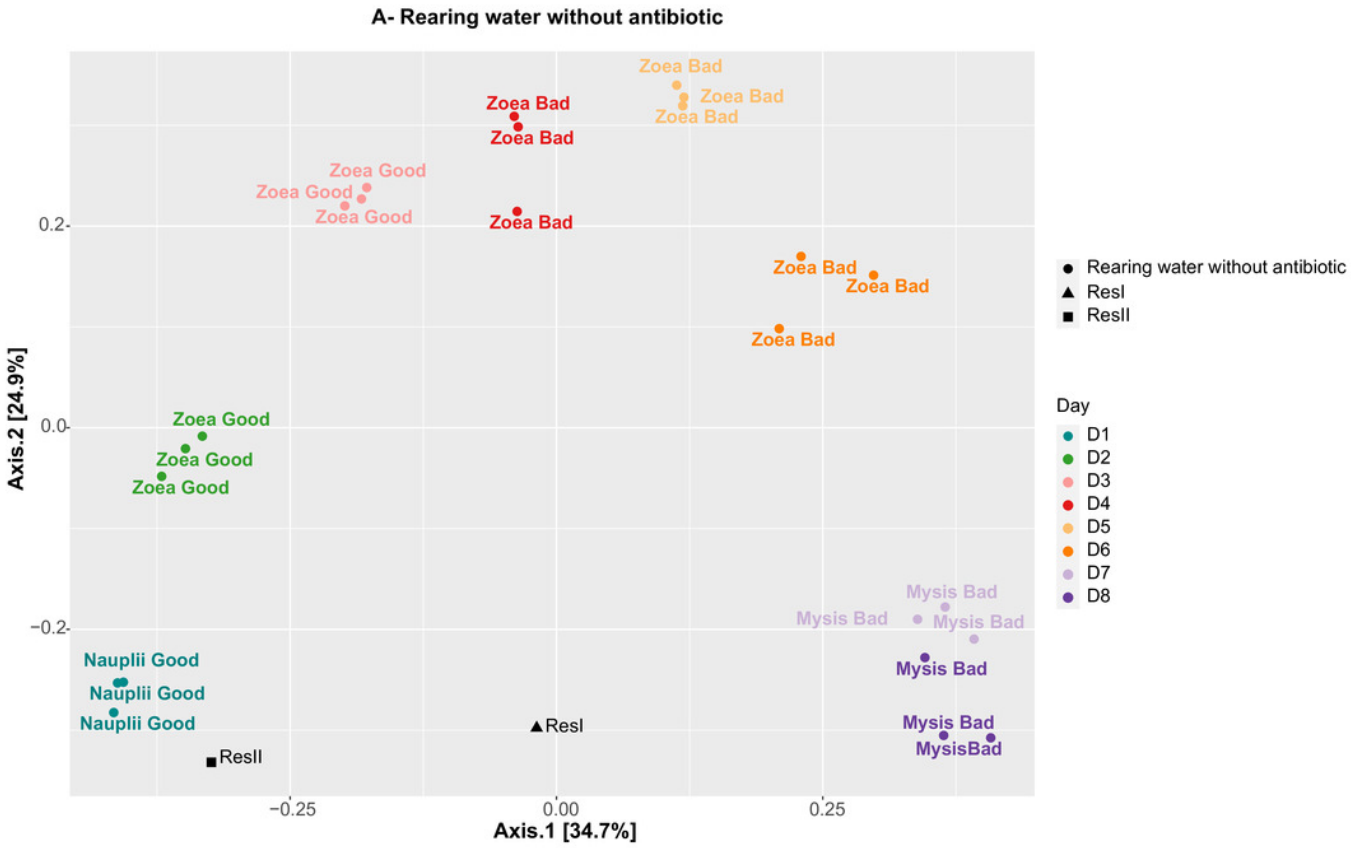
Ordination of the water samples based on the PCoA method and a Bray-Curtis dissimilarity matrix

A) PCoA of the rearing water samples without antibiotic according to the sampling day and the 2 water storage samples. **B**) PCoA of the rearing water samples with antibiotic regarding the sampling day and the 2 water storage samples. For each sampling day the larval stage and health were displayed with good for good survival rates and bad for low survival rates, each color correspond to a sampling day in the figure and the sampling day corresponding is noted in the same color in the caption on the right of each PCoA.


A- Rearing water without antibiotic
Axis.2 [24.9%]
Axis.1 [34.7%]
Rearing water without antibiotic
ResI
ResII
Day
D1
D2
D3
D4
D5
D6
D7
D8

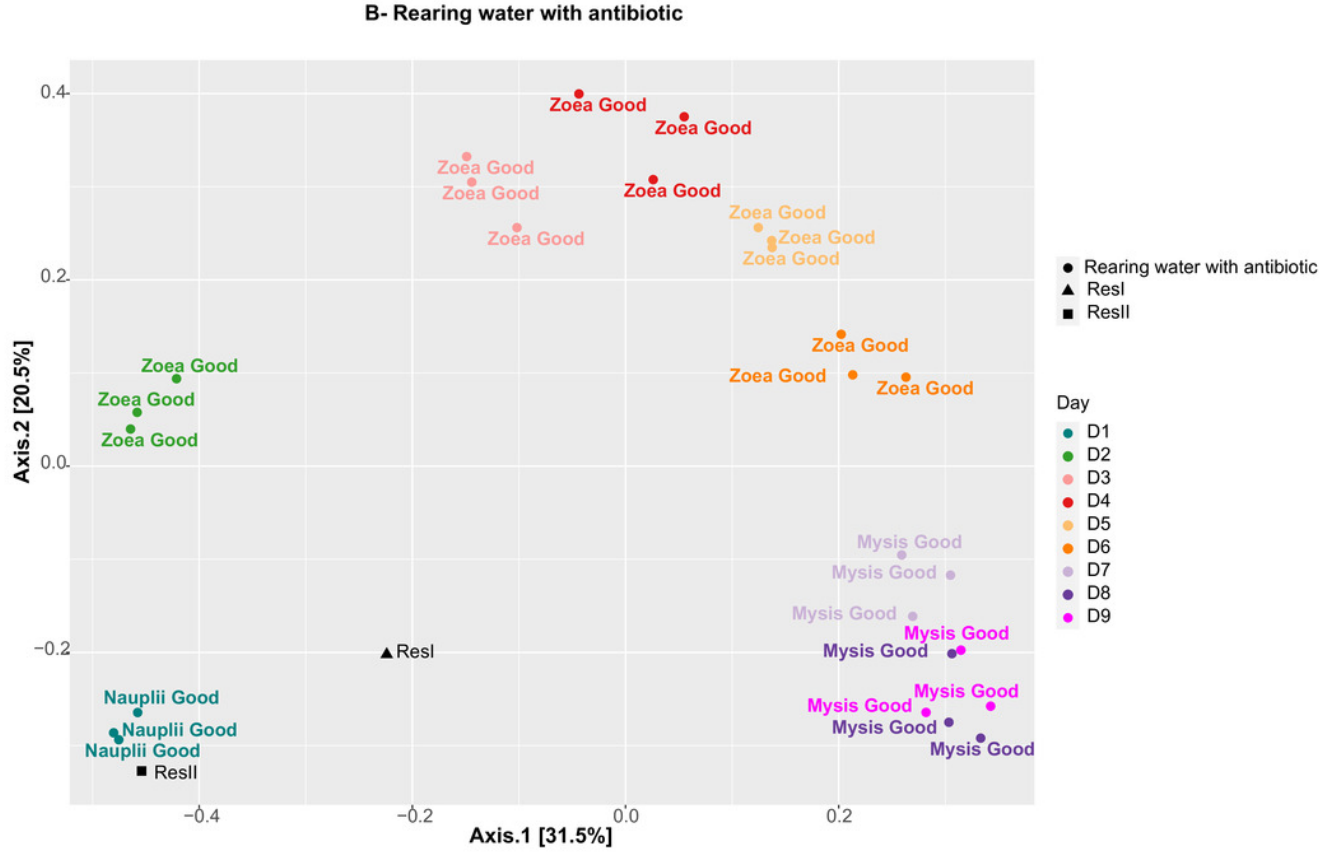

B- Rearing water with antibiotic
Axis.2 [20.5%]
Axis.1 [31.5%]
Rearing water with antibiotic
ResI
ResII
Day
D1
D2
D3
D4
D5
D6
D7
D8
D9

Figure 4

Microbial composition of the water samples

Relative abundance of the main prokaryotic families. The relative abundance is represented in terms of percentage of the total prokaryotic sequences per sample. Only families representing more than 1% of the overall abundance in at least 3 samples are displayed on the barplot. ResI stands for the primary reservoir sample, ResII for the secondary reservoir sample, Control stands for the control water without larvae, antibiotic and food; Without antibiotic for the rearing water without antibiotic, With antibiotic for the rearing water supplemented with antibiotic. D0 to D8 correspond to the sampling day. Sample day are followed by A, B or C which correspond to the replicate tanks for the rearing water with or without antibiotic.

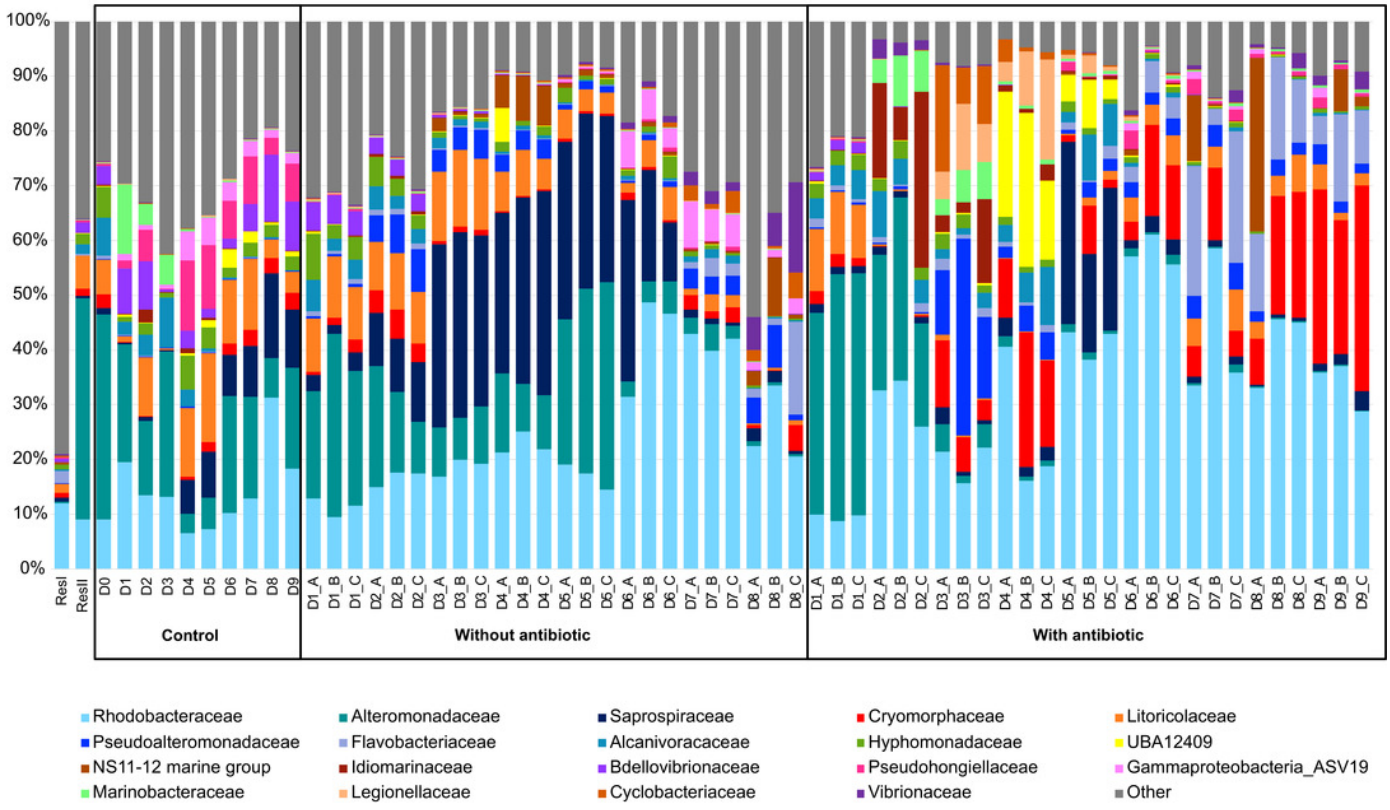


Figure 5

Microbial communities associated with the specific and core microbiomes of the whole rearing experiment in the rearing water and the water storages

A) Venn diagram of shared ASVs among all the rearing water samples. The light red ellipse corresponds to the ASVs common to all the rearing water hosting the nauplii. The light khaki ellipse corresponds to the ASVs common to all the rearing water hosting the zoea with a good survival rate (Zoea Good). The light green ellipse represents the ASVs common to the rearing water hosting the zoea with a bad survival rate (Zoea Bad). The light blue ellipse corresponds to the ASVs common to the rearing water hosting the mysis with a good survival rate (Mysis Good). The light purple ellipse represents the ASVs common to the rearing water hosting the mysis with a bad survival rate (Mysis Bad). The overlapping area between all the ellipses, corresponds to the core microbiome composed by the 137 ASVs common to all the samples. The numbers inside the ellipses and in the overlapping zones correspond to the total number of ASVs present in the condition. B) Venn diagram of shared ASVs between the specific ASVs of the nauplii in the light red ellipse and the ASVs of the water storages: in light yellow the primary reservoir and in light maroon the secondary reservoir. C) Venn diagram of shared ASVs between the specific ASVs of the Zoea Bad in the light green ellipse and the ASVs of the water storages: in light yellow the primary reservoir and in light maroon the secondary reservoir. D) Venn diagram of shared ASVs between the specific ASVs of the Mysis Good in the light blue ellipse and the ASVs of the water storages: in light yellow the primary reservoir and in light maroon the secondary reservoir. E) Venn diagram of shared ASVs between the specific ASVs of the Mysis Bad in the light purple ellipse and the ASVs of the water storages: in light yellow the primary reservoir and in light maroon the secondary reservoir. F) Venn diagram of the core microbiota of the rearing water and the ASVs of the water storages: in light yellow the primary reservoir and in beige the secondary reservoir.

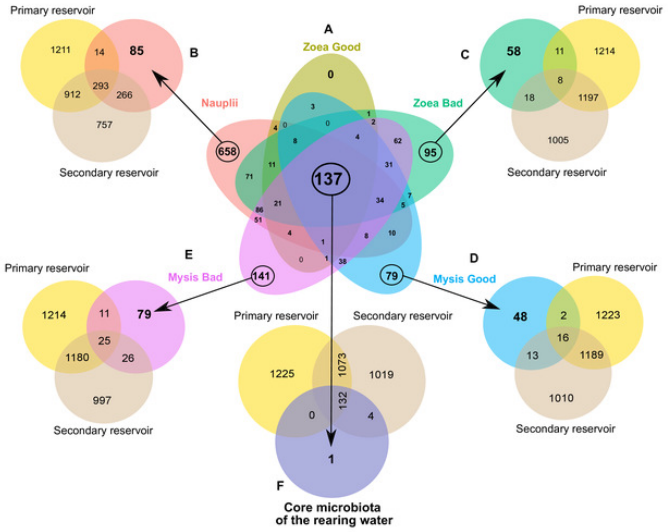

A
Zoea Good
0
B
Nauplii
658
C
Zoea Bad
95
137
Primary reservoir
1211
14
85
912
293
266
757
Secondary reservoir
Primary reservoir
58
11
1214
8
18
1197
1005
Secondary reservoir
E
Mysis Bad
141
Primary reservoir
1214
11
79
25
1180
26
997
Secondary reservoir
D
Mysis Good
79
Primary reservoir
Secondary reservoir
Primary reservoir
48
2
1223
16
13
1189
1010
Secondary reservoir
1225
1073
1019
132
0
4
1
F
Core microbiota
of the rearing water

Figure 6

Differentially abundant families according to the larval growth and survival status; and Pearson correlations between the biomarkers and larval growth and survival status

A) LEfSe, linear discriminant analysis (LDA) effect size, exhibiting the families significantly more abundant in the rearing water according to the larval growth and survival status; B) Correlogram between the biomarkers at the family level highlighted by the LEfSe and larval growth, survival status and both the larval stage and survival status, with high for high survival rate and bad for a low survival rate. Nauplii good do not appear in the correlogram as all the nauplii exhibited a high survival rate and the mortality occurred at the zoea stage.

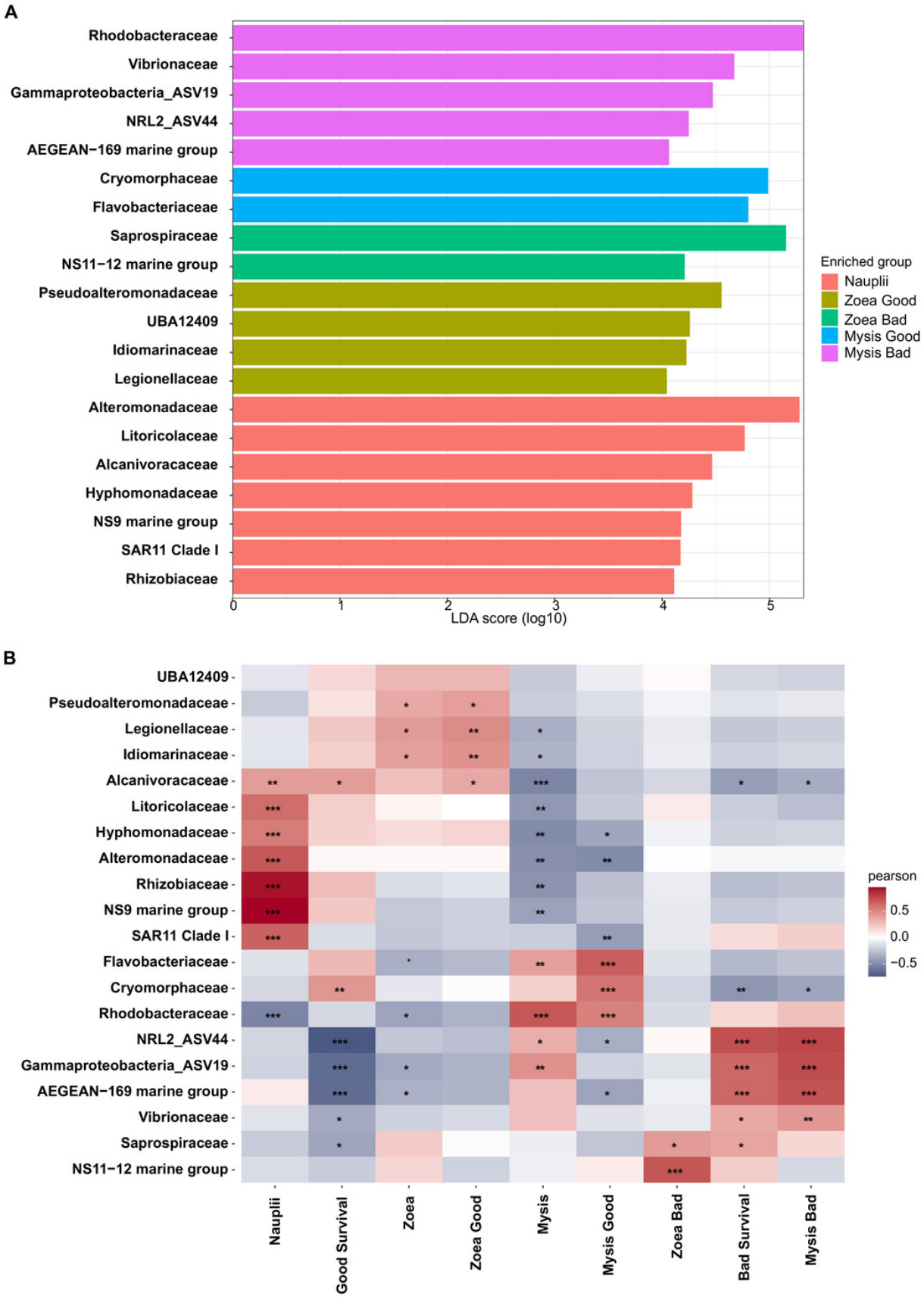

A
Rhodobacteraceae
Vibrionaceae
Gammaproteobacteria_ASV19
NRL2_ASV44
AEGEAN−169 marine group
Cryomorphaceae
Flavobacteriaceae
Saprospiraceae
NS11−12 marine group
Pseudoalteromonadaceae
UBA12409
Idiomarinaceae
Legionellaceae
Alteromonadaceae
Litoricolaceae
Alcanivoracaceae
Hyphomonadaceae
NS9 marine group
SAR11 Clade I
Rhizobiaceae
0
1
2
3
4
5
LDA score (log10)
Enriched group
Nauplii
Zoea Good
Zoea Bad
Mysis Good
Mysis Bad
B
UBA12409
Pseudoalteromonadaceae
Legionellaceae
Idiomarinaceae
Alcanivoracaceae
Litoricolaceae
Hyphomonadaceae
Alteromonadaceae
Rhizobiaceae
NS9 marine group
SAR11 Clade I
Flavobacteriaceae
Cryomorphaceae
Rhodobacteraceae
NRL2_ASV44
Gammaproteobacteria_ASV19
AEGEAN−169 marine group
Vibrionaceae
Saprospiraceae
NS11−12 marine group
Nauplii
Good Survival
Zoea
Zoea Good
Mysis
Mysis Good
Zoea Bad
Bad Survival
Mysis Bad
pearson
0.5
0.0
−0.5

Table 1(on next page)

Time series of larval stage

Time series of larval stage compared to the Reference larval stage index to reach each day (e.g.: usual larval stage obtained for a specific day; stage reference has been calculated for each day using data of 10 years of successful rearing; Ifremer data, Pham comm. Pers.); D0 to D9 correspond to the day of the rearing. Each color corresponds to a specific larval stage, when in black that means that the larvae were all dead in the tanks. Without-A, Without-B, Without-C, correspond to the rearing tanks without antibiotic; and With-A, With-B, With-C, stand for the rearing tanks with antibiotic. The considering larval stage was named, when more than 75% of observed the larvae were at this given stage.

Table 1: Time series of larval stage. Time series of larval stage compared to the Reference larval stage index to reach each day (e.g.: usual larval stage obtained for a specific day; stage reference has been calculated for each day using data of 10 years of successful rearing; Ifremer data, Pham comm. Pers.); D0 to D9 correspond to the day of the rearing. Each color corresponds to a specific larval stage, when in black that means that the larvae were all dead in the tanks. Without-A, Without-B, Without-C, correspond to the rearing tanks without antibiotic; and With-A, With-B, With-C, stand for the rearing tanks with antibiotic. The considering larval stage was named, when more than 75% of observed the larvae were at this given stage.

	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9
Without-A	Nauplii	Nauplii	Zoea 1	Zoea 2	Zoea 2	Zoea 3	Zoea 3	Mysis 1	Mysis 2	
Without-B	Nauplii	Nauplii	Zoea 1	Zoea 2	Zoea 2	Zoea 2	Zoea 3	Mysis 1	Mysis 1	
Without-C	Nauplii	Nauplii	Zoea 1	Zoea 2	Zoea 2	Zoea 2	Zoea 3	Mysis 1	Mysis 2	
With-A	Nauplii	Nauplii	Zoea 1	Zoea 2	Zoea 2	Zoea 3	Zoea 3	Mysis 1	Mysis 1	Mysis 2
With-B	Nauplii	Nauplii	Zoea 1	Zoea 2	Zoea 2	Zoea 3	Zoea 3	Mysis 1	Mysis 2	Mysis 3
With-C	Nauplii	Nauplii	Zoea 1	Zoea 2	Zoea 2	Zoea 3	Zoea 3	Mysis 1	Mysis 2	Mysis 2
Reference	Nauplii	Nauplii	Zoea 1	Zoea 2	Zoea 2	Zoea 3	Mysis 1	Mysis 2	Mysis 3	Post Larvae