Symbiont acquisition strategies in post-settlement stages of two co-occurring deep-sea *Rimicaris* shrimp.

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Short title

Symbiont acquisition of *Rimicaris* spp.

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Abstract

At deep-sea hydrothermal vents, deprived of light, most living communities are fuelled by chemosynthetic microorganisms. These can form symbiotic associations with metazoan hosts, which are then called holobionts. Among these, two endemic shrimp of the Mid-Atlantic Ridge (MAR), *Rimicaris exoculata* and *Rimicaris chacei* are colonized by dense and diversified chemosynthetic symbiotic communities in their cephalothoracic cavity and their digestive system. Although both shrimp harbor similar communities, they exhibit widely different population densities, distribution patterns at small scale and diet, as well as differences in post-settlement morphological modifications leading to the adult stage. These contrasting biological traits may be linked to their symbiotic development success. Consequently, key questions related to the acquisition of the symbionts and the development of the holobiont are still open. Here we examined symbiotic development in juveniles of *R. exoculata* and *R. chacei* from TAG and Snake Pit using 16S metabarcoding to identify which symbiotic lineages are present at each juvenile stage. In addition, we highlighted the abundance and distribution of microorganisms at each stage using Fluorescence *in situ* Hybridization (FISH) and Scanning Electron Microscopy (SEM). For the first time, *Candidatus* Microvillispirillaceae (midgut tube), *Candidatus* Foregutplasma rimicarensis and *Candidatus* BG2-rimicarensis (foregut) were identified in late juveniles stages. However, these lineages were absent in early juveniles stages, which coincides for the midgut tube with our observations of an immature tissue, devoid of microvilli. Conversely, symbiotic lineages from the cephalothoracic cavity were present from the earliest juvenile stages of both species and their overall diversities were similar to those of adults. These results suggest different symbiont acquisition dynamics between the cephalothoracic cavity and the digestive system, which may also involve distinct transmission mechanisms.

Introduction

Most hydrothermal vents occur at depths deprived of light where photosynthesis is not possible. In these habitats, chemosynthesis is instead the main biosynthetic process, performed by microorganisms, living free or in association with metazoan hosts (Kouris et al., 2007; Dubilier et al., 2008; Sogin et al., 2021). These associations between host and microbial symbionts, now called holobionts (Zilber-Rosenberg and Rosenberg, 2008), dominate vent communities. They usually involve large engineer species like *Bathymodiolus* spp. mussels or *Riftia* tubeworms (Nussbaumer et al., 2006; Duperron et al., 2006, 2008, 2010), which have developed diversified symbioses in specific organs. To ensure the maintenance of their symbiotic relationship over successive generations, symbionts are transmitted horizontally (Nussbaumer et al., 2006; Bright and Bulgheresi, 2010; Wentrup et al., 2014). Cases of vertical transmission (directly from the parents) or mixed mode of transmission (both vertical and horizontal) also exist respectively for *Calyptogena* clams and for the scaly-foot snail (Ikuta et al., 2016; Lan et al., 2022). These acquisition phases often go with marked changes in the external and/or internal anatomy of their hosts (Nussbaumer et., 2006; Chen et al., 2018; Franke et al., 2021), which can be triggered by the symbionts themselves (Chun et al., 2008). Hence, symbionts are now increasingly recognized as fundamental actors of their host developmental processes in most metazoan phyla (Carrier and Bosch, 2022).

The shrimp *Rimicaris exoculata* and *Rimicaris chacei* are also symbiotic species co-occurring at active vents along the Mid Atlantic Ridge (MAR) (Williams and Rona, 1986; Zbinden and Cambon Bonavita, 2020). *R. exoculata* live in dense aggregations (up to 3000 individuals per m^2) covering the substratum of active black smoker walls (Segonzac et al., 1993; Hernandez-Avila et al., 2022; Methou et al., 2022). This shrimp can be exposed to quite high temperatures (10-30°C) and to diverse chemical compounds (Schmidt et al., 2008a, b; Methou et al., 2022). *R. chacei* seems to be less abundant, and live in different ecological niches, usually with lower hydrothermal influence (Hernandez-Avila et al., 2022; Methou et al., 2022).

In adulthood, both species have distinct morphologies. *R. exoculata* exhibits a laterally inflated cephalothoracic cavity enclosing the two first pairs of pereiopods (including chelipeds) and hypertrophied mouthparts (such as scaphognathites and exopodites) (Van Dover et al., 1988; Segonzac et al., 1993; Komaï et Segonzac, 2008). On the contrary, the chelipeds of *R. chacei* remain free and functional as its cephalothoracic cavity and mouthparts are not as hypertrophied as *R. exoculata* (Casanova et al., 1993; Segonzac et al., 1993). Despite these morphological differences, both species harbor dense bacterial communities in their cephalothoracic cavity, colonizing the inner side of the branchiostegites as well as the scaphognathites and the exopodites and their setae (Apremont et al., 2018). In both *R. exoculata* and *R. chacei* adults, symbiotic communities of the cephalothoracic cavity are mainly composed of *Campylobacteria*, then *Gammaproteobacteria* followed by minor proportions of *Alphaproteobacteria*, *Desulfobulbia*, *Zetaproteobacteria* and *Bacteroidia* (Zbinden et al., 2008 ; Petersen et al., 2010 ; Hügler et al., 2011 ; Guri et al., 2012 ; Jan et al., 2014 ; Apremont et al., 2018 ; Jiang et al., 2020; Cambon et al., 2021). In *R. exoculata*, these ectosymbiotic communities are renewed every 10 days after each molt event (Corbari et al., 2008a, 2008b) and play a major trophic role for their host, as evidenced by their isotopic ratios (Polz et al., 1998; Gebruk et al., 2000; Methou et al., 2020). This was confirmed by experiments with radiolabelled inorganic carbon showing a direct transfer of the ^{14}C incorporated by the symbionts to the host through the cuticle of the cephalothorax (Ponsard et al., 2013). Conversely, *R. chacei* appears to have a mixotrophic feeding behavior (Gebruk et al., 2000; Methou et al., 2020).

As in other decapods, the digestive system of both *R. exoculata* and *R. chacei* is divided in three distinct regions: the foregut composed of the oesophagus and the stomach, the midgut that comprises the hepatopancreas and the midgut tube, and the hindgut that is the terminal excretion zone (Vogt, 2021). For both species, the foregut is a complex filtering structure made of several plates and setae (Guéganton et al., 2022). Contrary to the foregut and the hindgut which have an ectodermic origin, the midgut is devoid of a cuticle (endodermic origin) and is thus not subjected to molt (Vogt, 2021). The foregut and hindgut of *R. exoculata* are reduced whereas the midgut is very long (Komai and Segonzac, 2008; Durand et al., 2009; Guéganton et al., 2022). On the contrary, the digestive system of *R. chacei* is more similar to that of other caridean shrimp (Segonzac et al., 1993; Komai and Segonzac, 2008; Apremont et al., 2018) with a relatively large stomach, in agreement with its mixotrophic behavior.

Like the cephalothoracic cavity, the digestive system of *R. exoculata* and *R. chacei* hosts symbiotic microorganisms (Durand et al., 2009, 2015; Apremont et al., 2018). Two distinct communities were identified: the first one is located in the foregut and consists mainly of *Candidatus* Foregutplasma rimicarensis and *Candidatus* BG2_rimicarensis (Aubé et al., 2022). In adult shrimp, these symbionts have been observed on the setae of the oesophagus and in the pyloric chamber of the stomach (Guéganton et al., 2022). In the midgut tube, microbial communities consist mainly of *Candidatus* Rimicarispirillum spp. (Aubé et al., 2022) that form long thin "spaghetti-like" bacteria cells (Guéganton et al., 2022). These are inserted between the microvilli of the epithelial cells, colonizing the ectoperitrophic space (Guéganton et al., 2022), which is recognized as "sterile" in several other crustaceans (Martin et al., 2020). *Candidatus* Rimicarispirillum spp. are not subject to molt and their growth is believed to be under host control as they exhibit no cell division (Durand et al., 2009; Apremont et al., 2018), while having all the genes for chromosome replication and cell division (Aubé et al., 2022).

While symbioses are well characterized in *Rimicaris* adults, less is known about these bacterial partners at juvenile stages (Guri et al., 2012; Cowart et al., 2017), particularly for *R. chacei*. Juveniles are easily recognizable from the adults because of their red/orange color, which is due to lipid storage (Pond et al., 2000; Methou et al., 2020). Both species are relatively similar at these early stages, leading to potential misidentifications, but taxonomy of early stages was recently revised, clarifying the different juvenile stages of each species (Methou et al., 2020). In their environment, juveniles of *R. chacei* live in nurseries and are separated from their adults, whereas juveniles of *R. exoculata* gather in patches adjacent to adult assemblages (Hernandez-Avila et al., 2022; Methou et al., 2022). Whereas *R. exoculata* adults were in higher number than juveniles, juveniles of *R. chacei* showed a high abundance contrasting with a relatively low number of their adults (Methou et al, 2022). This limited number of *R. chacei* adults could be related to a collapse of the population during recruitment. Such difference in the demography of the two *Rimicaris* species at MAR vent sites might stem from differences in the niches they occupy and/or the symbiosis development in juveniles following settlement with metamorphoses differing according symbiont colonization level (Methou et al., 2023b).

However, the timing of symbiont acquisition and the colonization dynamic of each hosting organs remain unclear. Although a vertical transmission was suggested for midgut symbionts (Durand et al. 2015), these lineages could not be detected in egg broods of *R. exoculata* (Guri et al., 2012; Cowart et al., 2017; Methou et al., 2019). On the other hand, digestive symbionts could not be found in the environment of the shrimp either (Hügler et al., 2010; Flores et al., 2011). *Rimicaris* broods nevertheless exhibit bacterial communities developing on the envelop of eggs with similar lineages than those found in the cephalothoracic cavity (Methou et al., 2019). A transmission of these symbionts to larval stages by

ingestion/scrapping of the egg enveloppe is unlikely as *Rimicaris* larvae have undeveloped buccal organs preventing the ingestion of materials upon hatching (Hernandez-Avila et al., 2015). Moreover, nutrition of juveniles of both *Rimicaris* species before recruitment is largely based on photosynthetic derived organic matter and gradually shifts toward a chemosynthetic diet during metamorphosis (Methou et al., 2020). This trophic shift may reflect the development of symbioses suggesting that recruitment could be a key phase of symbiont acquisition for these shrimp.

Therefore, we focused here on juvenile stages of *R. exoculata* and *R. chacei* from two vent sites along the MAR (TAG and Snake Pit) using a combination of microscopy (Fluorescent *in situ* Hybridization (FISH) and Scanning Electron Microscopy (SEM)) and 16S rRNA metabarcoding to address the following questions: 1) Which are the earliest juvenile stages in which each of the symbiotic lineages can be detected? 2) What are the dynamics of the different symbiotic communities throughout the juvenile development? 3) Does these acquisition timing and dynamics vary between host species and vent sites? 4) Is the establishment of symbiotic communities linked to anatomical changes of the host organs?

Materials and methods

Sample collections

Samples were collected at two vent fields along the MAR: TAG (26°08' N - 44°49' W, 3660 m depth) and Snake Pit (23°23' N - 44°58' W, 3480 m depth) during the BICOSE cruise (10 January to 11 February 2014, DOI https://doi.org/10.17600/14000100), the HERMINE cruise (16 March to 27 April 2017, DOI https://doi.org/10.17600/17000200) and the BICOSE2 cruise (26 January to 10 March 2018, DOI http://dx.doi.org/10.17600/18000004). The different specimens were caught in shrimp aggregations or nurseries using the suction sampler of the HOV (Human Occupied Vehicle) Nautile, or the ROV (Remotely Operated Vehicle) VICTOR 6000, operated from the R/V *Pourquoi pas?*. Once on board, some shrimp were immediately dissected under sterile conditions and tissues were fixed for FISH studies in a 3% formalin seawater solution for 3 hours to keep cell integrity. Other specimens were fixed without dissection for whole individual observations. Samples were then rinsed with a phosphate buffered saline solution (PBS) and stored in a PBS/Ethanol (1:1) solution at -20°C (Durand et al., 2009). Tissues of some specimens were fixed in a 2.5% glutaraldehyde solution (16 hours at 4°C), and then rinsed and stored at 4°C in buffered filtered seawater containing a biocide (NaN_3 at 0.44g/L) to avoid bacterial development until use for scanning electron microscopy (SEM) observations. Other shrimp specimens were frozen at -80°C for later dissections at the laboratory for DNA extraction or fixation for FISH (**Appendix 1.**).

DNA extraction

In the laboratory, we defrosted and aseptically dissected on ice 50 specimens of *R. chacei* and *R. exoculata* from TAG and Snake Pit (stages A, B, and subadults) (**Appendix 1.**, **Appendix 2.**). For each specimen used in metabarcode analysis, species identity was first confirmed through *COI - cytochrome oxidase I* – sequencing using DNA extracted from pleopods. For prokaryotic metabarcoding, midgut tube, foregut, gills, branchiostegites, scaphognathites and exopodites were dissected (the three last were pooled together for DNA extraction and metabarcoding) (**Appendix 2.**).

Pleopods DNA was extracted using the E.Z.N.A® Tissue DNA Kit (Omega BIO-TEK), following instructions of the manufacturer. DNA was eluted in 100 µL (total) Elution Buffer and then stored at -20°C.

DNA for bacterial metabarcoding was extracted from dissected tissues using the Kit Genomic DNA from Soil: NucleoSpin® Soil kit (Macherey-Nagel) following instructions of the manufacturer, using SL2 lysis buffer. DNA was eluted in 30 µL or 50 µL (respectively digestive system tissues or cephalothoracic tissues) SE Buffer and then stored at -20°C.

COI sequencing

DNA amplification, using a Gene-Amp® PCR System 9700 (Applied Biosystems, Forster City, CA), was performed with the specific probes Cari-COI-1F (5'-GCAGTCTRGYGTCTTAATTTCCAC-3') and Cari-COI-1R (5'-GCTTCTTTTTTACCRGATTCTTGTC-3') (Hernández-Ávila, 2016) which produce a 891 bp fragment (**Appendix 3.**). The PCR products were sequenced (Sanger Sequencing) at Eurofins Genomics GmbH. Sequences were aligned with the MUSCLE (MUltiple Sequence Comparison by Log- Expectation) algorithm (Edgar, 2004) using Geneious software v9 (Kearse et al., 2012) and were compared to three *COI* sequences of *R. exoculata* adults (MT270775, MT270774 et MT270776) and three *COI* sequences of *R. chacei* adults (MT270739, MT270748 et MT270708) using the BLAST search program within the NCBI GenBank database (Altschul et al., 1990).

Bacterial 16S rRNA gene amplifications

Amplification of the bacterial 16S rRNA gene was performed with the universal primers Whoi341 (5'-CTTTCCCTACACGACGCTCTTCCGATCTCCTACGGGNGGCWGCAG-3') and Whoi785 (5'-GGAGTTCAGACGTGTGCTCTTCCGATCTGACTACHVGGGTATCTAATCC-3') with adapter (Herlemann et al., 2011), which targets the V3 - V4 regions of the bacterial 16S rRNA gene (444 bp), using a Gene-Amp® PCR System 9700 (Applied Biosystems, Forster City, CA). PCR reaction mixes and cycles are provided in **Appendix 3**. For each sample, 3 PCR reactions were performed with different DNA amounts to maximize chances to have good amplifications. Amplification products of the 3 PCRs were pooled for the following steps. We performed nested PCRs when amplifications did not yield enough material. A first PCR round was performed with primers E8F (5'- AGAGTTTGATCATGGCTCAG-3') and 907R (5'-CCGTCAATTCTTTGAGTTT-3') amplifying a 900 bp fragment. Again, for each sample, 3 PCR reactions were performed with different DNA amounts to maximize chances to have amplifications (**Appendix 3.**). A second nested PCR was performed on the amplicons of each of the 3 previous PCR using Whoi341 and Whoi785 (**Appendix 3.**). The amplicons of the three nested PCR were pooled and purified with the Nucleospin® Extract II (Macherey-Nagel) following instructions of the manufacturer. DNA was eluted in 60 µL (total) NE Buffer and stored at -20°C. Some negative PCR controls were also purified to be sequenced (**Appendix 3.**). Amplification products were sent to the GeT PlaGe platform (Castanet-Tolosan, France) for prokaryotic diversity sequencing using an Illumina MiSeq platform with a paired-end read length of 2 x 250 bp with chemistry V3.

Metabarcoding analyses

The DNA metabarcoding data were processed using SAMBA v3.0.1 (Standardized and Automated Metabarcoding Analyses (https://gitlab.ifremer.fr/bioinfo/workflows/samba) an

open-source workflow using NextFlow workflow manager (Di Tommaso et al., 2017) which automates and standardizes the analysis of metabarcoding data through a suite of "standard" sequence treatment software. SAMBA is built around three main steps: data integrity checking, bioinformatics processes and statistical analyses. First, it consists in a checking process that allows to verify raw data integrity. Next, sequencing primers are trimmed from reads and reads where primers are not found are removed, using QIIME 2 version 2019.10 (Bolyen et al., 2019). Then DADA2 (Callahan et al., 2016) is used to filter bad quality reads, correct sequencing errors, overlap paired reads, cluster sequences into ASV (Amplicon Sequence Variants) and remove chimeras. Here DADA2 were executed with the optimal paremeters. The dbOTU3 (Olesen et al., 2017) algorithm was used through QIIME 2. It clusters ASV according to sequence similarity and abundance profil in order to take into account the oversurestimation of diversity produced by DADA2. The *microDecon* R package (McKnight et al., 2019) is also used to remove contaminating ASVs present in control samples (extraction, PCR, purification blank). Taxonomic classification was achieved using the SILVA 138 reference database (Quast et al., 2012; Glöckner et al., 2017).

The final step of SAMBA consists in statistical analysis of α diversity and β diversity. We used observed richness (ASVs numbers) and Chao1 index (estimate the expected richness) to estimate the species richness within each sample. Statistical analyses were performed with both indexes. Only results with Chao1 are presented here since both gave identical results. ANOVA (Analysis of the Variance) tests were performed to compare the α diversity between species, sites and stages. β diversity analyses were achieved by ordination method using Non-metric Multidimensional Scaling (NMDS) based on Jaccard distances (reflects bacterial communities composition) calculated on a normalized dataset (DESeq2 using standardization based on negative binomial distribution). Permutational multivariate analyses of variance (PERMANOVA) were performed using the Adonis function of package vegan to assess the influence of site, species and life stage on bacterial community. Outside the SAMBA workflow, the tool ANCOM-BC v2.0.3 (ANalysis of COmposition of Microbiomes with Bias Correction) is used to detect differentially abundant taxa in different samples (Lin and Peddada, 2020). The values obtained with ANCOM-BC are characterized by "W" (W-statistic, the test statistic of ANCOM-BC) that represents the standard error of the mean difference of absolute abundance between groups in log scale, p represents the P-value obtaines from two-sided Z-test using the test statistic W and q that represents the adjusted p-value. The R software (version 4.2.2) was used to perform statistical analyses and produce data visualizations.

BLASTN in Blast+ v2.12.0 (Camacho et al. 2009) was used to control the dominant ASVs affiliation to *Candidatus*_Hepatoplasma and uncultured_Deferribacteres against the 16S rRNA sequences retrieved from MAGs obtained by Aubé et al., 2022 using a num_alignments of 1.

Fluorescent in situ hybridization

Fluorescent *in situ* hybridization analyses were performed on dissected branchiostegites, scaphognathites, exopodites, midgut tube and foregut of juveniles at different stages (**Appendix 1., Appendix 4.**). Entire juveniles were also used. Fixed tissues were progressively dehydrated in PBS/ethanol series at ambient temperature, then transferred to ethanol/resin series at 40°C (Duperron et al., 2007). Dissected organs or the entire juveniles were then embedded in polyethylene glycol-distearate -1-hexadecanol (9: 1) resin (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). After polymerization, resin blocks containing the samples were stored at -20°C. They were trimmed into 8-10 µm transversal tissue sections with a RM 2255 microtome (Leica Biosystems, Nussloch, Germany). Sections (between 3 and 7) were placed on slides (Menzel-Gläser Superfrost® Plus, USA). Before hybridization, resin was removed

with ethanol (3 times 5min in 96% ethanol) and tissues were partially rehydrated (5 min in 70% ethanol). Then tissues were hybridized in a reaction mix containing one or several FISH specific probes labelled with cyanine 3 (Cy3) or cyanine 5 (Cy5) dyes (**Appendix 5.**) (at 0.5µM final concentration) and the hybridization buffer [0.9M NaCl, 0.02M Tris-HCl [pH 7.5], 0.01% [w/v] sodium dodecyl sulphate (SDS), 20%, 30%, 35% or 45% deionized formamide], and incubated for 3 hours at 46°C. After hybridization, sections were briefly pre-rinsed in a washing buffer at 48°C, and washed in a rotary oven in the washing buffer at 48°C during 30 minutes. The washing buffer is adapted to the stringency condition used for hybridizations [0.215M, 0.102M, 0.07M, 0.03M NaCl respectively for 20%, 30%, 35% or 45% formamide, 0.02M Tris-HCl [pH 7.5], 0.005M EDTA [pH 8] and 0.01% [w/v] SDS]. After washing, the slides were briefly rinsed twice with distilled water: first at 48°C then at ambient temperature. Finally, sections were mounted with SlowFade™ Gold antifade reagent with DAPI (Invitrogen) and stored at -20°C in the dark. Observations of hybridized tissues were made using a Zeiss Axio Imager.Z2 microscope equipped with the Apotome.2® sliding module and Colibri.7 light technology (Zeiss, Oberkochen, Germany). Images were produced using the Zen software (Zeiss).

Scanning Electron Microscopy

Dissected foreguts and midgut tubes of *Rimicaris exoculata* and *Rimicaris chacei* stage A, B and subadult from TAG were used for electron microscopy. Glutaraldehyde fixed samples were dehydrated in ethanol series (10% to 100% in 8 steps), before being placed in a filter holder (superposition of stainless-steel washer and 0,2 µm polycarbonate filter) and critical-point dried (Leica EM CPD300). Once dried, they were affixed to a stub using carbon glue and then coated by golds puttering (60% gold/40% Palladium, Quorum Technologies SC7640). SEM observations were performed using a Quanta 200 MK microscope (FEI, Hillsboro, OR) and images were taken with the Xt Microscope Control acquisition program (Soft Imaging System, Munster, Germany).

Results

PCR comparison

The use of two distinct PCR approaches may biased our results (Kanagawa, 2003). Therefore, a set of samples was amplified with both direct and nested PCRs in order to evaluate potential bias. According to the analysis, no significant bias were observed (**Appendix 6., Supplementary Figure 1., Supplementary Tables 1., 2., 3.**). This suggests that the PCR procedure did not significantly change the bacterial DNA amplification obtained, and samples amplified with both PCR procedures were further analyzed together.

Global diversity of symbiotic tissues in juveniles of Rimicaris spp.

First, all samples were considered together. Richness diversity analyses showed significant influence of the tissue and site factors (respectively p = 5.63e-06 and p = 0.000356 with Chao1), the significant influence of tissue also appearing when interactions with host species were considered (**Figure 1., Supplementary Tables 4.**). β diversity analyses showed that the diversity of symbiotic communities varied significantly with all considered factors: site, tissue, host species and life stages (p = 0.0001 each with Jaccard) (**Supplementary Tables 4.**).

To study in more details how bacterial communities evolved through development of each species, we analyzed metabarcode data of each major symbiotic tissue separately: cephalothoracic cavity, foregut and midgut tube. According to the diversity of the bacterial

communities observed in adults, each organ/cavity can be considered as a proper functional entity within which we want to analyze the variations. In the following sections, an ASV table has been produced for each tissue separately.

Digestive anatomy and symbiotic communities of the foregut

Stereomicroscope observations of whole juveniles stomach of each species show they look similar and only differ in size, those of *R. chacei* being slightly larger than those of similar stage *R. exoculata*. The oesophagus, as for adults, is really short, curved and narrow. This structure links the anterior part of the stomach to the shrimp's mouth. The stomach becomes larger in older stages, being in subadults roughly twice the size it is in stage A juveniles (Methou et al., 2023b). In all juvenile stages, the stomach forms a single cavity with two distinct parts, as for adults (**Figure 2A.**). First, a large and smooth structure, the cardiac chamber (**Figure 2B., Supplementary Figure 2B.**), represents the most part of the stomach (little bit larger for *R. chacei* subadult specimens than *R.* exoculata subadult specimens). Between the cardiac chamber and the midgut tube, the pyloric chamber appears externally as a pair of striped "balls" (**Figure 2A., C.**). As for adults, the cardiac chamber is a simple structure covered of thin setae, well developed in stage A juveniles for both species. It is composed of a cardiac floor that is a complex filtration structure made of a superposition of ossicles and many setae (for complete description see Guéganton et al., 2022) (**Figure 2B., Supplementary Figure 2C.**). The pyloric chamber is also well developed in stage A juveniles for both species. It is composed of different complexe and filtering structures as the pylorus (pyramidal structure, **Figure 2A., Supplementary Figure 2D**.). This pyramid lies on the floor of the pyloric chamber and is made of different layers of plates, each covered with dense and serrulate setae (**Figure 2A., Supplementary Figure 2D.)**. This filter is not attached to the cardiac floor. Rather, it is located under the floor, suggesting that the minerals and nutrient may fall along the cardiac floor crest directly into this pyramidal filter. On the sides of the pyramidal filter – the inner side of the lateral walls of the striped "balls" – there is a dense mat of setae, which are of two types (**Figure 2A., Supplementary Figure 2E., F.)**. The most abundant look like the setae of the unpaired anterior ossicle – in the center of the mat – and the others are thinner and composed of several branches – on the periphery of the mat. There are minerals at the base of these mats (**Supplementary Figure 2E.)**. On the ceiling of the pyloric chamber, other setae – very long, simple and thin – are visible on the cuticle and hang over the pyramidal filter (**Supplementary Figure 2F.)**.

Our metabarcoding analyses showed that species richness of the foregut communities did not significantly vary with any of the considered factors (site, stage, species, respectively $p = 0.679$, $p = 0.666$ and $p = 0.634$, , Choa1 index, ANOVA) (**Supplementary Tables 5.**). Still, beta diversity with NMDS showed a clear separation between vent sites (**Figure 2D.**). In addition, it highlighted changes in foregut bacterial diversity with life stage and a separation between species that was clear for older juveniles (stage B and subadult) while bacterial diversities in stage A were more similar and partially overlapped between the two species (**Supplementary Figure 2A.**). PERMANOVA analyses confirmed these observations. The bacterial community associated with the foregut was significantly influenced by the site ($p = 0.0001$, Jaccard index, Adonis), the species ($p = 0.0001$, Jaccard index, Adonis), and the life stage ($p = 0.0001$, Jaccard index, Adonis) (**Supplementary Tables 5.**).

Vizualization with barplots (**Figure 2E.)** showed that bacterial taxonomic composition was diversified in the foregut. Whatever the species and the life stage, *Sulfurovum* spp. (*Campylobacteria* class) seemed to dominate the bacterial communities in juveniles. *Hepatoplasmataceae* were also one of the most represented lineages. Twenty-five ASVs were affiliated to this lineage in our samples. We compared the dominant ASVs affiliated to

Candidatus Hepatoplasma spp. to the 16S rRNA sequences retrieved from MAGs obtained by Aubé et al., 2022. Two main ASVs retrieved were affiliated to *Candidatus* Foregutplasma rimicarensis (with 99.77 % - 100% sequence identity) and one to *Candidatus* Bg2_rimicarensis (100% sequence identity) (Aubé et al., 2022). At TAG and Snake Pit, *Candidatus* Foregutplasma rimicarensis was mainly retrieved (**Table 1., Figure 2E.**). *Hepatoplasmataceae* were almost absent in stage A and subadults *R. chacei* at Snake Pit, but were detected in higher proportions in subadults at TAG, even being a dominant lineage for TAG individuals. In *R. exoculata*, *Hepatoplasmataceae* were almost not detected in stage A juveniles at Snake Pit, but not at TAG. Similarly to *R. chacei*, their proportions increased in later juvenile stages, but with high variability among individuals. In *R. exoculata* subadults, *Hepatoplasmatacae* were also detected in high proportion, similar to those found in stage B juveniles at Snake Pit, but not at TAG where subadults generally exhibited low proportions of this lineage (except one individual) (**Table 1., Figure 2E.**). This result was confirmed by ANCOM-BC analysis (**Supplementary Tables 6.**). At the genus level, *Candidatus* Hepatoplasma spp. was significantly more abundant in later juvenile stage (W = 31.74, q = 2.374708e-05, ANCOM-BC). Even if the metabarcoding analysis highlighted the presence of *Hepatoplasmatacae* (**Table 1**.), they were visualize in FISH with Myco378-1 neither in the oesophagus nor in the stomach in both *R. exoculata* and *R. chacei* juveniles (**Figure 2C.**). Neither in the oesophagus, nor in the stomach the probe gave a fluorescent signal. In some subadult individuals of *R. exoculata* from TAG, Myco378-1 (Guéganton et al., 2022) highlighted some coccoid bacteria in the alimentary bolus or near the tissues. Still, the high mineral content in the alimentary bolus may hide the bacteria and prevent hybridization signal visualization due to autofluorescence.

Digestive anatomy and symbiotic communities of the midgut tube

The structure of midgut tubes from stage A juveniles of both species clearly differs from midgut tubes of other stages with no visible microvilli (**Figure 3A.**). On the other hand, the midgut tube was well developed for stages B and subadults of *R. exoculata* and subadults of *R. chacei* with microvilli of the epithelial cells already visible (**Figure 3B., Supplementary Figure 3A, B.**). They form a thick and dense layer between the epithelium and the alimentary bolus that is full of minerals.

Our metabarcoding analyses showed no significant variation in bacterial richness with life stage, species or site (respectively p = 0.13002, p = 0.07731 and p = 0.14290, Chao1 index, ANOVA), but interaction between them had a significant impact on richness index (p = 0.00989, Chao1 index, ANOVA) (**Supplementary Tables 7.**). Similar to foregut communities, NMDS showed a clear separation of midgut community compositions between vent sites and also highlighted changes with life stages and a separation between species that was clearer for older juveniles (stages B and subadults) (**Figure 3C., Supplementary Figure 3C.)**. A PERMANOVA analysis confirmed that the site, the species and the stage all significantly influenced bacterial communities' structure (p = 0.0001, Jaccard index, Adonis) (**Supplementary Tables 7.**).

As for the foregut, *Sulfurovum* spp. (*Campylobacteria* class) were detected in all specimens, being in large proportion or even dominant in most cases (**Figure 3E.**). Two other lineages were also well represented: *Tyzzerella* spp. (*Clostridia* class) and *Deferribacteraceae* spp. (*Deferribacteres* class). As for the foregut, we compared the main *Deferribacteres* ASVs against the 16S rRNA sequences retrieved from MAGs obtained by Aubé et al., 2022. We identified that 5 main ASVs were affiliated to the genus *Candidatus* Rimicarispirillum spp. (with 99.5 % - 100% sequence identity) and 2 other ASVs were 96% identical to 16S sequences within the MAGs obtained by Aubé et al., 2022. These two ASVs belong to the *Candidatus* Microvillispirillaceae family. In all samples, most *Deferribacteraceae* spp. were affiliated to

Candidatus Rimicarispirillum spp.. Other *Candidatus* Microvillispirillaceae lineages were almost not found at TAG, but occurred in significant proportions at Snake Pit, although *Candidatus* Rimicarispirillum remained the dominant *Deferribacteraceae* at both sites (**Table 2., Figure 3E.**). *Candidatus* Microvillispirillaceae were almost not detected in stage A juveniles except in one out of 10 individuals of each species at TAG where they dominated midgut communities (**Table 2.**). These data were supported by SEM : no bacteria was visible whatever species or site, as well as by FISH (probe Eub338 and specific probe Def1229, Amann et al., 1990; Guéganton et al., 2022) with no *Candidatus* Microvillispirillaceae observed in any of the midgut tube sections of stage A juveniles (**Supplementary Figure 3D.**). In contrast, *Candidatus* Microvillispirillaceae were found in large proportions in almost all later juvenile stages and subadults at both sites (ANCOM-BC, W = 44.25, q = 3.982480e-08) (**Supplementary Tables 8.**). According to SEM observations, long and well-developed bacteria with the typical *Candidatus* Microvillispirillaceae morphology attached to the tissue, extending out and entangled within the microvilli, were visible in both stages B and subadults (**Figure 3B., Supplementary Figure 3A., B.)**. The same bacteria, appearing as long "spaghetti-like" cells, were observed in FISH with the specific probe Def1229 in stage B and subadut for *R. exoculata* and *R. chacei* (Snake Pit and TAG) (**Figure 3D., Supplementary Figure 3E., F.**). They were not visible all along the midgut tube, but were mostly observed in the posterior part of the midgut close to the hindgut, whereas the anterior part seemed uncolonized yet. They were separated from the alimentary bolus by the peritrophic membrane as observed for adults (Durand et al., 2009; Guéganton et al., 2022). *Tyzzerella* spp. were detected in varying proportions among individuals and tended to be more present in *R. exoculata*, and *Candidatus* Foregutplasma rimicarensis and *Candidatus* Bg2_rimicarensis were identified in the midgut of some specimens (mainly subadults at TAG) (**Figure 3E.**).

Symbiotic communities of the cephalothoracic cavity

In both species, we observed an increasing bacterial colonization of the cephalothoracic cavity along the host post-settlement development (**Figure 4.**). Indeed, the mouthparts and the branchiostegites were generally colonized by denser bacterial communities in subadults than in stage A juveniles. In addition, filamentous bacteria presented more subunits and were more abundant in subadults compared to juvenile stages (**Figure 4.**). Overall, *R. chacei* were less colonized than *R. exoculata* at equivalent life stages. Cephalothoracic cavities of *R. chacei* stage A juveniles were mostly uncolonized whereas dense bacterial coverage was observed in *R. exoculata* of similar stage (**Figure 4E., F.**). Mouthparts (scaphognathites and exopodites) and their setae were fully colonized in subadults of both species (**Figure 4A., B., C., D.**). However, only branchiostegites of *R. exoculata* subadults were fully colonized, whereas those of *R. chacei* exhibited a limited bacterial colonization (**Figure 4A., B., C., D.**).

Richness diversity analyses showed that the site and species factors (respectively p = 0.00128 and p = 0.04765, Chao1 index, ANOVA) significantly impact bacterial richness in the cephalothoracic cavity, the interaction of species and life stages being also significant (p = 0.00377, Chao1 index, ANOVA) (**Supplementary Tables 9.**). NMDS plots showed a clear separation between vent sites and between species, but changes among life stages were less marked and overlapped largely within each *Rimicaris* species (**Figure 5B., Supplementary Figure 4A.**). Accordingly, β diversity analyses showed that site, species and life stage all had a significant influence on the bacterial community composition in the cephalothorax (p = 0.0001 each, Jaccard index, Adonis; R2 : 0.06441 for stage, against 0.22364 and 0.12972 for site and species; **Supplementary Tables 9.**).

Taxonomic composition of bacterial communities from the cephalothoracic cavity in juveniles was similar those found in adults (**Figure 5A.**). *Campylobacteria* (*Nitratifractor* spp.

and *Sulfurovum* spp.) were retrieved whatever the site, the stage and the species. The main lineage was *Sulfurovum* spp. for both species but *R. exoculata* (each stage and site) showed more *Nitratifractor* spp. related sequences in their microbial communities than *R. chacei* (ANCOM-BC, W = 8.19, q = 1.121996e-14) (**Table 3., Supplementary Tables 10.**). Using FISH (specific probe Epsy549, Lin et al., 2006), as for adults, *Campylobacteria* were mostly observed on the inner side of the branchiostegites or on setae (filament composed of multiple cell units, **Figure 4A., B., C., Supplementary Figure 4.**). *Gammaproteobacteria*, *Bacteroidia* and *Alphaproteobacteria* (class), were retrieved whatever the site, life stage or species. Genus affiliated to *Gammaproteobacteria* including *Cocleimonas* spp. were significantly more retrieved at TAG (ANCOM-BC, respectively W = 3.39, q = 2.599282e-02) (**Table 3., Supplementary Tables 11.**). Using FISH (specific probe GAM42a, Manz et al., 1992) at subadult stage for both species, they were observable (thin filaments, sometimes baccili or coccoids) mainly on the setae of scaphognathites and exopodites (low abundancy) whereas branchiostegites were almost deprived of them at Snake Pit. On the contrary at TAG, they were found in higher abundancy, mainly on the setae and on the branchiostegites (**Figure 4., Supplementary Figure 4.)**. *Alphaproteobacteria* were also detected mostly in TAG samples, whatever the species (but mostly in *R. chacei*) mainly affiliated to the *Rhodobacteraceae* family (ANCOM-BC, W = 4.95, q = 2.563687e-05) (**Table 3., Supplementary Tables 12.**). *Bacteroidia* were retrieved whatever the site, the species and the stage, even if they were mostly retrieved at TAG compared to Snake Pit (**Table 3.)**. Moreover, ANCOM highlighted a differenciation according to species (W= 5.70387705, q = 5.035840e-07 for *Maritimimonas* spp.). The results were confirmed using FISH (specific probe CF319a, Manz et al., 1996). Most of the time, *Bacteroidia* were located on the scaphognathites, exopodites and their setae, or on the inner side of the branchiostegites. Moreover, they were always close to *Campylobacteria* (**Figure 5 C., Supplementary Figure 4B**.), often at the base of the tissues or of the setae where the *Campylobacteria* grow. *Mariprofondus* spp. belonging to *Zetaproteobacteria* were detected mainly at TAG (but slightly in some juveniles from Snake Pit). The ANCOM-BC analysis also revealed a different abundance of these *Zetaproteobacteria* between the two sites (ANCOM-BC, W = 2.25 and q = 2.053967e-03) (**Supplementary Tables 11.**). At TAG, these were identified on all specimens of every stages of *R. chacei* but only in some for *R. exoculata* (all stage A, 4/5 stage B and 2/5 subadults) (**Table 3.**). Using FISH (specific probe Zeta709, Hoshino et al., 2016), *Zetaproteobacteria* were visible in stage B juveniles and subadults of *R. exoculata* and in *R. chacei* subadults at both sites (less visible at Snake Pit) but could not be observed in stage A juveniles of both species. They were rod-shaped bacteria, found in low numbers along the setae and tissues of scaphognathites and exopodites as well as on the inner side of the branchiostegites (**Figure 5E., Supplementary Figure 4D.**). *Desulfobulbia* were identified in *R. exoculata* at each stage and site (except for one subadult from Snake Pit and one stage B and one stage A from TAG), and for *R. chacei*, in 1 and 2 stage A juveniles from TAG and Snake Pit respectively, and in 2 subadults from each site (**Table 3.**). For some specimens, especially for *R. chacei*, *Desulfobulbia* ASV account for only a few sequences (ANCOM-BC, W =5.60, q = 9.190496e-07 for *Desulfocapasa* spp.) (**Supplementary Tables 11.**). Using FISH (specific probe DSB706, Lucker et al., 2007), *Desulfobulbia* could be only found in stage B juveniles at Snake Pit and subadults at both sites for *R. exoculata*. They were little coccoids (**Figure 5D., Supplementary Figure 4C.**). As *Bacteroidia*, they were mixed with the *Campylobacteria* and *Gammaproteobacteria*. They were visible at the bottom of the microbial mat, and more particularly at the base of the filamentous bacteria both on branchiostegites and all around the setae of the scaphognathites and exopodites (**Figure 5D., Supplementary Figure 4C.**).

Discussion

Our study provides a first comprehensive dataset of bacterial communities colonizing each symbiotic organ (midgut tube, foregut, cephalothoracic cavity) of the distinct juvenile stages of *Rimicaris chacei* and *Rimicaris exoculata*. While previous studies were limited in the number of specimens available as well as by the lack of a precise identification of the distinct juvenile stages (Guri et al., 2012; Cowart et al., 2017; Apremont et al., 2018), our dataset with 5 replicates per species/stage/site, using the revised identification of *Rimicaris* juveniles (Methou et al., 2020), allows us to investigate the inter-individual variability and the influence of biotic (species, stage) and environmental (site) factors on symbiotic development in different host organs.

Anatomical change of digestive organs upon symbiont acquisition

Morphologically, our observations show that stomach structures of juveniles are entirely developed even if it is of smaller size than in adults (Guéganton et al., 2022). On the contrary, the midgut tube was devoid of microvilli in the earliest stage (stage A) of both species, these structures only appearing in older juveniles (respectively stage B juveniles for *R. exoculata* and subadult for *R. chacei*). The formation of these microvilli was concomitant with the midgut symbionts colonization. Involvement of symbionts in metamorphosis and maturation of animal tissues has been observed in many species across nearly all metazoan phyla suggesting that symbionts act as "the other cells" of their host developmental machinery (Carrier and Bosch, 2022). In the squid *Euprymna scolopes* symbiosis with the bioluminescent bacteria *Vibrio fischeri*, aposymbiotic hatched juveniles are immediately colonized by the free living *Vibrio* symbionts leading to the maturation of the hosting light organ in parallel with the regression of its ciliated epithelium, preventing a latter colonization by other symbionts (Chun et al., 2008). These tissue modifications are triggered by molecules produced by the symbionts which are recognized by the host, hence modifying its transcription patterns. In mammals, during the first phase after birth, midgut is able to establish a stable host-bacterial symbiosis that stimulates the intestinal epithelium maturation and also its regeneration (Huang et al., 2013; Hill et al., 2017; Walker, 2017; Nigro et al., 2018). Further investigations are required to decipher whether the maturation of the intestinal epithelium with the formation of microvilli is a process entirely controlled by the host cells and is a prerequisite for symbiont colonization, or if this developmental process is triggered by symbionts upon their acquisition.

Acquisition and transmission of each symbiotic lineage

Our study suggests a fast colonization of symbiotic organs in both *R. exoculata* and *R. chacei* specimens right after their settlement, although with an offset between the primary colonization of digestive symbionts and cephalothoracic symbionts. For the cephalothoracic cavity, the symbiotic relationship is established in all individuals at the earliest juvenile stages (stage A juveniles). On the other hand, the digestive symbionts *Hepatoplasmataceae* and *Candidatus* Microvillispirillaceae, were only detected in the foregut and the midgut of a few stage A juveniles for both species. A presence of these digestive symbionts in all individuals analyzed was only observed in later stages, from stage B juveniles for *R. exoculata* and from subadults for *R. chacei*. Therefore we suggest that symbiont acquisition starts first in the cephalothoracic cavity, right at settlement, and only begins after for the resident digestive symbionts, during the transition between stage A and stage B juveniles for *R. exoculata* and between stage A and subadult juveniles for *R. chacei*.

These observations for the cephalothoracic cavity are in line with previous hypotheses suggesting a horizontal transmission of these symbionts. Indeed, the cephalothoracic symbiotic communities are very similar to the adult ones wich are renewed at each molt (Zbinden et al., 2004, 2008; Corbari et al., 2008a, 2008b ; Cambon-Bonavita et al., 2021). This is also supported by the fact that the cephalothoracic related lineages were retrieved as free-living in the shrimp environment (Hügler et al., 2011; Guri et al., 2012 ; Jan et al., 2014).

Conversely, transmission of the digestive symbionts is less clear. For now, no OTUs related to *Candidatus* Microvillispirillaceae and only one related to *Mycoplasmatales* (order) has been found in hydrothermal fluids around shrimp aggregates (Hügler et al., 2010; Flores et al., 2011). The data, associated with the presence of one OTU related to *Mycoplasmatales* on a few egg broods of *R. exoculata* (Methou et al., 2019) may have suggested a vertical transmission from mother to embryos at this life stage. However, the results of this study revealed that whatever the site and the shrimp species, the first stage after settlement did not systematically have *Candidatus* Microvillispirillacea nor *Candidatus* Foregutplasma in their digestive system or only a few for some individuals. Consequently, a vertical acquisition at egg stages, maintained all along the lifecycle can be dismissed. A more likely scenario would be that digestive symbionts are acquired horizontally after juvenile settlement. In this case, the apparent absence of the digestive symbionts in the environment may be linked to a spatially restricted niche in specific areas and/or substrates which have not been explored yet. For instance, a presence restricted to nurserie habitats (Methou et al., 2022) or on rocks substrates rather than in the surrounding water could be possible.

As hypothetized by Durand et al. 2015, we also cannot exclude an inter-generational transmission between individuals from adults to juveniles by trophallaxis, or by another mean. In terrestrial isopods, which also host similar lineages of *Hepatoplasmataceae*, an horizontal transmission by ingestion of inoculated food sources, either through coprophagy or cannibalism, has been proposed (Bouchon et al., 2016). The transmission mechanisms of *Hepatoplasmataceae* and *Candidatus* Microvillispirillacea might also differ as their hosting organs are submitted to different constraints. *Hepatoplasmataceae* within the foregut are for instance submitted to a renewal of the cuticle at each molt, due to the ectodermic origin of the foregut (Vogt, 2021), requiring a renewed aquisition from the environment at each molt. On the other hand, *Candidatus* Microvillispirillacea within the midgut tube (endodermic origin) are not constrained by host exuviation and could have only one acquisition phase during the metamorphosis in first juvenile stages. Also, the cell division of these lineages appears to be controlled by their shrimp host (Aubé et al., 2022) limiting their proliferation and possibly a release in the environment to colonize other shrimp congeners. Still, this cell division control was observed in adults but could vary during host life cycle or through molt phases. Indeed, many examples of variations in the host-symbiont communications and interactions exist, offering possibilities for the symbiont to escape the host control (Gross et al., 2009; Jacobovitz et al., 2021). So, *Candidatus* Microvillispirillaceae would be ejected from adult midgut tube with the feces or the cuticle (hindgut) during exuviation and released in the environement. Then, they could be captured by juveniles with the flow of fluids from the posterior (Martin et al., 2020) to proximal regions of the midgut tube.

Still, on an evolutionary time scale, several other evidences are pointing instead towards a vertical transmission of these digestive symbionts, particularly for *Hepatoplasmataceae*. The presence of related symbiont lineages in alvinocaridid shrimp from other regions but also in crustaceans from other ecosystems (Eberl, 2010 ; Bouchon et al., 2016; Methou et al., 2023a) are indicative of an ancient and conserved association. Moreover, the highly reduced size of

their genomes (0.48 to 0.83 Mbp for *Hepatoplasmataceae* and 1.25 to 1.36 Mbp for *Candidatus* Microvillispirillacea) (Aube et al., 2022), a low GC content and the loss of several essential genes (single copy core genes), all suggest a genome reduction which is generally associated with vertical inheritance (a process known as Muller's ratchet, McCutcheon and Moran, 2012). For comparison, genome sizes of symbionts from vesicomyid clams, that follow a strict vertical mode of transmission, are comprised between 1.0 and 1.25 Mbp (Russell et al., 2020). Future investigations of the acquisition of these digestive symbionts in other alvinocaridids, or in other crustaceans for the case of *Hepatoplasmataceae,* should help to clarify these apparent discrepancies between our ecological observations and the evolutionary patterns.

Dynamic of symbiotic communities along the post-settlement metamorphosis

Candidatus Foregutplasma rimicarensis and *Candidatus* Bg2_rimicarensis, *i.e*. the main colonists of the foregut, were also detected in the midgut tube of older juveniles (stage B and subadults) in both *Rimicaris* species. However, in adults, these symbiotic lineages are known to be more abundant in the foregut (Aubé et al., 2022; Guéganton et al., 2022). This could reflect an initial colonization of the entire digestive system and not specifically of the foregut at juvenile and subadult stages. Such colonization process has similarities with what is observed in the tubeworm *Riftia pachyptila* which endosymbiotic bacteria are also acquired after settlement, at the post-larval stage. At the adult stage their symbionts are hosted in a dedicated organ - the trophosome - but during the acquisition phase, they colonize all host tissues after entering through the worm epidermis, before a migration at later life stages towards the tissue layer that give birth to the adult trophosome (Nussbaumer et al., 2006). Even with a constant symbiont acquisition throughout their adult life (Wentrup et al., 2014), a similar phenomenon has also been observed in bathymodioline mussels with a first colonization by symbiotic lineages between the pediveliger and metamorphosis stages in all of their organs before being restricted to gill tissues at older stages (Wentrup et al., 2013; Franke et al., 2021). Similarly, in *Rimicaris* shrimp from the MAR, the compartimentalization of *Hepatoplasmatacae* symbionts only within their foregut could be acquired progressively and finalized after the metamorphosis with an aspecific colonization of both organs at juvenile and subadult stages.

Interestingly, in *Rimicaris variabilis* and *Nautilocaris saintlaurentae*, two alvinocaridid shrimp that host similar lineages of digestive symbionts, both *Candidatus* Microvillispirillaceae and *Hepatoplasmatacae* symbionts were found within the foregut and the midgut even at adult stages, suggesting that compartimentalization of these symbionts might exist in some alvinocaridids but not all (Methou et al., 2023a). Potentially, this could be due to differences in their diets with an organ partitioning of digestive symbionts in species relying, at least in part, on their cephalothoracic symbiosis – *i.e*., *R. exoculata* and *R. chacei* – and a aspecific colonization in species relying on a bacterivory/scavenging diet – *i.e*., *R. variabilis* and *N. saintlaurentae* – even if a wider comparison including more alvinocaridid species would be required to confirm this pattern. At last, we cannot exclude either the hypothesis of contamination during our dissections, or leakage of stomach bacteria towards the midgut tube due to animal stress during their sampling.

Variability of symbiotic communities among host species and vent sites

At equivalent stage, the cephalothoracic cavity was more colonized in *R. exoculata* than in *R. chacei*. Indeed, even at the subadult stage, branchiostegites of *R. chacei* were barely colonized, contrary to the mouthparts which were well colonized by *Gammaproteobacteria* and *Campylobacteria* as observed in adults (Apremont et al., 2018). This contrasts with the dense bacterial colonization observed on both mouthparts and branchiostegites of *R. exoculata*

already in stage B juveniles. Even stage A juveniles of *R. exoculata* exhibit denser bacterial colonisation in the cephalothoracic cavity than stage A of *R. chacei*. This is in line with the gradual transitions towards distinct diets of the two species during their post-settlement metamorphosis, respectively to a chemosymbiotic diet for *R. exoculata* and to a mixotrophic behavior for *R. chacei* (Methou et al., 2020). These differences in the colonization patterns of the two species as well as in their trophic transitions are also reflected at the anatomical level during this post-settlement metamorphosis phase. As evidenced by Methou et al. (2023b) and our observations, enlargement of the mouthparts and the branchiostegites is more marked for *R. exoculata* whereas foregut size exhibited a drastic increase in *R. chacei*. Just as the appearance of microvilli in the digestive tract of juveniles was concomitant with the symbiotic colonization of this organ, it is interesting to see that the absence of branchiostegite enlargement in *R. chacei* coincides with the absence of colonization of this anatomical structure in subadults. Conversely, complete colonization of these branchiostegites at an equivalent stage in the genetically similar shrimp *R. hybisae* gives rise to significant hypertrophy of their cephalothoracic cavity at adult stages (Methou et al., 2023b). Although a causal link cannot be clearly established yet, all these observations question the respective role of the symbionts and their host in the developmental processes during the metamorphosis phase of these shrimp.

The symbiotic communities hosted in both *Rimicaris* spp. seem to also be influenced by the vent field of origin and could be related to known differences in the fluid composition of TAG and Snake Pit (Fouquet et al., 2010). In the cephalothoracic cavity, the same dominant lineage is present at each stage for both species (*Campylobacteria*) but variations are visible for other lineages, as seen in adult stages or on the surface of eggs (Methou et al., 2019; Cambon-Bonavita et al., 2021). For instance, the iron-oxidizing *Zetaproteobacteria* are much more abundant at TAG and almost absent at Snake Pit which can be linked to the higher iron concentration in TAG vent fluids (Fouquet et al., 2010).

Conclusion

Upon settlement on sites, juveniles of each *Rimicaris* species occupy a different habitat, which is adjacent to adults for *R. exoculata* but on diffusing areas without adults for *R. chacei*. Such spatial segregation could have an impact for the acquisition of symbionts in each species. Overall, our data do not support previous hypotheses predicting vertical transmission for some symbiont lineages, but rather highlight an horizontal symbiont acquisition from local bacterial pools following the dispersal phase both for the cephalothoracic and digestive symbioses. However, different pathways of transmission could occur for each symbioses. Cephalothoracic symbionts are likely acquired from an environmental free-living pool, while digestive symbionts, showing streamlined genomes, probably also involve specific mechanisms of symbiont release from older stages with established symbiosis allowing colonization of early juveniles. The two symbioses also differ by their colonization dynamic with a cephalothoracic cavity rapidly colonized with diverse bacterial lineages present from the earliest juvenile stages, while digestive systems seem to develop afterwards with only well established symbiont communities at later juvenile stages. The colonization of symbionts in the cephalothoracic cavity is also different for the two host species. Whatever the stage, *R. exoculata* juveniles harbor a denser bacterial colonization than *R. chacei* juveniles. This difference may be in line with the absence of branchiostegite enlargement in *R. chacei* and with the habitat. This difference in colonization could potentially explain, at term, the collapse of *R. chacei* population during recruitment.

Still, larval stages remain a major gap to fully understand acquisition of symbiotic lineages all along the host life cycle and confirm if these life stages are aposymbiotic. However

technical difficulties to sample or to rear larvae at laboratory preclude detailed study of the larval biology of these shrimp at the moment.

Authors contribution

MG contributed to data acquisition and analysis, wrote the first draft of the manuscript, review and editing of this manuscript. PM contributed to sampling, review and editing the manuscript. OR contributed for FISH microscopy data acquisition and review of this manuscript. NG contributed for electron microscopy data acquisition. JA, CN and VC-G contributed for metabarcode analysis. JA and LD helped for dissection. FP and M-AC-B contributed to the conception and the design of this study, review and editing of the manuscript, and the supervision of the project. M-AC-B contributed to funding. All authors contributed to the article and approved the submitted version.

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Data availability statement

The raw reads of the metabarcode are available in the European Nulceotide Archive under Bioproject Accession Number PRJEB71821. The configuration files for the processes options used in SAMBA are avalaible in the Appendix (Appendix 7., 8., 9., 10., 11.)

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Figure caption

Figure 1 : Boxplot for Chao1 richness estimator according to the species, the tissue, the stage and site.

Figure 2 : The foregut of *R. exoculata* and *R. chacei* juveniles. (A) Dorsal view of the pyloric chamber (opened) of a *R. exoculata* subadult with the different filtering structures showed by white arrows (SEM). **(B)** Dorsal view of the cardiac floor crest of the cardiac chamber of *R. exoculata* subadult specimen (SEM). **(C)** Section of the pyloric chamber of a *R. exoculata* stage A observed by autofluorescence thanks to the Filter Set 43 HE Cy3 with cube (orange). Tissue cell nuclei are labelled with DAPI (blue). Scale bars = 50 µm (FISH). **(D)** NMDS plot of the bacterial diversity of the foregut samples based on Jaccard distance colored according to the

site**.** Ellipses represent 95% confidence interval for each group. **(E)** Bacterial taxonomic composition at the genus-level in foregut samples of juvenile shrimp at different life stages.

Figure 3 : The midgut tube of *R. exoculata* and *R. chacei* juveniles. (A) General structure of a section of the midgut tube of a *R. chacei* stage A specimen. No microvilli is visible (SEM). **(B)** Zoom on a section of the epithelium of the midgut tube of a *R. exoculata* subadult specimen. The white arrow shows the insertion of bacteria between the microvilli (SEM). **(C)** NMDS plot of Jaccard distance between midgut tube samples colored according to the site. Ellipses represent 95% confidence interval for each group. **(D)** *Candidatus* Microvillispirillaceae of the midgut tube of a *R. exoculata* subadult from Snake Pit hybridized with the specific probes Def1229-Cy3 (yellow) and Eub338-Cy5 (red). The superposition of the probe gives orange colored bacteria. Tissue cell nuclei are labelled with DAPI (blue). Scale bars = 20 µm (FISH). **(E)** Bacterial taxonomic composition at the genus-level in midgut samples of juvenile shrimp at different life stages.

Figure 4 : FISH observations of cephalothoracic symbionts evolution for both species during metamorphosis. *Gammaproteobacteria* were hybridized with the specific probe GAM42a and *Campylobacteria* with Epsy549. **(A)** Entire cephalothoracic cavity of a *R. exoculata* from TAG hybridized with the specific probes GAM42a-Cy3 (yellow) and Epsy549-Cy5 (red). **(B)** Entire cephalothoracic cavity of a *R. chacei* from Snake Pit hybridized with the general probe Eub338-Cy3 (yellow). **(C)** Bacteria on branchiostegites of a *R. exoculata* subadult from TAG hybridized with the specific probes Epsy549-Cy5 (pink) and GAM42a-Cy3 (yellow). **(D)** Bacteria on branchiostegites and setae of the scaphognathite of a *R. chacei* subadult from TAG hybridized with the specific probes Epsy549-Cy5 (red) and GAM42a-Cy3 (yellow). **(E)** Bacteria on branchiostegites of a *R. exoculata* stage A from TAG hybridized with the specific probes Epsy549-Cy5/Eub338-ATTO488 (green-blue) and GAM42a-Cy3/Eub338-ATTO488 (red). **(F)** Bacteria on branchiostegites of a *R. chacei* stage A from Snake Pit hybridized with the specific probes Epsy549-Cy5/Eub338-ATTO488 (green-blue) and GAM42a-Cy3/Eub338-ATTO488 (red). Tissue cell nuclei were labelled with DAPI (blue). **(A, B)** were mosaics. Scale bars = 20 µm **(C, D, E, F)**, 500 µm **(A,B)**.

Figure 5 : The cephalothoracic cavity of *R. exoculata* and *R. chacei* juveniles. Using FISH, *Gammaproteobacteria* were hybridized with the specific probe GAM42a, *Campylobacteria* with Epsy549, *Zetaproteobacteria* with Zeta709, *Bacteroidia* with CF319a and *Desulfobulbia* with DSB706. **(A)** Bacterial taxonomic composition at the genus-level in cephalothorax samples of juvenile shrimp at different life stages. **(B)** NMDS plot of Jaccard distance between cephalothorax samples colored according to the site. Ellipses represent 95% confidence interval for each group. **(C)** Bacteria on scaphognathites of a *R. exoculata* stage B from Snake Pit hybridized with the specific probes Epsy549-ATTO488 (green), GAM42a-Cy5 (red) and CF319a-Cy3 (yellow, pink arrows). **(D)** Bacteria on setae of scaphognathites of a *R. exoculata* stage B from Snake Pit hybridized with the specific probes Epsy549-ATTO488 (blue), GAM42a-Cy5 (red) and DSB706-Cy3 (yellow). **(E)** Bacteria on branchiostegites of a *R. exoculata* stage B from Snake Pit hybridized with the specific probes Epsy549-ATTO488 (green-blue), GAM42a-Cy5 (red) and Zeta709-Cy3 (yellow). Tissue cell nuclei were labelled with DAPI (blue). Scale bars = 20 µm.

Tables

Table 1 : Proportion of sequences obtained and affiliated to *Candidatus* Foregutplasma rimicarensis or *Candidatus* Bg2_rimicarensis of the total of bacterial communities of

foregut samples in juveniles of the two *Rimicaris* spp. at different life stages (average ± standard deviation).

	R. exoculata stage A	***R. exoculata stage B***	***R. exoculata subadult***	***R. chacei stage A***	***R. chacei subadult***
Candidatus Foregutplasma rimicarensis *(%) – TAG*	12.86 ± 13.16 (n=5)	35.17 ± 36.10 (n=5)	8.16 ± 13.55 (n=5)	0 ± 0 (n=5)	35.04 ± 24.97 (n=5)
Candidatus Bg2_rimicarensis *(%) – TAG*	0.0014 ± 0.0031 (n=5)	0.34 ± 0.46 (n=5)	0.37 ± 0.52 (n=5)	0.56 ± 1.25 (n=5)	3.7 ± 4.91 (n=5)
Candidatus Foregutplasma rimicarensis *(%) – Snake Pit*	0 ± 0 (n=5)	18.87 ± 15.62 (n=4)	12.27 ± 4.86 (n=5)	0.078 ± 0.11 (n=5)	0.093 ± 0.16 (n=3)
Candidatus Bg2_rimicarensis (%) *– Snake Pit*	0.06 ± 0.13 (n=5)	1.88 ± 2.94 (n=4)	1.3 ± 2.35 (n=5)	0 ± 0 (n=5)	2.37 ± 3.82 (n=3)

Table 2 : Proportion of sequences obtained and affiliated to *Candidatus* Rimicarispirillum spp. and the two others dominant ASVs identify as *Candidatus* Microvillispirillaceae of the total of bacterial communities of midgut tube samples in juveniles of the two *Rimicaris* spp. at different life stages (average ± standard deviation).

	R. exoculata stage A	***R. exoculata stage B***	***R. exoculata subadult***	***R. chacei stage A***	***R. chacei subadult***
Candidatus Rimicarispirillum spp. *(%) – TAG*	14.9 ± 30.13 (n=5)	40.16 ± 23.93 (n=5)	55.16 ± 26.15 (n=5)	13.82 ± 29.95 (n=5)	26.62 ± 15.5 (n=5)
Others dominant ASVs *(%) – TAG*	0.0012 ± 0.0027 (n=5)	0 ± 0 (n=5)	0 ± 0 (n=5)	0 ± 0 (n=5)	0 ± 0 (n=5)
Candidatus Rimicarispirillum spp.*(%) – Snake Pit*	0.0076 ± 0.017 (n=5)	40.08 ± 35.43 (n=5)	29.69 ± 9.69 (n=5)	0.0032 ± 0.0072 (n=5)	24.01 ± 24.3 (n=5)
Others dominant ASVs *(%) – Snake Pit*	0 ± 0 (n=5)	5.41 ± 8.09 (n=5)	5.83 ± 9.70 (n=5)	0 ± 0 (n=5)	16.37 ± 19.42 (n=5)

Table 3 : Proportion of sequences obtained and affiliated to the main bacterial communities of the cephalothoracic cavity samples in juveniles of the two *Rimicaris* spp. at different life stages (average ± standard deviation).

	R. exoculata stage A	***R. exoculata stage B***	***R. exoculata subadult***	***R. chacei stage A***	***R. chacei subadult***
Bacteroidia (%) – TAG	5.88 ± 3.96 (n=5)	4.55 ± 2.67 (n=5)	15.24 ± 7.83 (n=5)	6.22 ± 9.15 (n=5)	2.40 ± 1.37 (n=5)
Bacteroidia (%) – Snake Pit	1.57 ± 1.08 (n=5)	2.39 ± 2.11 (n=5)	1.19 ± 0.70 (n=5)	1.43 ± 2.27 (n=5)	0.92 ± 0.63 (n=5)
Alphaproteobacteria (%) – TAG	2.12 ± 1.11 (n=5)	1.81 ± 1.31 (n=5)	0.77 ± 0.52 (n=5)	6.48 ±10.65 (n=5)	16.87 ± 10.24 (n=5)
Alphaproteobacteria (%) – Snake Pit	0.38 ± 0.30 (n=5)	0.05 ± 0.06 (n=5)	0.02 ± 0.03 (n=5)	0.78 ± 1.52 (n=5)	0.51 ± 0.66 (n=5)
Desulfobulbia (%) – TAG	0.73 ± 1.12 (n=5)	1.34 ± 2.72 (n=5)	1.33 ± 2.79 (n=5)	0.007 ± 0.01 (n=5)	0.02 ± 0.03 (n=5)
Desulfobulbia (%) – Snake Pit	0.11 ± 0.06 (n=5)	1.06 ± 1.84 (n=5)	0.09 ± 0.08 (n=5)	0.009 ± 0.02 (n=5)	0.02 ± 0.03 (n=5)
Zetaproteobacteria (%) – TAG	1.63 ± 2.19 (n=5)	0.13 ± 0.12 (n=5)	0.003 ± 0.005 (n=5)	0.24 ± 0.28 (n=5)	0.75 ± 0.74 (n=5)
Zetaproteobacteria (%) – Snake Pit	0.01 ± 0.02 (n=5)	0 ± 0 (n=5)	0 ± 0 (n=5)	0.004 ± 0.009 (n=5)	0 ± 0 (n=5)
Gammaproteobacteria (%) – TAG	18.55 ± 8.05 (n=5)	17.87 ± 9.32 (n=5)	14.05 ± 3.27 (n=5)	9.33 ± 7.17 (n=5)	14.16 ± 5.36 (n=5)
Gammaproteobacteria (%) – Snake Pit	2.34 ± 1.24 (n=5)	2.85 ± 1.94 (n=5)	2.29 ± 2.01 (n=5)	0.89 ± 1.21 (n=5)	2.01 ± 2.35 (n=5)
Campylobacteria (%) – TAG	63.03 ± 13.26 (n=5)	72.53 ± 14.13 (n=5)	80.16 ± 7.16 (n=5)	75.66 ± 25.26 (n=5)	50.66 ± 22.43 (n=5)
Campylobacteria (%) – Snake Pit	93.51 ± 2.46 (n=5)	93.01 ± 4.82 (n=5)	96.53 ± 2.62 (n=5)	92.91 ± 7.41 (n=5)	93.51 ± 5.69 (n=5)

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