



# Blow Your Nose, Shrimp! Unexpectedly Dense Bacterial Communities Occur on the Antennae and Antennules of Hydrothermal Vent Shrimp

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In crustaceans, as in other animals, perception of environmental cues is of key importance for a wide range of interactions with the environment and congeners. Chemoreception involves mainly the antennae and antennules, which carry sensilla that detect water-borne chemicals. The functional importance of these as exchange surfaces in the shrimp's sensory perception requires them to remain free of any microorganism and deposit that could impair the fixation of odorant molecules on sensory neurons. We report here the occurrence of an unexpected dense bacterial colonization on surface of the antennae and antennules of four hydrothermal vent shrimp species. Microscopic observation, qPCR and 16S rRNA barcoding reveal the abundance, diversity and taxonomic composition of these bacterial communities, that are compared with those found on a related coastal shrimp. Bacterial abundances vary among species. Bacteria are almost absent in coastal shrimp, meanwhile they fully cover the antennal flagella in some hydrothermal vent species. *Epsilon*- and *Gammaproteobacteria* dominate the hydrothermal shrimp-associated communities, whereas *Alphaproteobacteria* and *Bacteroidetes* are dominant in the coastal ones. Bacteria associated with vent shrimp species are most similar to known chemoautotrophic sulfur-oxidizers. Potential roles of these bacteria on the hydrothermal shrimp antennae and antennules and on sensory functions are discussed.

**Keywords:** hydrothermal, shrimp, Alvinocarididae, chemosensory perception, antennules, bacteria, grooming

## INTRODUCTION

Information derived from environmental cues is essential for animals to make behaviorally and ecologically relevant decisions. For mobile fauna inhabiting deep-sea hydrothermal vents, the environment presents several challenges. One of these is to accurately detect active fluid emissions in the absence of light. Hot fluids can indeed be deadly, meanwhile a certain level of exposure is often necessary in order for animals to gain the compounds that their bacterial symbionts use as

substrates. Decapod shrimp of the family Alvinocarididae are among the most abundant vagile macrofauna around chimneys at many hydrothermal vents (Desbruyères et al., 2000).

The most studied species, *Rimicaris exoculata*, lives in the immediate vicinity of fluid exits, forming highly dense and spectacular swarms sheltering thousands of highly motile individuals that constantly swim in and out of the swarm (Segonzac et al., 1993). It relies on chemoautotrophic symbiotic bacteria that use reduced compounds from the fluid and ensure hosts nutrition (Zbinden et al., 2004; Ponsard et al., 2013). Other species including *Rimicaris chacei* (formerly *Chorocaris chacei*, reassigned to its initial genus by Vereshchaka et al., 2015), *Mirocaris fortunata* and *Alvinocaris markensis*, live at lower densities or as solitary individuals, in areas of weak fluid emissions (Casanova et al., 1993). Shrimp species need to evaluate their environment in order to interact with their congeners, find food, or to find the most suitable habitat. This particular habitat is characterized by steep physicochemical gradients, and deadly risk of exposure to high-temperature fluids (Sarradin et al., 1999; Sarrazin et al., 1999; Le Bris et al., 2006). In crustaceans, sensory perception is mediated by sensilla distributed over almost the entire body (i.e., first and second antennae, mouthparts, legs, cephalothorax, abdomen and telson) (Ache, 1982). Chemoreception is more specifically mediated by two types of chemosensory sensilla: unimodal olfactory sensilla (the aesthetascs) and bimodal sensilla (containing mechano- and chemo-receptor neurons), mainly located on the first and second antennae (Ache, 1982; Derby et al., 2016). The sense of smell is associated with intermittent interaction between the odor environment and these chemosensory organs (Schmitt and Ache, 1979). Aesthetascs sensilla are characterized by a thin cuticle (Kamio and Derby, 2017; Machon et al., 2018), permeable to environment chemicals which will cross the cuticle and bind to the olfactory sensory neurons. Bimodal sensilla, on the other hand, have a thick and non-permeable cuticle, such that environmental chemicals enter them only via a terminal pore, and bind to chemo- and mechano-receptor (Kamio and Derby, 2017).

A recent study describing the general structure of chemosensory organs of vent shrimp (Zbinden et al., 2017) highlighted a previously unsuspected bacterial colonization on the surface of the antennae and antennules, nevertheless without characterization of the microorganisms. If associations with bacteria are widespread in hydrothermal vent ecosystems, including on the surface of arthropod cuticle (Dubilier et al., 2008; Goffredi, 2010), occurrence of bacteria on the chemosensory organs is surprising and was not reported before. Crustaceans living in aquatic environments are under constant exposure to a wide variety of microbial and micro- or macroscopic fouling organisms, and thus spend considerable time and energy in grooming or cleaning their body (Bauer, 1989). Grooming of the antennules, the primary olfactory organs, is the most commonly observed grooming behavior in crustaceans. The antennules are indeed frequently wiped by the setal combs of the third pair of maxillipeds, in order to remove the material and odorants that have accumulated on or between the aesthetascs (Bauer, 1989; Barbato and Daniel, 1997). According to Barbato and Daniel (1997), anything enhancing

the level of microbial fouling is detrimental to the structural integrity of the antennules, and presumably to its functional role as a chemoreceptor organ. Experiments preventing shrimp from grooming their antennules indeed lead to extensive structural damage of the aesthetascs, sometimes up to the breakage and loss of all of them within 2 weeks (Bauer, 1977). Identical findings were made on insects, for which preventing antennal grooming lead to the obstruction of sensilla pores, thus impairing olfaction (Böröczky et al., 2013). This result was confirmed by electroantennogram measurements, which show that insect antennae that were prevented from being groomed were significantly less responsive to the pheromones than groomed ones (Böröczky et al., 2013). Other, previously described, associations between *R. exoculata* and bacteria harbored in the gill chamber (Segonzac et al., 1993; Zbinden et al., 2004) are, on the contrary, beneficial for shrimp and considered as true mutualism: bacteria nourish shrimps through transcuticular transfers of small organic molecules they produce, and/or contribute to the detoxication of the environment (Ponsard et al., 2013).

Observation of bacterial coverage, sometimes very dense, on the chemosensory organs of different species of hydrothermal shrimp raises the question of the potential impact of bacteria on chemoreception and their possible role(s) in shrimp physiology. We propose here a detailed description of the occurrence of bacteria on antennae and antennules of four hydrothermal species (*Rimicaris exoculata*, *Rimicaris chacei*, *Mirocaris fortunata*, and *Alvinocaris markensis*) and a related species sampled in coastal waters (*Palaemon elegans*). We assess the coverage, relative abundance and diversity of bacteria associated with antennules and antennae of shrimp using electron microscopy, qPCR, and a high-throughput metabarcoding approach based on the investigation of the bacterial 16S rRNA-encoding gene. This study is the first to investigate the microbial populations associated with sensory organs in deep-sea shrimp, and yields insights on their potential metabolisms and roles for shrimp.

## MATERIALS AND METHODS

### Animal Collection, Maintenance, and Conditioning

Specimens of the Alvinocarididae *Mirocaris fortunata*, *Rimicaris exoculata*, *Rimicaris chacei*, and *Alvinocaris markensis* were collected during the Biobaz, 2013 and BicoSe, 2014 cruises, on hydrothermal vent sites located on the Mid-Atlantic Ridge (see Table 1 for cruises and sites). In each sampling event, shrimp were collected using the suction sampler of the ROV 'Victor 6000' operating from the RV 'Pourquoi Pas?' The ROV then surfaced within a few hours, and living specimens were processed immediately. Specimens were dissected and tissues of interest (see below) were fixed for morphological observations (see below), or frozen in liquid nitrogen for molecular biology experiments. Specimens of the Palaemonidae *Palaemon elegans* were collected from Saint-Malo region in 2016 (France), using a shrimp hand net. Those specimens were dissected and fixed 1 day

after field sampling and used for all molecular biology analyses and some electron microscopy. Other *P. elegans* specimens used for electron microscopy were collected in 2013, and transferred to aerated aquaria with a 12 h:12 h light:dark cycle, a salinity of 35 g.l<sup>-1</sup>, and a water temperature of 18°C prior to dissection.

## Scanning Electron Microscopy (SEM)

For morphological observations, antennae and antennules (both medial and lateral flagella), as well as branchiostegites (lateral part of the carapace covering the gills), were used. Samples were fixed in a 2.5% glutaraldehyde/seawater solution, rinsed and post-fixed in osmium tetroxide 1%. For SEM, they were then dehydrated through a series of baths of increasing ethanol concentrations, critical-point-dried with an Emitech K850 or a CPD7501 critical point drying apparatus (Quorum Technologies, Laughton, United Kingdom) and platinum-coated in a Scancoat six Edwards sputter-unit or gold-coated with a JEOL JFC-1200 fine coater. Observations were carried out with a scanning electron microscope (Cambridge Stereoscan 260 or Hitachi SU3500), operating at 15 kV.

## DNA Extraction and Estimation of Bacterial Densities Using qPCR

From each individual specimen, the antennae, the medial and lateral flagella of the antennules were separated, flash frozen and stored in liquid nitrogen until further processing. Due to small size, the antennae/antennular organs from three to five specimens were pooled (Table 1). DNA was extracted using the QIAGEN Blood and Tissue Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, United States), and visualized on an agarose gel.

The host gene encoding GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a reference gene, as previously recommended (Cottin et al., 2010). GAPDH is a single copy-gene in vertebrates, and possibly also in crustaceans based on some work (Camacho-Jiménez et al., 2018), although very few studies have investigated this in detail. It was amplified using two distinct primer sets for the hydrothermal vent shrimp (RimGAPDH-F 5'-CCCCCTTCATTGCTCTGGACTAC-3' and RimGAPDH-R 5'-ACCACCTTGGAAGTGAGCAGA-3', originally designed for *Rimicaris exoculata*; Cottin et al., 2010), and for the coastal shrimp *Palaemon elegans* (PalGAPDH-F 5'-GTGAGGTGAAGGCTGAGGAC-3' and PalGAPDH-R 5'-CGGATGGAGCAGAGATGATT-3', this study). The bacterial 16S rRNA encoding gene was amplified using primers Eub-1052F (5'-TGCATGGYTGTCTGTCAGCTCG-3') and Eub-1193R (5'-CGTCRTCCCRCTTCC-3', Wang and Qian, 2009). The specificity of primer sets was tested by gel electrophoresis and the sequencing of PCR products. The qPCR reactions were performed using the LightCycler® 480 SYBR I Master reaction mix on a LightCycler® 480 II Real-Time PCR Detection System (Roche, France) using 45 cycles consisting of denaturation (95°C, 10 s), hybridization (60°C, 15 s) and elongation (72°C, 15 s). Each reaction was run in triplicates for each gene and shrimp

specimen, and replicates with C<sub>T</sub> differing by more than 0.5 were discarded. A dilution series of an equimolar mixture of DNA extracted from all of the samples used in this study was included in each run and used to evaluate the amplification efficiency. The quality of qPCR runs was assessed based on melting curves and measured efficiencies. The bacterial 16S rRNA results were normalized and expressed as “-folds” versus host GAPDH by the  $\Delta C_T$  method. The results for the vent shrimp species were compared with the *P. elegans* data using the  $\Delta \Delta C_T$  method.

## 16S rRNA-Based Metabarcoding of Bacterial Communities Associated With Antennae and Antennules

A control PCR using the standard bacterial 16S rRNA gene-targeting primers 27F and 1492R was performed to test for the presence of bacteria. A ~500 bp fragment of the 16S rRNA-encoding gene corresponding to the V1–V3 variable regions of *Escherichia coli* was then amplified using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 534R (5'-ATTACCGCGCTGCTGG-3', Watanabe et al., 2001). This primer set was used to produce product for sequencing on an Illumina HiSeq platform (2 × 300 bp, paired-end sequencing, GATC Biotech, Germany). Raw reads were deposited into the GENBANK Sequence Read Archive (SRA) database under accession number SRP136751.

## Sequence Analysis

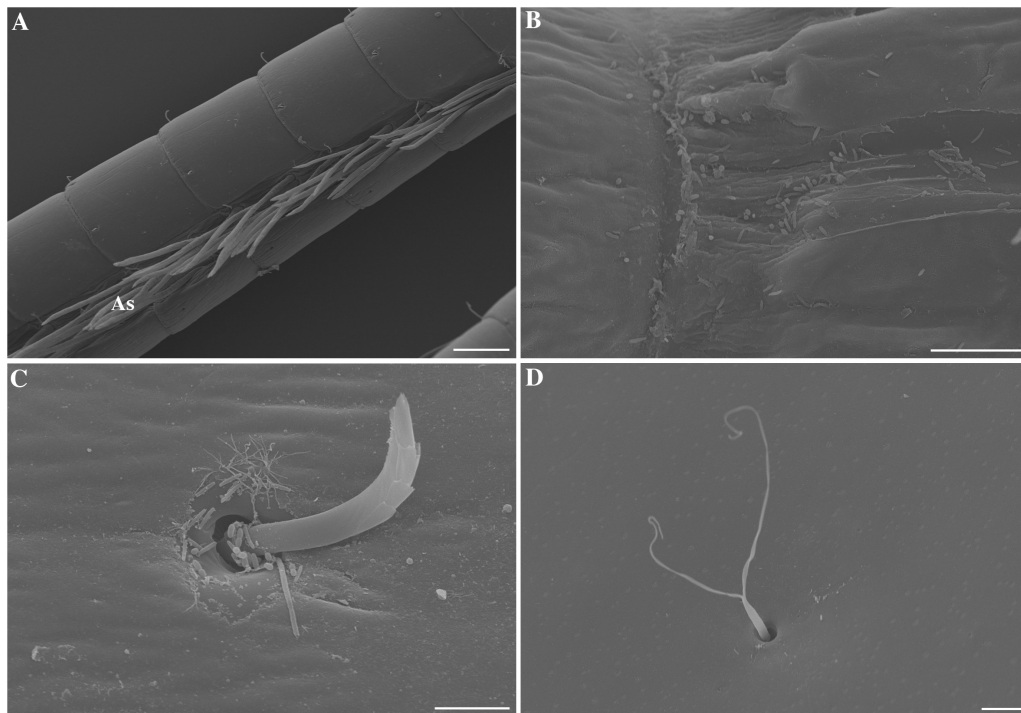
Sequence analyses were performed using QIIME2 (Caporaso et al., 2010). Paired-end sequences were imported and demultiplexed. Sequences quality control was performed using the DADA2 plugin (Callaghan et al., 2016). Sequences were trimmed to 280 bp before paired-ends were assembled. The error model was trained using 1,000,000 sequences. Reads displaying “N,” quality score below 2, more than two expected errors were discarded. Assembled sequences below 450 and above 600 bp were discarded. Chimeric sequences were removed based on a consensus approach, and a feature table was built. Identified unique amplicon sequence variants were then used to generate a guide phylogenetic tree that was used to compute UniFrac distances between samples (Lozupone and Knight, 2005). Rarefaction curves and diversity indices were also generated with QIIME2 using a sampling depth of 1,400,000 reads, corresponding to the lowest number of reads obtained for one sample (*Alvinocaris markensis* A1lat, see Table 1). Variants were classified using a naive Bayesian classifier using the SILVA (release 119-99) database (Quast et al., 2013) and bar-plots were generated. Community composition was compared between samples using alpha diversity indexes (observed variants and the Shannon index). A Venn diagram including all variants except singletons was drawn using the web-based tool available at <http://bioinformatics.psb.ugent.be/webtools/Venn/>. Community composition in the different species was compared using the PERMANOVA test implemented in QIIME2.

TABLE 1 | Samples used for qPCR and metabarcoding analyses.

Species (pooled specimens)	Sample ID	Site (coordinates)	Appendage	Vent	Depth (m)	Raw reads (RR)	Quality -filtered reads (QFR)	Percentage QFR/RR	Observed variants	Shannon index	BioSample accession
<i>Alvinocaris markensis</i> (5)	Alvinocaris – A1lat	Rainbow (36.23N 33.9W)	A1lat	Yes	2,320	2,521,768	1,429,593	56.7	434	3.98	SAMN08818885
	Alvinocaris – A1med	BioBaz 2013	A1med	Yes	2,320	3,005,232	1,802,922	60.0	649	4.29	SAMN08818886
<i>Rimicaris chacei</i> (3)	R. chacei - A1lat	TAG (23.14N 44.83W)	A1lat	Yes	3,650	4,730,004	2,106,792	44.5	235	4.97	SAMN08818887
	R. chacei – A1med	BICOSE 2014	A1med	Yes	3,650	4,632,202	2,110,099	45.6	245	4.98	SAMN08818888
<i>Mirocaris fortunata</i> (5)	R. chacei – A2		A2	Yes	3,650	4,845,753	2,238,252	46.2	234	4.84	SAMN08818889
	Mirocaris – A1lat	Menez Gwen (37.84N 31.53W)	A1lat	Yes	850	4,284,478	2,185,635	51.0	253	4.82	SAMN08818890
	Mirocaris – A1med	BioBaz 2013	A1med	Yes	850	4,946,794	2,519,179	50.9	383	5.53	SAMN08818891
	Mirocaris – A2		A2	Yes	850	5,673,435	2,751,293	48.5	276	4.91	SAMN08818892
<i>Rimicaris exoculata</i> (5)	R. exoculata – A1lat	Rainbow (36.23 N 33.9 W)	A1lat	Yes	2,320	3,864,343	2,209,124	57.2	205	3.94	SAMN08818896
	R. exoculata – A1med	BioBaz 2013	A1med	Yes	2,320	4,919,599	2,793,367	56.8	212	4.09	SAMN08818897
<i>Palaemon elegans</i> (5)	R. exoculata - A2		A2	Yes	2,320	3,907,040	2,330,298	59.6	214	4.03	SAMN08818898
	Palaemon – A1lat	Saint-Malo	A1lat	No	1	3,494,243	1,661,446	47.5	1,105	7.97	SAMN08818893
	Palaemon – A1med	(48.64N 2.00W)	A1med	No	1	3,551,358	1,922,298	54.1	1,123	7.75	SAMN08818894
	Palaemon – A2		A2	No	1	4,849,400	2,474,122	51.0	1,711	8.18	SAMN08818895

The number of pooled specimens is specified for each species (identical for each of the three appendages). The appendages correspond to the medial (A1med) and lateral (A1lat) flagella of the antennule, and to the antennae (A2). The number of raw and quality-filtered reads, as well as the percentages are indicated. The number of observed variants and the Shannon index are provided as measures relevant to Alpha-diversity using datasets rarefied to 1,400,000 reads, corresponding to the lowest number of reads obtained for one sample.





**FIGURE 1** | Scanning electron micrographs of *Palaemon elegans* antennules, antennae and branchiostegite. **(A)** Lateral antennular flagellum bearing the aesthetascs (As). **(B)** Close-up of an intersegment of the antennae colonized by few rod-type bacteria. **(C)** Close-up of the base of a non-aesthetasc beaked scaly seta on the antennae. **(D)** Close-up of a bifid seta on the outer side of the branchiostegite showing no bacterial colonization. Scale bars: **(A)** = 100  $\mu\text{m}$ ; **(B)**, **(D)** = 10  $\mu\text{m}$ ; **(C)** = 5  $\mu\text{m}$ .

## RESULTS

### Microscopic Observation of Microbial Communities on Antennules and Antennae

Important inter-species variations in the amount of bacteria were observed among the five shrimp species observed. Inter-individual variations were also observed, possibly linked, in part, to the molting stage which was not determined in this study. In the coastal species *Palaemon elegans*, antennules, antennae and branchiostegites were almost devoid of bacteria (**Figures 1A,D**), which occurred only as few scattered rods, mainly in intersegment furrows (**Figure 1B**) and at the base of the sensilla (**Figure 1C**). The most colonized samples were those from *Mirocaris fortunata*, where bacteria settled down the whole length of the antennular and antennal flagella (**Figures 2A,B**). Bacteria occurred either as dense plates of rods which could cover the entire circumference of the flagella (usually toward the base, **Figure 2C**), or in dense filamentous bacterial mats, which could be as long as the sensilla (toward the apex) (**Figure 2D**). In between these two extremes, a gradation was observed. *Alvinocaris markensis* resembled *P. elegans* in terms of density of bacteria present. Its bacterial cover was globally very modest (**Figure 3A**), with just a few rods at the base of the sensilla and in the intersegments. Increasing densities were observed in *Rimicaris exoculata* (**Figure 3C**), and even more in

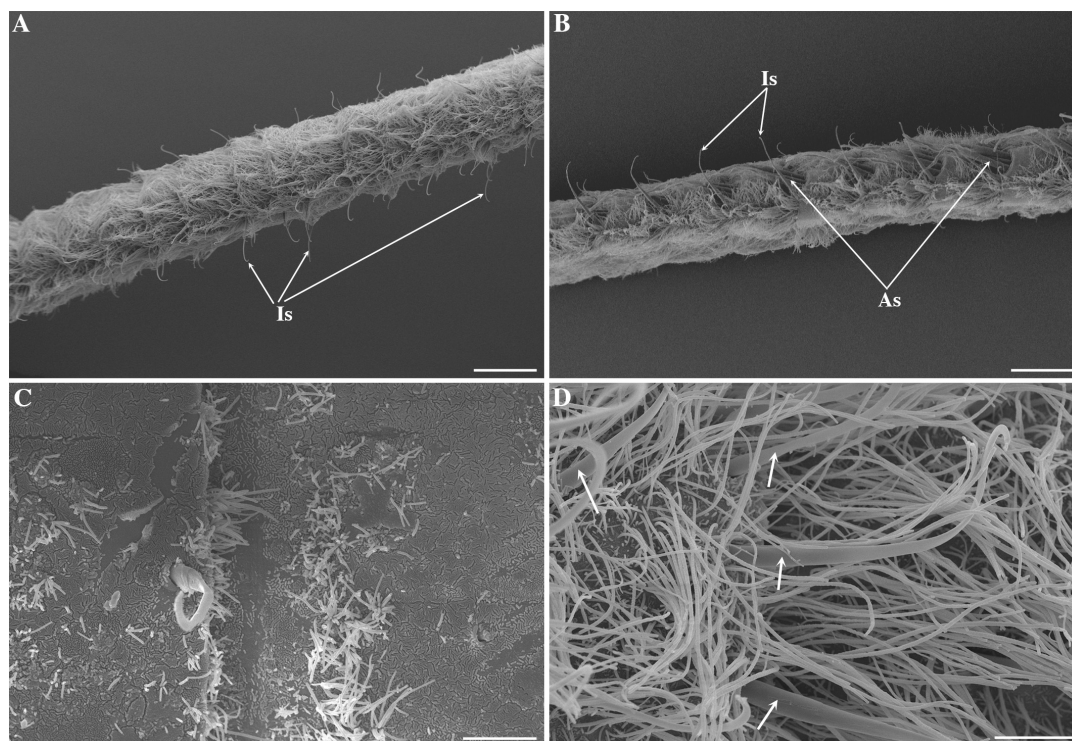
*R. chacei* (**Figure 3D**). On the less colonized samples, bacteria were present as patches of rods on the surface of the flagella, and in the intersegment troughs, with more filamentous bacteria and longer ones when the colonization increased. Aesthetascs were sometimes completely covered by rods, and sometimes by filamentous bacteria (**Figures 1D, 3E,F**).

The branchiostegites were also observed to test if the bacterial coverage of the antennal flagella was specifically located on these appendages. They revealed bacterial colonization in hydrothermal species, quite in accordance with what was observed on the antennae and antennules (**Figures 3G,H**): the more the antennae were colonized, the more the branchiostegites were, too.

Morphotypes observed were very similar among the different species, and limited to thin and wide rods and filaments. *A. markensis* was the only species that displayed a broader diversity, with rods of different sizes, cocci, and a morphotype characteristics of rods tapered at their extremity, found on the antennae, the antennules and branchiostegites (see **Figure 3B**).

### qPCR-Based Comparison of Bacterial Densities Among Species and Appendages

The qPCR-estimated ratio between the bacterial 16S rRNA-encoding and the host GAPDH-encoding gene (used as a host reference gene) was used as a proxy for bacterial density



**FIGURE 2 |** Scanning electron micrographs of *Mirocaris fortunata* antennules. Medial (A) and lateral (B) antennular flagellum completely covered by filamentous bacteria. Intermediate seta (Is) and aesthetasc (As) are visible among the bacterial filaments. (C) Close-up of the base of the antenna showing patches of rod-shaped bacteria and some bacterial filaments. (D) Close-up of the medial flagella of the antennule, showing non-aesthetasc setae (arrows) in the midst of the bacteria. Scale bars: (A) = 200  $\mu\text{m}$ ; (B) = 100  $\mu\text{m}$ ; (C,D) = 20  $\mu\text{m}$ .

on appendages. We assumed that the higher the ratio of bacterial-to-host DNA, the higher the density of bacteria on the host appendage. All 15 samples were compared. Results were normalized versus the “-fold” value obtained in the coastal species *Palaemon elegans* in order to compare all appendages and species (Figure 4). Within a host species, values in the three appendages were within an order of magnitude (10-fold). The highest ratios were obtained for *Rimicaris chacei* and *Mirocaris fortunata*, with average 16S rRNA values representing 181 and 138 times those found in *P. elegans*. These values were an order of magnitude above those in *Alvinocaris markensis* and *Rimicaris exoculata* (15 and 10, respectively).

### Diversity of Bacterial Communities of the Antennules and Antennae

A total of 59,225,649 raw sequences were obtained from the 14 samples comprising the antenna, the medial and the lateral flagella of antennules of five host species. No sequence was obtained from the antenna (A2) of *Alvinocaris markensis*. After quality filtering, 30,534,420 were retained. They clustered into 4,550 variants averaging 500 bp length. Out of these, 99 variants were considered abundant as they displayed a relative abundance above 1% in at least one of the samples (Supplementary Material S1), and were representing a total of 24,786,444 sequences (81.2%). Ten variants were considered dominant

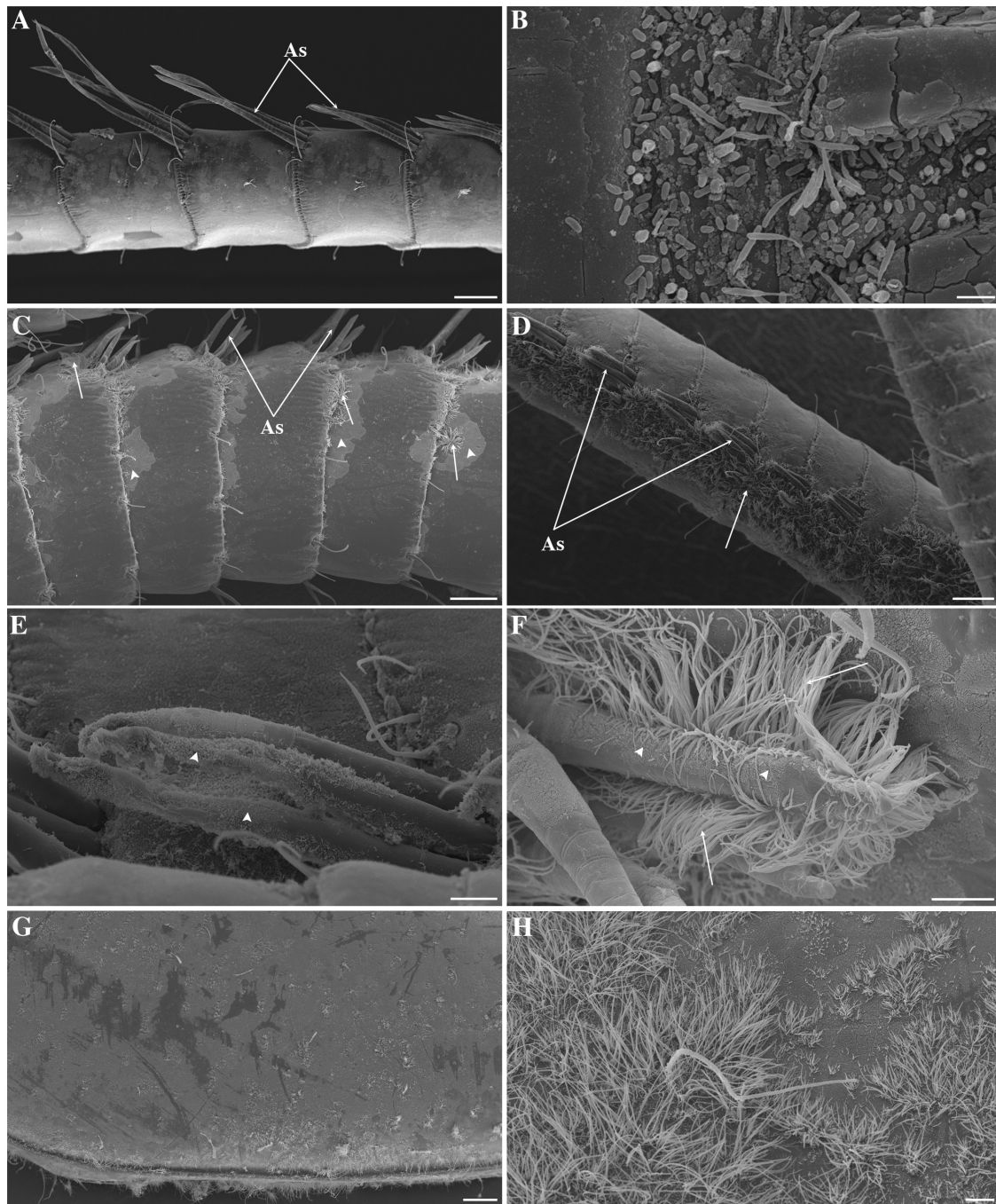
because they represented at least 10% of the reads in at least one sample, overall representing 15,845,985 sequences (63.9%). Between 0.14 and 2.89% of the reads were unassigned.

Rarefaction curves indicate that the coverage was sufficient to properly assess the community composition in all samples (Supplementary Material S2). The highest numbers of observed variants were obtained for *Palaemon elegans*, and the lowest in *Rimicaris exoculata*, the two extreme numbers of variants differing by a ratio of 8.3 (Table 1). The highest values of the Shannon Diversity index were also obtained for *P. elegans*, suggesting more heterogeneous communities, while lowest values obtained for *Rimicaris exoculata* and *Alvinocaris markensis* suggest a more homogeneous bacterial community (Table 1). Within a given shrimp species, the diversity levels observed and the indexes (observed variants and Shannon index) of the three samples (medial and lateral flagella of the antennules, and antennae) were overall similar (Table 1). *Palaemon elegans* displayed notably higher observed variants (1,105–1,711 versus 205–649) and Shannon index (7.75–8.18 versus 3.94–5.53, respectively) compared to vent species altogether.

### Comparison of Bacterial Communities Among Appendages and Species

The principal coordinates analysis plot based on unweighted UniFrac distances shows that the bacterial communities

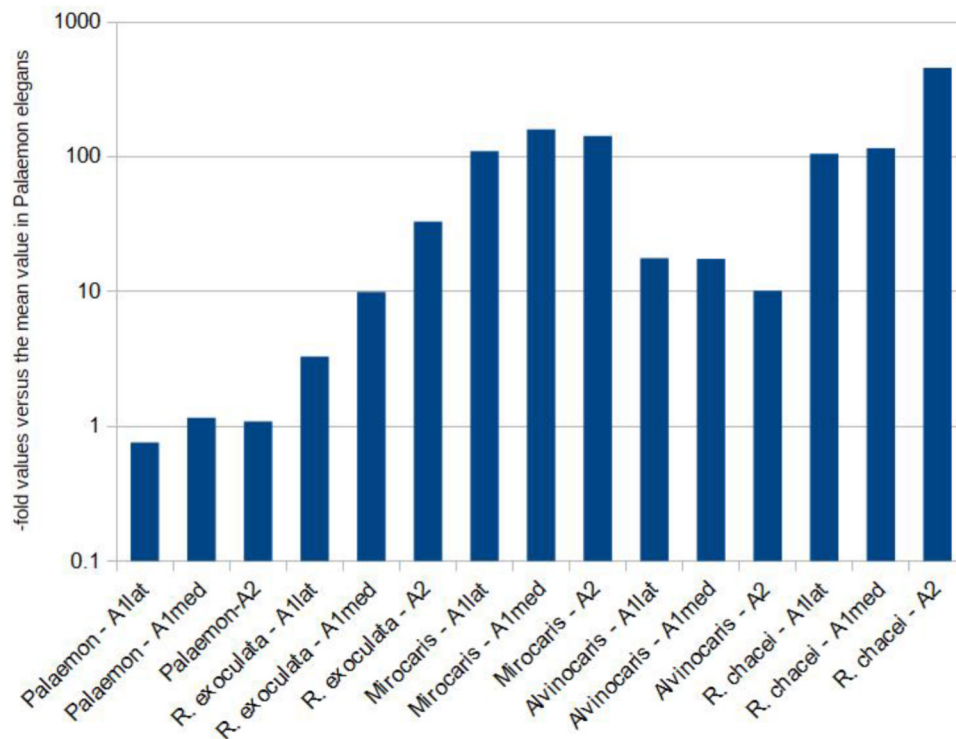




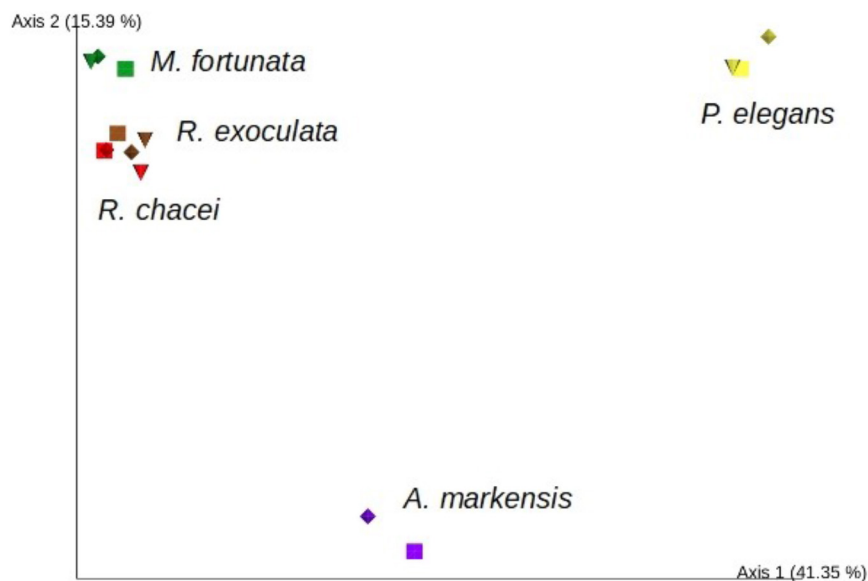
**FIGURE 3 |** Scanning electron micrographs of *Alvinocaris markensis*, *Rimicaris chacei* and *Rimicaris exoculata* antennae and branchiostegite. **(A)** *Alvinocaris markensis* lateral antennular flagellum bearing the aesthetascs (As) and almost devoid of bacteria. **(B)** Close-up on an intersegment of the antennae of *A. markensis* colonized by rods and rods tapered at their extremity. **(C)** *Rimicaris exoculata* lateral antennular flagellum bearing the aesthetascs (As). Patches of rods are visible (arrow heads) along with some short filamentous bacteria (arrows). **(D)** *Rimicaris chacei* lateral antennular flagellum bearing the aesthetascs (As), surrounded by patches of filamentous bacteria (arrows). **(E,F)** Close-ups on the aesthetascs (As) of *M. fortunata* **(E)** and *R. exoculata* **(F)**, covered by rods (arrows heads) and filamentous bacteria (arrows). Scale bars: **(A,C,D)** = 100  $\mu\text{m}$ ; **(B)** = 2  $\mu\text{m}$ ; **(E,F,H)** = 20  $\mu\text{m}$ ; **(G)** = 200  $\mu\text{m}$ .

associated with the three appendages of a given shrimp species (i.e., their medial flagella, the lateral flagella of the antennule, and the antennae), tend to cluster together, suggesting overall similar compositions (**Figure 5**). The first and second axes

(together representing 56.7% of the variance) clearly separated several groups: *P. elegans* and *A. markensis* were well separated from the rest. The three other species displayed more similar communities, with *Rimicaris chacei* and *R. exoculata* being the

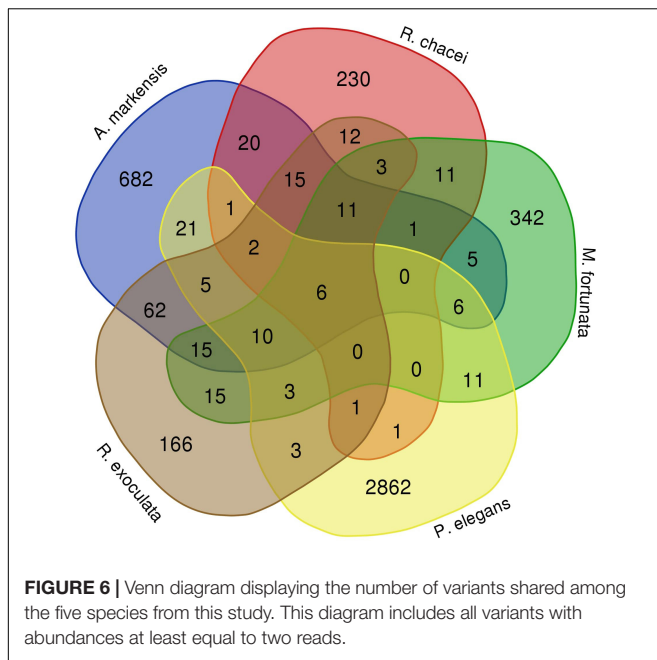


**FIGURE 4 |** Results from the qPCR-based comparison among samples. The bacterial 16S rRNA gene was the target, and host GAPDH was used as a reference gene. Results are expressed as “-folds” versus the average value obtained from the coastal species *Palaemon elegans* in order to compare the results among samples. Notice the logarithmic y-axis.



**FIGURE 5 |** Principal coordinates analysis, 2D plot of the distribution of samples from *Alvinocaris markensis* (purple), *Rimicaris chacei* (red), *Mirocaris fortunata* (green), *Palaemon elegans* (yellow), and *Rimicaris exoculata* (brown) according to the composition of associated bacterial communities estimated by 16S rRNA metabarcoding. Symbols correspond to the different appendages, namely A1lat (diamonds), A1med (square) and A2 (triangle). Axes 1 and 2 explained 41.35 and 15.39% of the observed variance, respectively.





closest. Differences exist between community compositions among species [PERMANOVA test, 14 samples, 5 groups, 999 permutations, pseudo-F = 6.71,  $p < 0.001$  (unweighted UniFrac), pseudo-F = 86.96,  $p < 0.001$  (weighted UniFrac)]. Only six variants were shared among the five species, and additional 11 were shared among the four species from hydrothermal vents (Figure 6). *A. markensis* and *R. exoculata* shared the highest number of variants with 126 sequences, out of which 62 were unique to these two species. As a comparison, between 166 and 2,862 variants were unique to a single host shrimp species, indicating a relatively modest level of inter-species overlap at the variants level of resolution.

## Taxonomic Composition of Bacterial Communities and Dominant Variants

Datasets of variants obtained for bacterial communities associated with the antennules and antennae of *Palaemon elegans* were dominated by members of the *Alphaproteobacteria* (31.0–40.0% depending on appendage, Figure 7) and *Bacteroidetes* (41.8–47.2%). *Epsilonproteobacteria* dominated the datasets obtained from the four vent species, representing the majority of the reads in *Rimicaris exoculata* (84.6–90.5%) and *Mirocaris fortunata* samples (67.1–80.0%). In *R. chacei* and *Alvinocaris markensis*, *Epsilon*- and *Gammaproteobacteria* reads were present in similar relative abundances (respectively, 37.3–53.3% and 36.9–47.2%).

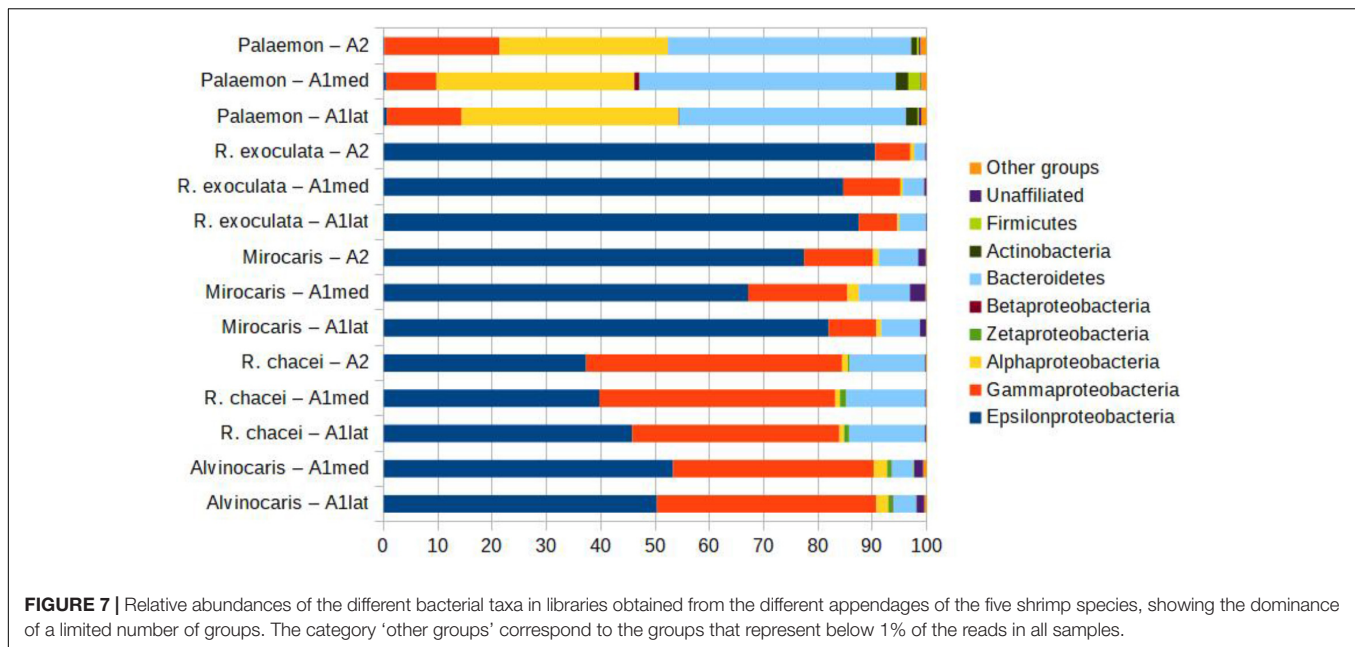
Within the whole dataset, 10 variants were considered dominant because they represented at least 10% of the reads in at least one of the 14 samples. They are listed alongside their best BLAST hits in Table 2. None of these was abundant (>1%) in *P. elegans*. In hydrothermal vent shrimp, the different samples from a given host species shared the same dominant variants in roughly similar abundances, and the dominant

variants belong to the *Epsilon*- and *Gammaproteobacteria* (8 and 2, respectively). In *R. exoculata* samples, between 48 and 56.2% of the sequences belonged to one of three variants, namely 1, 2, and 4. According to a phylogenetic reconstruction, these are closely related and they cluster with various epsilonproteobacterial sequences obtained from the gill chamber of *R. exoculata* (Table 2 and Supplementary Material S3). In *M. fortunata*, two closely related variants (namely 5 and 8) altogether represented between 21.5 and 25.3% of the reads. They are similar to sequences from vent *Epsilonproteobacteria* including *Rimicaris* epibionts (Table 2). With the notable exception of variant 10, which is identical to the sulfur-oxidizing gill endosymbiont of the MAR vent mussels *Bathymodiolus azoricus* and *B. puteoserpentis*, all dominant variants are highly similar to sequences of gill chamber epibionts of arthropods (*R. exoculata*, *R. chacei* or the galatheid *Kiwa* sp.), which appear among their best BLAST hits (Table 2). Although eight of the dominant variants occurred (i.e., above 0 reads) in at least another sample, only variant 1 from *R. exoculata* was abundant (>1%) in another species, namely *M. fortunata* (Table 2).

## DISCUSSION

### Hydrothermal Vent Shrimp Harbor Dense Bacterial Communities on Their Antennules and Antennae

In a previous study describing for the first time the chemosensory organs of hydrothermal shrimp (Zbinden et al., 2017), some of us pointed out that bacteria profusely colonized the antennae and antennules of several hydrothermal vent shrimp (i.e., *Rimicaris exoculata*, *Rimicaris chacei*, *Mirocaris fortunata*, and *Alvinocaris markensis*). The occurrence of filamentous bacteria had also previously been reported on the antennules and antennae of *R. chacei* by Casanova et al. (1993). The present study confirms these preliminary observations, highlighting bacterial populations on the antennae and antennules of the four hydrothermal shrimp species. Present in varying quantities depending on the species and the individuals, bacteria can completely cover the antennae or antennules including the whole surface of chemosensory sensilla, and especially the aesthetascs, specialized in olfaction. A stark contrast exists with the coastal species *Palaemon elegans* for which only anecdotal bacterial coverage was observed. The higher bacterial densities observed under the electron microscope, in particular in *M. fortunata* and *R. chacei*, compared to other species, are in agreement with the qPCR results. Indeed, using the bacterial-to-host DNA ratios estimated by qPCR, *M. fortunata* and *R. chacei* appendages display values an order of magnitude above those obtained in *R. exoculata* and *A. markensis*, and two orders of magnitude above those in *P. elegans*. This supports that densest bacterial communities occur in *M. fortunata* and *R. chacei*, the less dense in *P. elegans*, whereas *R. exoculata* and *A. markensis* are somewhere in between. Different bacterial groups harbor different numbers of copies of their 16S rRNA gene, so qPCR results need to be taken with great caution as they provide only rough estimates



of bacterial densities, and by no means true quantification (Větrovský and Baldrian, 2013). However, in this study we used qPCR to compare bacterial densities among shrimp antennae, and the fact that the species displaying the highest ratios, namely *M. fortunata* and *R. chacei*, are those for which SEM suggested the densest bacterial cover (and vice versa with *P. elegans*) supports the hypothesis that the former actually display denser communities. Inter-species and inter-individual differences could be species-specific, possibly related to the molt cycle. A quick molt cycle was estimated for *R. exoculata* (Corbari et al., 2008), which renews its cuticle twice as fast as a coastal shrimp of the same size (in 10 days for *R. exoculata* vs. 21 for *Penaeus japonicus*, Corbari et al., 2008). In many crustaceans, a high molting rate is considered as an antifouling mechanism allowing the regular elimination of epibiotic bacteria (Bauer, 1989), however, the re-colonization process (at least for the ectosymbionts of the cephalothorax) is very fast in *R. exoculata* and the bacterial community is completely renewed within 2 days after the molt (Corbari et al., 2008). The molt stage would thus be of little influence as an antifouling mechanism and on the inter-species and inter-individual variations herein observed.

## Grooming Behavior in Hydrothermal Vent Shrimp

When their exchange surfaces (i.e., gills or olfactory organs) are subjected to fouling by microbial and micro- or macroscopic organisms, crustaceans frequently wipe and groom them with specialized appendages (Bauer, 1989). Indeed important microbial fouling was shown to be potentially detrimental to the structural integrity of the antennules, and presumably to its functional role as a chemoreceptor organ (Barbato and Daniel, 1997). In the four hydrothermal species, we did not observe any structural damage that could be linked to this bacterial coverage, even on the most colonized samples. Moreover, while *R. exoculata*

first pereopods were shown to lack the grooming apparatus necessary to clean its antennae (Komai and Segonzac, 2008), the three other hydrothermal species possess well-developed antennal grooming apparatus on the carpus of the first pereopod. The four species also possess functional pairs of third maxillipeds that should allow the grooming of the antennules (Komai and Segonzac, 2003, 2005, 2008). They should thus be able to efficiently clean these appendages.

Direct *in situ* observations usually do not allow to observe grooming behavior in hydrothermal shrimp due the high mobility of the animals. We nevertheless performed some observations of isolated individuals of *Mirocaris fortunata* and *Rimicaris exoculata* in aquaria (authors observation from incubation experiments performed on other specimens during the same cruise). Grooming behavior was seen in the former, but not in the latter. Grooming behavior thus seem to be present in at least *M. fortunata*, however, it does not prevent a significant bacterial colonization.

To test whether colonization of the antennae is specific to this part of the body, we observed the surface of the cuticle elsewhere on the shrimp (i.e., the branchiostegites). These body parts also exhibit bacterial colonization. Even if bacterial colonization occurs on other body parts, the very particular role of antennae and antennules in sensory perception raises the question of the presence of these bacteria, of their potential impact on olfaction, and their possible role in shrimp physiology.

## Sensory Capacities in Hydrothermal Vent Shrimp

Detection of odorant molecules was tested on *Rimicaris exoculata* (Renninger et al., 1995) and *Mirocaris fortunata* (Machon et al., 2018) through electrophysiology approaches. These studies showed that these two species can detect sulfide and organic stimuli (such as bacterial homogenates or dead shrimp

**TABLE 2** | Percentages of the reads corresponding to the dominant variants identified in this study, best BLAST hits and sequence similarities.

Variant ID and number (1–10)	Best BLAST hits and sequence similarity (%)									
	<i>R. exoculata</i> – A2	<i>R. exoculata</i> – A1med	<i>R. exoculata</i> – A1lat	<i>Palaemon</i> – A2	<i>Palaemon</i> – A1med	<i>Palaemon</i> – A1lat	<i>Mirocaris</i> – A2	<i>Mirocaris</i> – A1med	<i>Mirocaris</i> – A1lat	
5fa9cd2e5ab13715cddce17de2e9928f	1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Epsilonproteobacteria from <i>Rimicaris exoculata</i> HE805989
ce6f21c8cc56d51582ea17b5b75d2d2e	2	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Epsilonproteobacteria from <i>Rimicaris exoculata</i> FN658969
0ea707d8047463ae62e01a1153f739e57	3	<b>35.5</b>	<b>39.0</b>	0.0	0.0	0.0	0.0	0.0	0.0	Epsilonproteobacteria from <i>R. exoculata</i> and Lost City DQ270633
3fdec649d9c8412b2cf598906047ce8b	4	<b>0.0</b>	<b>0.0</b>	0.0	0.0	0.0	0.0	0.0	0.0	Epsilonproteobacteria from <i>Rimicaris exoculata</i> HE805996
eb40aa9bdc55ac2698f6606407f7a50	5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Epsilonproteobacteria from Rainbow HQ894293
597d4e2ba99ad1d400b9fb2f8be57c59	6	<b>0.1</b>	<b>0.1</b>	0.0	0.0	0.0	<b>12.9</b>	<b>13.9</b>	<b>14.9</b>	Epsilonproteobacteria from <i>Chorocaris chacei</i> LT855327
78d3c100deb219fc03445861bbeec6a	7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Epsilonproteobacteria from <i>Chorocaris chacei</i> LT855338
7772995e18aec26399ac503bfb28489	8	0.0	0.0	0.0	0.0	0.0	<b>9.1</b>	<b>7.7</b>	<b>10.4</b>	Various Epsilonproteobacteria including <i>Rimicaris</i> symbionts
59317dd2bd7c38da03cfd6c6cf85e7b8f	9	<b>20.1</b>	<b>11.8</b>	0.0	0.0	0.0	0.0	0.0	0.0	Gammaaproteobacteria from Lost City FJ792012 and Kiwa epibionts
c11419fb7d01ac895901f84ae074d2c1	10	<b>6.3</b>	<b>10.7</b>	0.0	0.0	0.0	0.0	0.0	0.0	Symbiont of <i>Bathymodiolus azoricus/puteoensis</i> AY945758 – AY 951931

Dominant variants are defined as representing at least 10% of the reads in at least one of the sample. Percentages in bold indicate that the variant was properly identified as a non-singleton in the sample, even if it was present in very low numbers (close to 0.0% abundance).

extract), but no clear behavioral response could be highlighted (Machon and Ravaux, personal communication). If dense bacterial colonization really impairs the olfactory perception, the occurrence of the bacterial coverage on hydrothermal shrimp antennae and antennules can plead for a secondary role of this sense at adulthood, and rather suggests that the hydrothermal shrimp uses other abilities to orient in their environment. Shrimp might detect the dim light emitted by vents (Pelli and Chamberlain, 1989) through their highly modified eyes; they might detect temperature gradients created by the mixing of the hot hydrothermal fluid and the cold background seawater (2–3°C) (Segonzac et al., 1993; Ravaux et al., 2009); shrimp might detect acoustic vibrations induced by hydrothermal fluid emissions (Segonzac et al., 1993). Crustacean hearing abilities and reaction to acoustic or hydrodynamic vibrations, involving antennal or antennular mechanoreceptors, have previously been demonstrated (Lovell et al., 2005). Crone et al. (2006) recorded the sounds emitted by black smokers and suggest that vent sounds may provide some local organisms with navigational cues. To explain the occurrence of bacteria on the antennae and antennules, we can also raise the hypothesis that the bacteria have a specific role for the shrimp, which could thus cultivate them on purpose.

## Bacterial Diversity and Potential Metabolisms

Bacterial communities associated with the three investigated appendages, namely the medial and lateral flagella of the antennules and the antennae, display similar taxonomic composition within a given species. This suggests that these structures are being colonized by the same types of bacteria. None of the tested appendages seems to display a different response to bacterial colonization (for example the exclusion of bacteria via appendage-specific grooming by the shrimp). Communities from the four vent species are on the other hand very different from those found in the non-vent species *Palaemon elegans*. Only six variants are shared between the two groups, and despite that four of the 10 variants that are dominant in vent shrimp occur in *P. elegans*, they represent negligible number of reads in the latter. Higher Shannon Diversity index suggests a more even distribution of variant abundances in *P. elegans*, as does the absence of any dominant variant. Communities from vent shrimp are on the other hand dominated by a limited number of variants belonging to the *Epsilon*- and *Gammaproteobacteria*. These are highly similar or identical to sequences already reported as crustacean epibionts in previous studies on *Rimicaris exoculata* gill chamber, on *R. chacei*, and on the cuticle of the galatheid *Kiwa* sp. (Zbinden et al., 2008; Goffredi, 2010; Petersen et al., 2010; Apremont, 2017). *Epsilonproteobacteria* are among the most efficient colonizers at vents and are very abundant as free-living bacteria as well as epibionts of metazoans (Corre et al., 2001; Lopez-Garcia et al., 2003; Nakagawa et al., 2005). Those associated with the *R. exoculata* gill chamber were shown to oxidize reduced sulfur and hydrogen and use the energy to fix carbon using the reverse TCA cycle based on direct experiments, as well as metagenomics-based approaches that confirmed



the presence of relevant genes (Zbinden et al., 2008; Hügler et al., 2011; Jan et al., 2014). *Gammaproteobacteria* epibionts of *R. exoculata* and *Kiwa* sp. similar to those from the present study are also reported as autotrophic sulfur- and hydrogen-oxidizers that use the Calvin–Benson–Bassham cycle for carbon fixation, while some may instead use methane (Goffredi et al., 2008; Zbinden et al., 2008; Hügler et al., 2011; Jan et al., 2014). A particularly interesting finding is the identification of a variant, dominant in *Alvinocaris markensis*, which sequence is identical to that of the sulfur-oxidizing gill endosymbiont of the vent mussels *Bathymodiolus puteoserpentis* and *B. azoricus*. It may correspond to the free-living form of the symbiont, already suspected to occur on shell-associated biofilms and on an annelid (Petersen et al., 2012), or to a non-symbiotic close relative. Whatever is its status as a potential mussel symbiont, it is likely that it can perform sulfur-oxidation-fuelled autotrophy. Overall, it can be concluded that the most dominant bacteria found on the sensory appendages of vent shrimp are similar to bacteria already documented to engage in associations with metazoans, that have the ability to perform autotrophy and sulfur oxidation. The fact that different host shrimp species display different community compositions dominated by different bacterial OTUs is harder to interpret at this stage, given the limited dataset, but may be linked to their specific sites or microhabitats. The two species that display the most similar communities, namely *R. exoculata* and *R. chacei* (see **Figure 5**), both live in close proximity to the vent fluid emissions and both harbor symbionts in their cephalothorax from which they derive their organic carbon. *Mirocaris fortunata* and *A. markensis* live further away from fluids, likely experiencing different locale conditions.

## Do Bacteria Have a Specific Role for Shrimp?

Based on their unexpectedly high densities, their localization close to chemosensory organs, and their hypothesized metabolisms, the bacterial communities identified on sensory organs of vent shrimp may play several roles. Some episympioses are indeed reported to confer protection or defense from predators or the environment itself, while other are nutritional (Goffredi, 2010). At hydrothermal vents, major recognized roles for metazoan-associated bacteria are nutrition through chemosynthesis and detoxication (Haddad et al., 1995; Jan et al., 2014). Sulfide is abundant in the vent fluids, and necessary to the metabolism of symbionts on which *Rimicaris exoculata*, and partially *R. chacei*, rely for their nutrition (Gebruk et al., 2000; Ponsard et al., 2013). But it is also toxic to animal cells, in particular by interfering with the cytochrome oxidases in mitochondria (Powell and Somero, 1986). Sulfide removal by the dominant bacteria may thus reduce sulfide levels in the vicinity of host sensory cells and confer a certain protection. Another possible important role could be chemosynthetic primary production. Carbon molecules produced by gill chamber epibionts have been shown to be transferred through the thin (0.5  $\mu\text{m}$ ) inner branchiostegite cuticle of *R. exoculata*, providing *trans*-tegumental nutrition (Ponsard et al., 2013). The very thin cuticle of the aesthetascs sensilla (0.5–1.5  $\mu\text{m}$  at the apex in

*M. fortunata*), as well as the pore-like structures occurring in the aesthetasc cuticle (Machon et al., 2018) could permit such transfer, allowing host cells to benefit from bacterial primary production. This would be of limited significance to the whole animal, but could be significant for nutrition of sensory cells. In other contexts, bacteria are known to produce volatile odorants that contribute to host odor or other recognition cues, such as mate choice or social cohesion (Archie and Theis, 2011). Other, rarer, bacterial OTUs may thus have major influence on the system thanks to their metabolisms, emitting odorant molecules perceived by the olfactory receptor neurons of the shrimp, but these are much more difficult to demonstrate.

Besides this direct influence, bacterial communities could influence shrimp in other ways. The localization of bacteria places them at the interface between the organism and its immediate environment. Dense bacterial communities coat the surface of appendages. This coating could, to a certain extent, isolate the appendage from the environment, and could be filtering the thermal information (thermal insulation), as well as vibrations (shock absorption). Bacteria could also alter the chemical cues that actually reach the physically close host sensory cells through selective removal or release of chemical substances. This could influence the way environmental cues are perceived by the nervous system, with eventual consequences on host behavior. Microbiote influence on chemical signals is well documented and should not be under-estimated (Archie and Theis, 2011). The physical proximity between bacterial communities and sensory cells provides a potential route for direct molecule transfer from bacteria to the shrimp nervous system. Overall, the possible influence of bacterial epibionts on the sensory apparatus of vent shrimp cannot be ignored and should be considered in further studies dealing with sensory organs.

## AUTHOR CONTRIBUTIONS

MZ conceived the study on sensory perception in hydrothermal shrimp, performed SEM observations, and drafted the paper. AG and JM performed SEM observations. KS performed molecular analyses. JR conceived the study on sensory perception in hydrothermal shrimp. NL performed BM analyses. SD performed molecular analyses and drafted the paper. MZ, AG, KS, JM, JR, and SD participated in data analysis and wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2018.00357/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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