Animal Microbiome

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Hemolymph microbiota and immune effectors' expressions driven by geographical rearing acclimation of the aquacultured *Penaeus stylirostris*

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Abstract

Background In holobiont, microbiota is known to play a central role on the health and immunity of its host. Then, understanding the microbiota, its dynamic according to the environmental conditions and its link to the immunity would help to react to potential dysbiosis of aquacultured species. While the gut microbiota is highly studied, in marine invertebrates the hemolymph microbiota is often set aside even if it remains an important actor of the hemolymph homeostasis. Indeed, the hemolymph harbors the factors involved in the animal homeostasis that interacts with the microbiota, the immunity. In the Southwest Pacific, the high economical valued shrimp *Penaeus stylirostris* is reared in two contrasted sites, in New Caledonia (NC) and in French Polynesia (FP).

Results We characterized the active microbiota inhabiting the hemolymph of shrimps while considering its stability during two seasons and at a one-month interval and evidenced an important microbial variability between the shrimps according to the rearing conditions and the sites. We highlighted specific biomarkers along with a common core microbiota composed of 6 ASVs. Putative microbial functions were mostly associated with bacterial competition, infections and metabolism in NC, while they were highly associated with the cell metabolism in FP suggesting a rearing site discrimination. Differential relative expression of immune effectors measured in the hemolymph of two shrimp populations from NC and FP, exhibited higher level of expression in NC compared to FP. In addition, differential relative expression of immune effectors was correlated to bacterial biomarkers based on their geographical location.

Conclusions Our data suggest that, in Pacific shrimps, both the microbiota and the expression of the immune effectors could have undergone differential immunostimulation according to the rearing site as well as a geographical adaptative divergence of the shrimps as an holobiont, to their rearing sites. Further, the identification of proxies such

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as the core microbiota and site biomarkers, could be used to guide future actions to monitor the bacterial microbiota and thus preserve the productions.

Keywords Active microbiota, Shrimp hemolymph, Biomarkers, Immune effectors, Host-microbiota interactions, Immunostimulation, Geographical divergence

Background

Microbial communities are known to colonize many multicellular organisms from plankton to plants, vertebrates, or invertebrates, and therefore form the host microbiota [1] with which they are in constant relationships [1–3]. In holobionts, microbiota is known to play a key role on host health, by having a great importance in nutrient intake, physiology, fitness or even in the host immunity [2, 4–12]. In addition, the microbiota can be linked to host pathogenic events or act as probiotics to prevent pathogenesis [4–8]. The importance of the microbiota within a specific host have been reported to be linked to selection mechanisms of its microbiota through the host's immunity effectors activities [13], through vertical transmission [2, 9] and/or horizontal transmission [5, 14, 15]. The microbiota is known to be influenced by various variables inherent of the host such as for example its age [10], the considered tissue [11], the molt stage [16], or the sex [17, 18]. Indeed, the microbiota of the mud crab exhibited a gender partitioning, with higher abundance of taxa related to *Escherichia-Shigella* in the males while *Rhodococcus* were more abundant in females [17]. Environmental factors can also modulate the host microbiota, such as CO_2 concentrations [19], pH [20], temperature [21], food intake [22] and even geographical origin [23]. Up to date, many studies have been done on exploring the microbiota associated with various aquacultured animals such as oyster, shrimp, trout, salmon etc [24]. However, specificities of the microbiota in different rearing conditions were less considered. In addition, many studies have considered only the gut microbiota which is linked to immunity [6, 25–27], but set aside another important immunocompetent site in aquatic crustacean: the hemolymph [17, 28]. It has been established that, in crustacean, the hemolymph is an open-circulatory fluid that harbors both the host immune system (hemocytes) and its own microbiota [28]. Like for the gut microbiota, many hemolymph microbiota studies described the whole microbiota, based on DNA analysis [29–31]. However, the active microbiota and its functions can differ from whole microbiota community [32–34].

Furthermore, besides its microbiota, the hemolymph harbors the hemocytes (immunocompetent cells) that are involved in the animal immunity through the expression of several immune effectors. Among these effectors, antimicrobial effectors which were expressed by all organisms and some of them are specific to marine crustaceans [35–38]. Among them, the peptides such as the anti-lipopolysaccharide factors (anti-LPS factors), the cryptdin, the crustin, and enzymes such as the superoxide dismutase and the lysozyme are widely studied for their role in immunity [38–42]. Other antimicrobial effectors are more lineage-specific such as the peneidin, specific of the penaeid shrimps [42]. The enzymes superoxide dismutase and lysozymes have been respectively identified as antioxidative [43] and as antibacterial [44, 45]. The anti-LPS factors, the cryptdin, the crustin, the peneidin, were reported to have antimicrobial activities depending on the model against both pathogenic and non-pathogenic bacteria, viruses and fungi [37, 46].

Aquaculture is a worldwide growing sector that has to provide sufficient amount of food to fit with the rising human consumption [47]. Among the farmed animals, shrimps rearing is among the main seafood providers [48]. In both New Caledonia (NC) and in several islands of French Polynesia (FP), the aquaculture *of P. stylirostris* is of a great socio and economical interest as this sector allows for example in NC the employment of 500 persons and is the first agri-food exporter. Contrary to FP, in NC high mortalities caused by vibriosis outbreak impact regularly the rearing. The epizooties are due to two *Vibrio* species: *V. penaecida* and *V. nigripulchritudo* [38, 49]. In addition since the past decades, in New Caledonia, larval mortality occurred in hatcheries, with no evidence of causes [4, 5, 50–52]. *Penaeus stylirostris*, originated from Central America, was imported in the 70s and is nowadays reared in several farms New Caledonia and in French Polynesia. Then, geographical divergence of the holobiont considering both the shrimp's microbiota and immune effectors of the shrimps could have occurred. In this context, the objectives of this study were to assess the active microbiota inhabiting the hemolymph of shrimp breeders, *P. stylirostris*, reared in 2 distinct geographical areas: one earthen pond in New Caledonia (NC) and another pond located in Tahiti Island, French Polynesia (FP), along with the expression of the immune effectors. To evaluate the stability of the active microbiota and of the immune status of the shrimps, 2 seasons were considered: cold season (June-September in NC and July-October in FP) and warm season (October-May in NC and November-June in FP), and 2 sampling times at one month apart. By characterizing the active microbial diversity of the hemolymph, we identified that the circulatory system exhibited an important microbial variability between the shrimps according to the rearing conditions and the sites. In each site, specific biomarkers

have been identified along with a common core microbiota composed of 6 ASVs. Interestingly, differential relative expression of immune effectors measured among the two shrimp populations from NC and FP, was correlated to bacterial biomarkers based on their geographical location. We showed bacterial lineages correlating positively with the expression of the immune effectors in NC and not in FP that suggest differential immunostimulation according to the site as well as an acclimation of the shrimps as an holobiont, to their rearing sites.

Results

To investigate the active microbiota inhabiting the shrimp's hemolymph, the active prokaryotic communities were analyzed using amplicon sequencing of the V4 region of the 16 S rRNA gene, across two geographical sites in the Southwest Pacific, New Caledonia (NC) and French Polynesia (FP) (Fig. 1A). The active microbiota of the rearing water was also analyzed to explore the rearing environment. The sample collection was realized, for each site, during the warm and the cold seasons (respectively named WS and CS) to consider seasonal variability. In addition, to investigate if the hemolymph microbiota from a same batch of animals reared in the same condition change within a month, the hemolymph and seawater were collected one month apart for each season (T0 and T1, Fig. 1B). A total of 9,935,705 reads were obtained from 66 libraries (58 hemolymph and 8 seawater samples), distributed in 8,002 distinct ASVs with at least one read. The number of reads per sample varied between 20,631 and 285,199 reads. Finally, six libraries were removed from subsequent analysis after quality filtering step (sample 134) or qPCR analysis failure (samples 16, 28, 37, 42, and 165) (see detailed Table S1).

Microbial diversities in the hemolymph and seawater samples in two geographical sites of the Pacific

To evaluate difference in microbiota diversity between samples, ordinations of the all the samples were constructed using the Principal Coordinates Analysis (PCoA) method and the four beta diversity indices (Bray Curtis, Jaccard, UniFrac and weighted UniFrac) (Fig. 2A and Fig. S1). The four beta diversity indices highlighted the presence of four clusters corresponding to the hemolymph NC, the hemolymph FP, the seawater NC and the seawater FP (Fig. 2A and Fig. S1). These clusters were validated by PERMANOVA, with the type of sample (hemolymph and seawater) being the first explicative variable of microbial diversity, and the site (NC and FP) as second explicative variable of microbial diversity (Table 1). The other variables (season, sampling time referred as time, molt stage, volume of hemolymph sampled) were less or not explicative of the variability of beta diversity between samples (Table 1). The alpha diversity of the samples was measured with six indices according to the four clusters identified in the PCoA (hemolymph NC, hemolymph FP, seawater NC and seawater FP). For Observed, ACE and Chao1 diversity indices, there were the same significant comparisons (Table S2), with lower alpha diversity in the hemolymph samples in NC compared to the hemolymph samples in FP; and higher alpha diversity in seawater samples compared to both NC and FP hemolymph samples (Fig. 2B and Fig. S2A-B). No statistical differences between the alpha diversity indices of the seawater samples in NC and in FP were detected. Pielou (Fig. 2C), Inverse Simpson and Shannon indices were significantly lower in the hemolymphs sampled in NC compared to hemolymph samples in FP (Table S2), but no difference between the hemolymph and the seawater samples

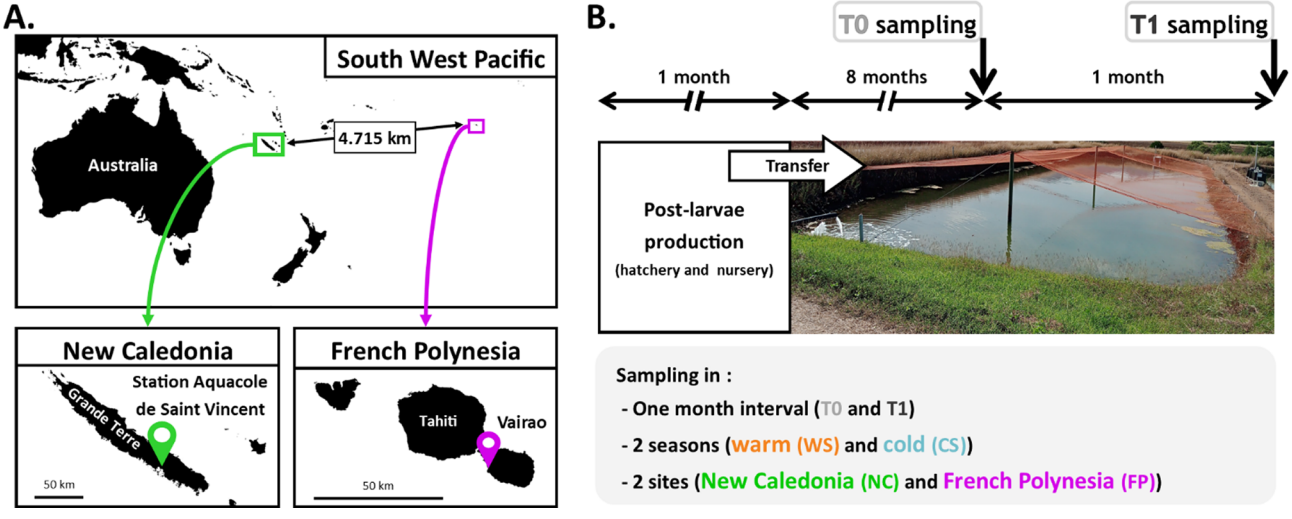

Fig. 1 Experimental design. **A**. Relative position and distance of the island of New Caledonia (NC) and French Polynesia (FP) **B**. Schematic representation of the experimental protocol. Water and hemolymph sampling. Maps were modified from the images source available at: commons.wikimedia.org.: (World pacific 0001.svg, Blank map of New Caledonia.svg, Blank map of Tahiti.svg)

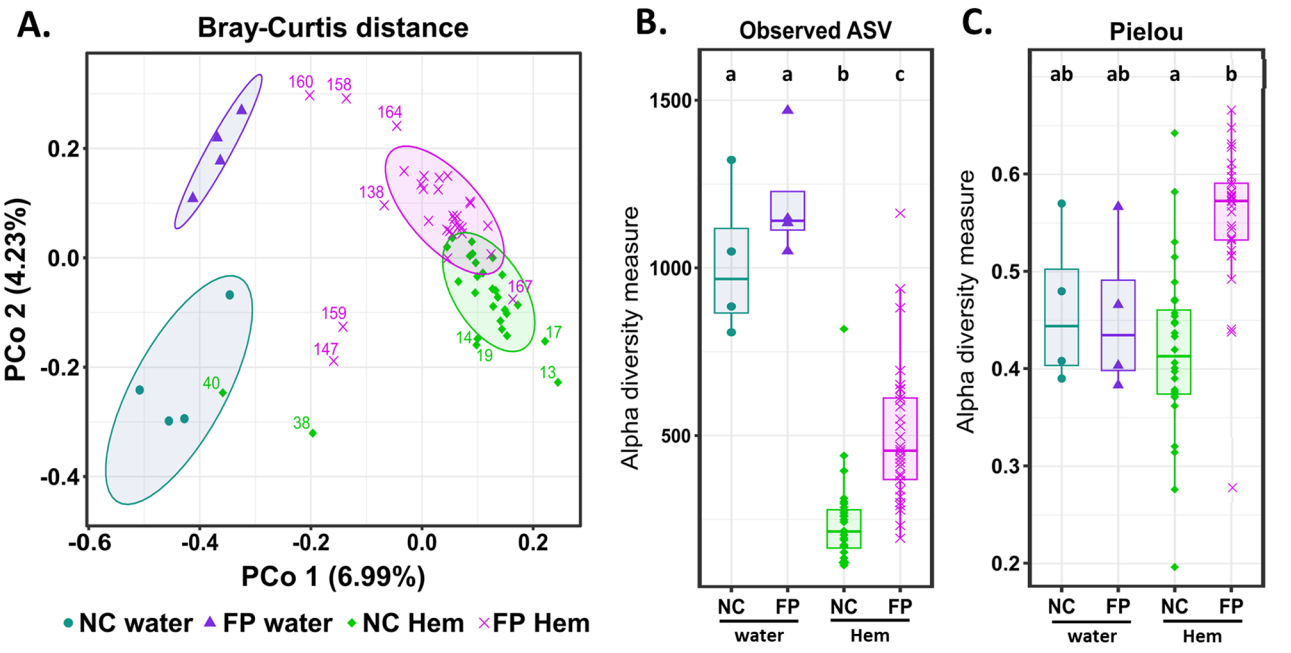

Fig. 2 Microbial diversity distribution among hemolymph and seawater samples according to the site. **(A)** PCoA representation of the beta diversity based on Bray-Curtis dissimilarity matrix. Ellipses regrouped 80% of the samples around the centroid per sample type (hemolymph, water) and site (NC, FP). **(B)** Alpha diversity based on Observed ASVs index. **(C)** Alpha diversity based on Pielou index. The conditions without common letter are significantly different, p-values < 0.05 according to pairwise Wilcoxon tests. Turquoise points stand for the seawater (water) samples collected in New Caledonia (NC); green lozenges for the hemolymph (Hem) samples collected in NC; purple triangle for the seawater sample collected in French Polynesia (FP); pink crosses for the hemolymph samples collected in FP

Table 1 Explicative percent of the dissimilarity between the samples according to four beta diversity indexes based on PERMANOVA[a]

Impacting variables [b]	Bray-Curtis	Jaccard	UniFrac	Weighted UniFrac
Type	5.47%	4.06%	9.64%	11.63%
Site	3.34%	2.56%	5.12%	6.56%
Season	1.90%	1.73%	0%	0%
Type and site	2.53%	2.40%	1.92%	0%
Site and time	1.88%	1.71%	0%	0%
Site and season	1.97%	1.78%	0%	0%
Time and season	1.82%	0%	0%	0%
Sum of the explained variance	**18.90%**	**14.24%**	**16.68%**	**18.19%**

a Calculated using the whole ASVs table

*b*The variables with no significative impact based on either beta diversity indices were not shown

(except the NC hemolymph and water samples based on Shannon index, Fig. 1B and Fig. S2C-D). In addition, Pielou index (Fig. 2C) indicated for each sample, the uniformity of the distribution of each ASV among the whole ASV Table [53] and displayed that the ASV distribution was more homogeneous in the hemolymphs sampled in FP compared to NC.

Bacterial order abundances in water samples

In the NC water samples, the 3 most abundant orders were the *Synechococcales*, the *Rhodobacterales*, the *Rhodospirillales* while in FP water samples, the most abundant orders were the *Synechococcales*, the *Rhodobacterales* and the *Chitinophagales*. Among the 20 most abundant orders, there were 11 orders common to both sites. Detailed results are available in the supplementary data (Fig. S3 and Supplementary results).

Bacterial abundances and relationships between samples

Concerning the microbiota in hemolymph samples, intra-site variability of microbiota at the order level was detected in the two sites (based on the ASVs counts, pairwise Wilcoxon p-value < 0.05, Table S3). In NC, several orders were more abundant in a specific condition such as the *Burkholderiales* in the condition NC T1 CS or the *Rhodobacterales* in the condition NC T0 WS (Fig. 3A). Likewise, in FP, *Flavobacteriales* were found across all conditions but were more abundant in the condition FP T1 CS (between 2.4 and 5.5 times, depending on the condition, Fig. 3B), *Phormidesmiales* were also more abundant in this condition and the *Enterobacterales* in the condition FP T0 CS (Fig. 3B).

Beyond the intra-site variability, at least 5 most abundant orders were found in all conditions for NC, with the *Pseudomonadales*, the *Enterobacterales*, the

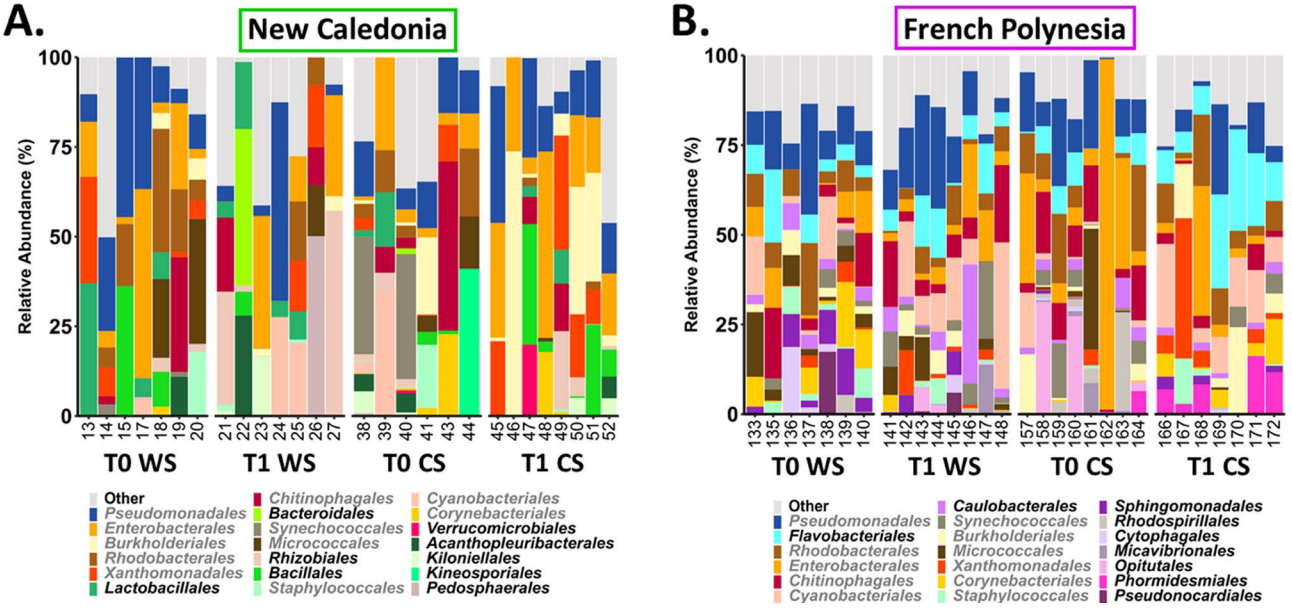

Fig. 3 Relative abundance of the 20 most abundant bacterial orders in the hemolymph samples. **(A)** Hemolymph samples in New Caledonia. **(B)** Hemolymph samples in French Polynesia. Each sample match to a given conditions corresponding to the sampling time: beginning of the experimentation (T0), 1 month later (T1) and season: warm season (WS), cold season (CS). Orders written in grey were common in both sites

Table 2 Explicative percent of the dissimilarity between the samples by site according to the four beta diversity indexes based on PERMANOVA

Impacting variables[a]	Bray-Curtis	Jaccard	UniFrac	Weighted UniFrac
New Caledonia				
Season	0%	0%	4.57%	0%
Time and season	0%	0%	0%	5.63%
Sum of the explained variance	**0%**	**0%**	**4.57%**	**5.63%**
French Polynesia				
Time	5.16%	4.37%	4.29%	7.67%
Season	5.01%	4.27%	5.02%	0%
Molt	14.61%	14.21%	15.63%	0%
Sum of the explained variance	**24.78%**	**22.84%**	**24.94%**	**7.67%**

[a]The variables with no significative impact based on either beta diversity indices were not shown

Burkholderiales, the *Rhodobacterales* and the *Xanthomonadales*; and for FP, with the *Pseudomonadales*, the *Flavobacteriales*, the *Rhodobacterales*, the *Enterobacterales* and the *Chitinophagales* (Fig. 3A-B). Plus, 11 orders were common to both sites among their 20 most abundant orders (in grey in Fig. 3A-B). Interestingly, some orders such as *Rhodobacterales* and *Pseudomonadales*, were detected as most abundant orders in both hemolymph and seawater samples collected in each site (Fig. 3A-B, and Fig. S3A-B). The *Flavobacteriales* and *Chitinophagales* were shared only between FP hemolymph and seawater samples, while no specific order was shared between the hemolymph and water samples in NC.

In addition, according to the PERMANOVA made with the beta diversity indices based on microbial diversity of the hemolymph by site (Table 2), the microbiota in NC was significantly impacted by the season (4.57%) based on UniFrac distance. While it was influenced by sampling time and season combined (5.63%) when using Weighted UniFrac distance, indicating that the phylogeny of the active microbiota was affected by these experimental variables in NC. In addition, we noticed that the variable time itself had no significant influence on the microbiota variability. The other beta diversity indices (Bray-Curtis and Jaccard) were not significantly influenced by the experimental variables (0%). Thus, the experimental variables did not have any strong influence on the microbial variability (presence and abundance). In FP, the PERMANOVA analysis displayed the impact of different experimental variables on the beta diversity for the four indices (Bray-Curtis, Jaccard, UniFrac and Weighted UniFrac, Table 2). Sampling time, season and molt stage explained significantly between 7.67 and 24.94% of the variability. Thus, the presence, abundance and phylogeny of the ASVs between samples were influenced by these variables.

Identification of shared features of the active microbiota inhabiting the hemolymph according to the rearing conditions and the site

Venn diagrams were built to identify specific and common ASVs between experimental conditions (Fig. 4).

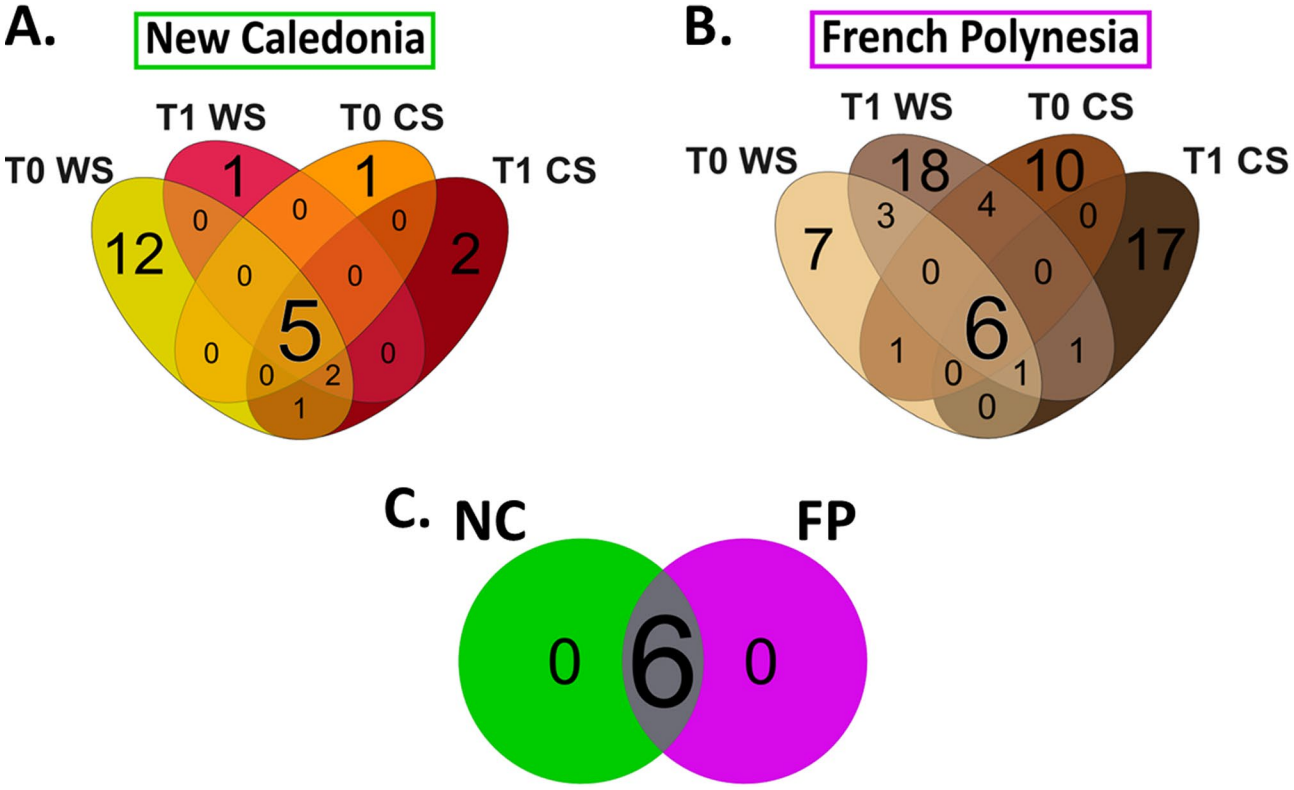

Fig. 4 Specific and common ASVs. Venn diagrams showing the common ASVs and specific ASVs in: **(A)** the NC conditions; **(B)** the FP conditions; **(C)** comparison of the core microbiota found in NC and in FP. Sampling time: beginning of the experimentation (T0), 1 month later (T1); Season: warm season (WS), cold season (CS). For all the Venn diagrams, ASVs present in at least 75% samples were considered

Venn diagrams were generated using all conditions per site (NC and FP, Fig. 4A-B) and between sites (Fig. 4C). ASVs were considered specific to one condition when they were found in at least 75% of the sample of this given condition. In NC, the diagram displayed that 12 ASVs were specific to the condition NC T0 WS and were related to *Escherichia-Shigella* (8 ASVs), *Halomonas*, *Clostridium sensu stricto*, *Synechococcus* CC9902 and *Bacteroides* (Fig. 4A and Table S4). One ASV affiliated to *Blautia* was specific to the condition NC T1 WS; and one other, related to *Staphylococcus* was specific to the condition NC T0 CS. Finally, 2 ASVs were specific to the condition NC T1 CS and were affiliated to *Pseudoalteromonas* and *Massilia* genera. Where all conditions overlapped in the Venn diagram, that displayed the core microbiota made by the active microorganisms that were shared by all the NC conditions. This core microbiota was composed of 5 ASVs affiliated to *Blautia*, *Collinsella*, *Escherichia-Shigella*, *Peptoclostridium*, and *Streptococcus* (Fig. 4A and Table S4). In FP, all conditions showed specific ASVs. There were 7 ASVs specific to the condition FP T0 WS: 6 affiliated to *Micrococcus*, and 1 to *Photobacterium* (Fig. 4B and Table S3). There were 18 ASVs specific to the condition FP T1 WS among which 15 were related to *Escherichia-Shigella* and the 3 others were *Ligilactobacillus*, *Micrococcus*, and *Bacteroides*. In the condition FP T0 CS, there were 10 ASVs considered as specific with 3 related to *Rhodobacteraceae*, 3 to *Saprospiraceae*, 2 to *Pseudoalteromonadaceae*, 1 to *Cyanobiaceae* and 1 to AEGEAN-169. There were 17 ASVs specific to the condition FP T1 CS with, 14 affiliated to the genus *Tenacibaculum* and the 3 others respectively to *Holdemanella*, *Streptococcus* and NS9 marine group. The Venn diagram of the FP samples by condition exhibited a core microbiota made of 6 ASVs affiliated to *Blautia*, *Collinsella*, *Enterococcus*, *Escherichia-Shigella*, *Peptoclostridium*, and *Streptococcus* (Fig. 4B and Table S3). Overall, there were more specific ASVs per condition in FP than in NC (1 to 12 ASVs *versus* 7 to 18 ASVs, Fig. 4A-B). Considering the ASVs present in at least 75% samples both in NC and in FP, 6 ASVs were identified as core microbiota between the two sites: *Blautia*, *Collinsella*, *Enterococcus*, *Escherichia-Shigella*, *Peptoclostridium* and *Streptococcus* (Fig. 4C and Table S3). There was no ASVs specific in the hemolymph samples collected in NC nor in FP meaning that they shared the same core microbiota. The same trend was observed when OTUs were used, with a restricted core microbiota (9 OTUs) and no specific OUT for NC nor FP (using FROGS method, data not showed) [54].

Biomarkers or statistically enriched taxa at all phylogenetic levels, were identified using an LEfSe analysis, considering each condition per site and between sites with LDA score threshold at 4 (Fig. 5). In NC, three of the four conditions showed specific biomarkers enriched at four taxonomic levels (phylum, class, order and family) (Fig. 5A). In the condition NC T0 WS, 2 biomarkers were highlighted: the order *Rhodobacterales* and the family *Rhodobacteraceae*. There were 3 biomarkers in the condition NC T0 CS: the phylum *Cyanobacteria*, the class *Cyanobacteriia*, and the order *Staphylococcales* and 1 biomarker in the condition NC T1 CS, the order *Burkholderiales*. No biomarker was enriched in the condition NC T1 WS. In FP, all conditions showed specific biomarkers (Fig. 5B). In the condition FP T0 WS, 2 biomarkers were identified and were the class *Actinobacteria* and the phylum *Actinobacteriota*. In the condition FP T1 WS, 2 biomarkers were identified: the family *Oscillatoriaceae* and the genus *Phormidium ETS-05*. In condition FP T0 CS, 1 biomarker was enriched: the family *Micrococcaceae*. In the condition FP T1 CS, 7 biomarkers were identified including the order *Flavobacteriales*, the family *Flavobacteriaceae* and the genus *Tenacibaculum*, that had the highest LDA scores. Overall, there were more biomarkers per condition in FP than in NC with lower LDA scores (Fig. 5A-B). Interestingly, there were no common biomarkers to both sites associated with common condition nor any common biomarker associated with a same season (Fig. S4). Then, biomarkers were identified according to each site to highlight site specificity (Fig. 5C). In NC, 18 enriched taxa were identified and taxa with the highest LDA score were the kingdom *Bacteria*, the phylum *Proteobacteria*, the class *Gammaproteobacteria*, and the order *Pseudomonadales*. In FP, only 4 enriched taxa were identified with an LDA score cutoff at 4, and were the order *Flavobacteriales*, the family *Flavobacteriaceae*, the genus *Tenacibaculum*, and the family *Nostocaceae*.

Two co-occurrence networks, one per site, were built at the order level using only the hemolymph samples. One was made for samples collected in NC using 116 orders; and the other one for the FP using 134 orders (Fig. 6A-B). Both networks showed only significative negative co-occurrences. In NC, the center node of the network was *Francisellales* (Fig. 6A), an order composed of the family *Francisellaceae* that include the genus *Francisella*. The *Francisellales* co-occurred with 37 orders including *Myxococcales*, *Desulfovibrionales*, and *Oscillospirales* which had the second higher number of co-occurrences with other orders. In FP, the main node was the *Bifidobacteriales*, linked with 15 other orders including *Streptomycetales*, *Oscillospirales* and *Kiloniellales* which had the higher number of co-occurrences with other orders (Fig. 6B). In addition, both networks, in NC and FP, had 9 similar orders: *Bifidobacteriales*, *Emcibacterales*, *Halanaerobiales*, *Ignavibacteriales*, *Nitrosopumilales*, *Peptococcales*, *Streptomycetales*, *Synergistales*, and *Oscillospirales* (in grey in Fig. 6A-B). To verify the higher level of co-occurrences in NC compared to FP, co-occurrences networks were also constructed at the genus level and showed 216 co-occurrent genera in NC (among 413 genera) and 174 co-occurrent genera in FP (among 557 genera) (Table S5).

Microbiota predicted functions are different depending on the site

To approach the functions of the active hemolymph microbiota, metabarcoding data were processed in

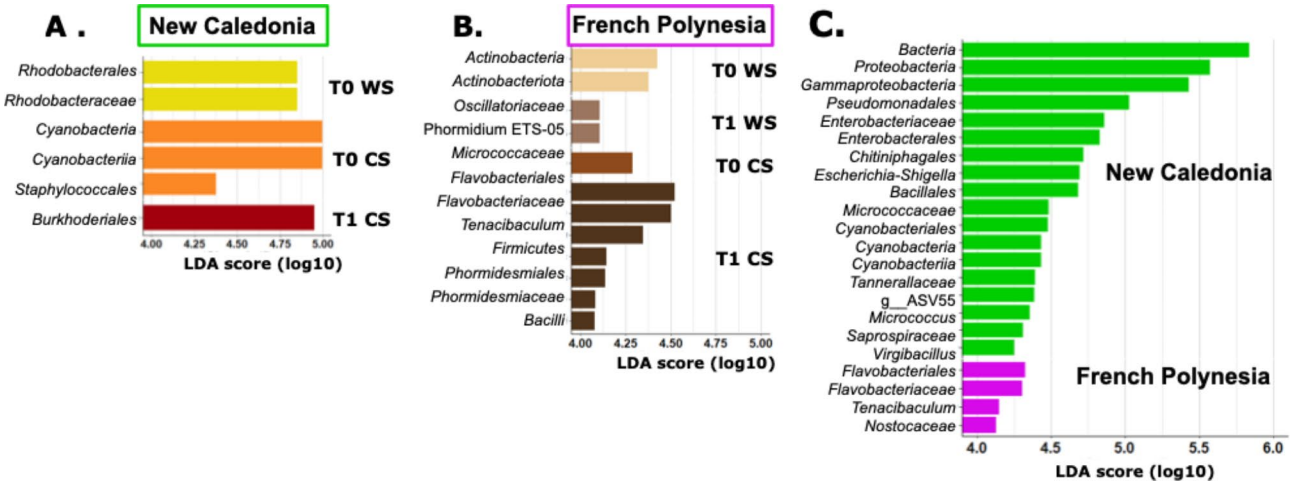

Fig. 5 Biomarkers of enriched taxa at different taxonomic ranks. Biomarkers were identified by LEfSe with an LDA cutoff score of 4 for **(A)** NC conditions, with no biomarker in the condition NC T1 WS; **(B)** FP conditions; **(C)** each site. g__ASV55 meant the ASV55 was affiliated to an unknown genus of the *Tannerellaceae* family, then the g__ in front of the ASV55 indicate that it is not a *stricto sensu* genus. Sampling times: beginning of the experimentation (T0), 1 month later (T1); Seasons: warm season (WS), cold season (CS)

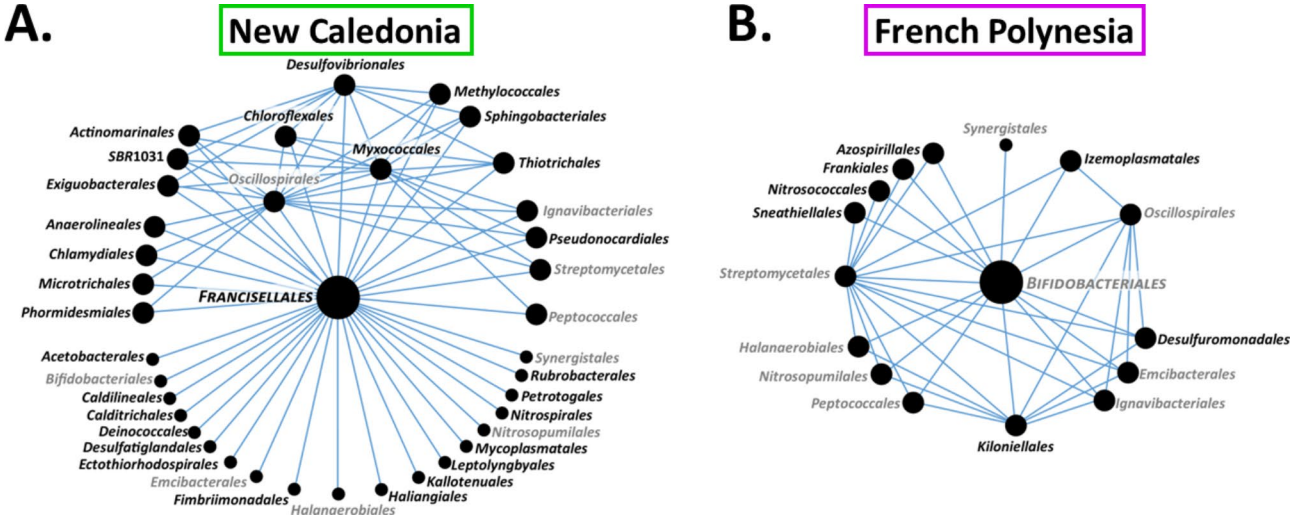

Fig. 6 Co-occurrence networks of the bacterial orders in the hemolymph samples by site. **(A)** in NC; **(B)** in FP. All co-occurrences *(p-values* < 0.05) identified were negatives and were represented with blue lines. Three sizes of dots were used, the smaller dots correspond to orders with only one interaction, the medium dots correspond to orders with multiple interactions and the bigger dot corresponds to the center node of interactions. In grey the 9 orders commons to both sites, the central node of each network is noted in capital letter

Tax4Fun, jointly with the public prokaryote database FAPROTAX and Silva KEGG Orthology (KO) references (SILVA123) [55], to predict putative functions of microbiota in each site at KO level 2 (Fig. 7 and Fig. S5). As displayed in the figure S6, the relative abundances of the putative functions were maintained between conditions as the profiles were neither considered different with Kruskal-Wallis nor with the pairwise Wilcoxon test. Even though the putative functions relative abundances cannot solely discriminate the shrimps' microbiotas between conditions, some predicted enriched functions (KO markers) were identified with an LEfSe analysis made between NC and FP. There were 10 enriched functions in NC and 6 in FP with an LDA threshold set at 3 (Fig. 7). In NC, the putative functions were mostly associated with bacterial competition, infections and metabolism, with the statistically enriched functions related to the two-component system, signal transduction, infectious disease: bacterial, replication and repair, starch and sucrose metabolism, glycan biosynthesis and metabolism. While in FP the enriched functions were only associated with the cell metabolism such as amino acid metabolism, energy metabolism, glycine, serine and threonine metabolism.

Variability of the relative expression of the immune effectors in shrimp hemocytes

In shrimp hemocytes from NC and FP, the relative expressions of 6 immune effectors were measured by qPCR and normalized with the expression of the reference gene, the elongation factor (EF1α), a housekeeping gene already used as a reference gene in shrimp [56]. For all immune effectors but also for each gene independently, differential expressions between NC and FP were significant (pairwise Wilcoxon test, p-values < 0.01) (Fig. 8 and Table S6). For all immune effectors, the relative expressions were higher in NC compared to FP (Fig. 8). In addition, using the relative expression of all genes, all NC conditions were significantly different from all the FP conditions (Kruskal-Wallis p-value < 0.005, pairwise Wilcoxon *p*-value < 0.001) (Table S7). Intra-site analysis displayed no significant difference of the relative expression of the immune effectors between the 4 conditions in NC (pairwise Wilcoxon); while in FP the condition FP T0 WS solely was significantly different from the others (p-value < 0.05) (Table S7). Finally, a PERMANOVA analysis was used to identify the correlations between the relative expressions of the immune effectors, environmental and experimental variables (time, season, molt stage, shrimp weight, hemolymph volume) (Table 3). In NC, the variables time, and season explained 41.23% of the difference in the relative expression of the immune effectors, while in FP, the variables time, season, molt stage, and time with hemolymph volume explained 55.60% of this difference. Notably, variables that explained the more variability of the relative expression were different between sites: the season for NC (33.54%) and the molt stage in FP (27.60%).

Interactions between the microbiota biomarkers and the immune effectors

To further identify potential interactions between the immune effectors and the microbiota, in each site, spearman correlations were done between the relative expression of immune effectors and the biomarkers previously identified (Fig. 9). The correlogram showed significant

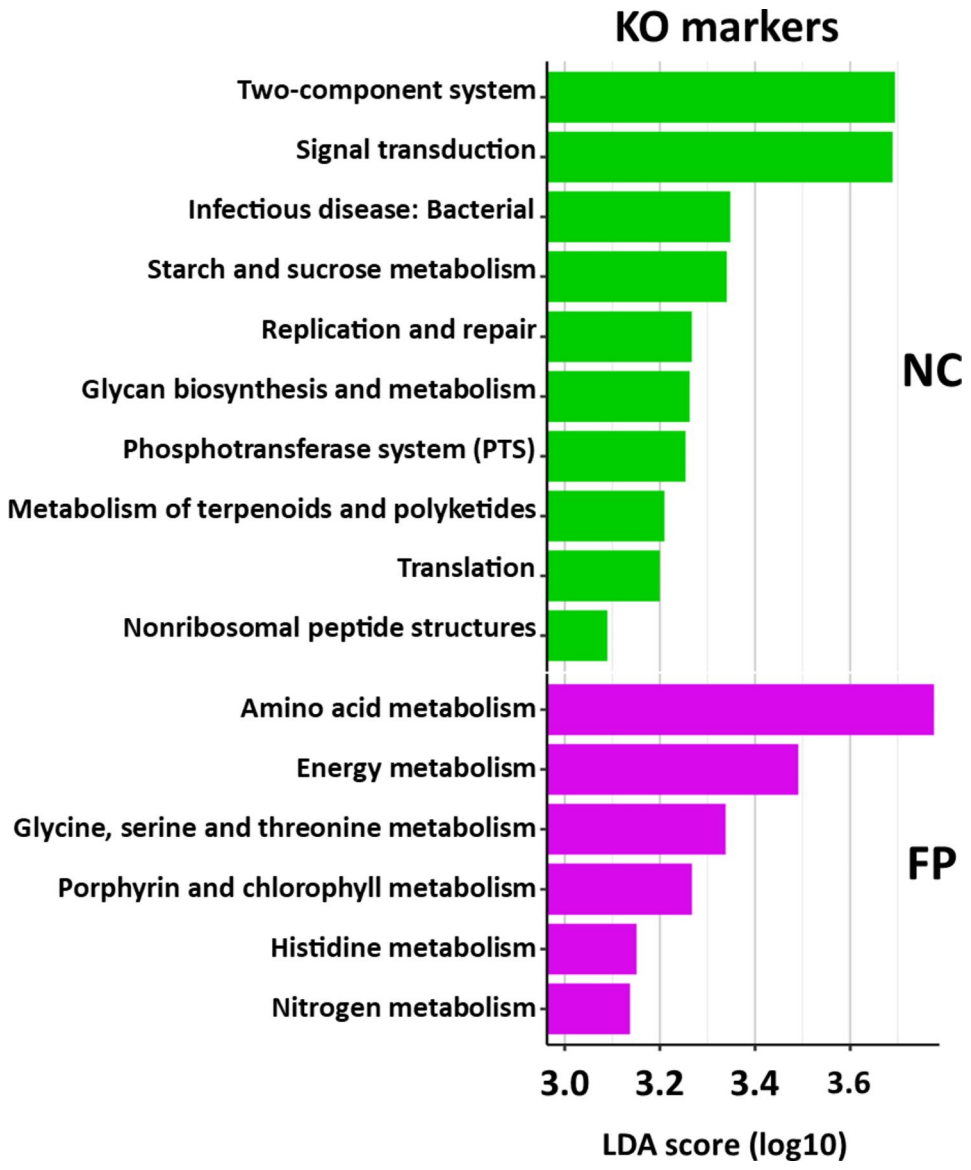

Fig. 7 Putative enriched functions of the microbial diversity inhabiting the hemolymph samples per condition and site. KO markers at the KEGG orthology (K.O) level 2, enriched putative functions in each site, determined by LEfSe with an LDA cutoff score of 3. The putative functions of the host associated-microbiota were identified and assigned with the R package Tax4Fun, the public prokaryote database FAPROTAX and the Silva KO references [55], according to the variables. Site: New Caledonia (NC), French Polynesia (FP)

correlations (p-values ranging between 7.53×10^{-5} and 0.049), in NC with 15 of the 18 biomarkers and in FP with the 4 identified biomarkers significantly correlated with the immune effectors (Table S8). In NC, the *Pseudomonadales*, the *Bacillales* and the *Virgibacillus* had no significant correlation with the relative expressions of the immune effectors. Eight biomarkers correlated positively while 7 correlated negatively with the expressions of the immune effectors. The correlations with the highest absolute coefficient values were the class *Gammaproteobacteria* that correlated positively with the anti-LPS factor and with the lysozyme, and the phylum *Cyanobacteria* and the class *Cyanobacteriia* that correlated negatively with the relative expression of the crustin. Interestingly, all biomarkers in FP compared to NC as depicted in Fig. 5 correlated negatively with the expression of the immune effectors, meaning that the higher abundances of the *Flavobacteriales*, *Flavobacteriaceae*, *Tenacibaculum* and *Nostocaceae* correlated with the lower expressions of the immune effectors (Fig. 9). Also, none of the biomarkers in FP correlated with the anti-LPS factor.

Discussion

The Pacific Blue shrimp *Penaeus stylirostris* represents a high economic and social value in the Southwest Pacific, especially in the two French rearing sites located in New

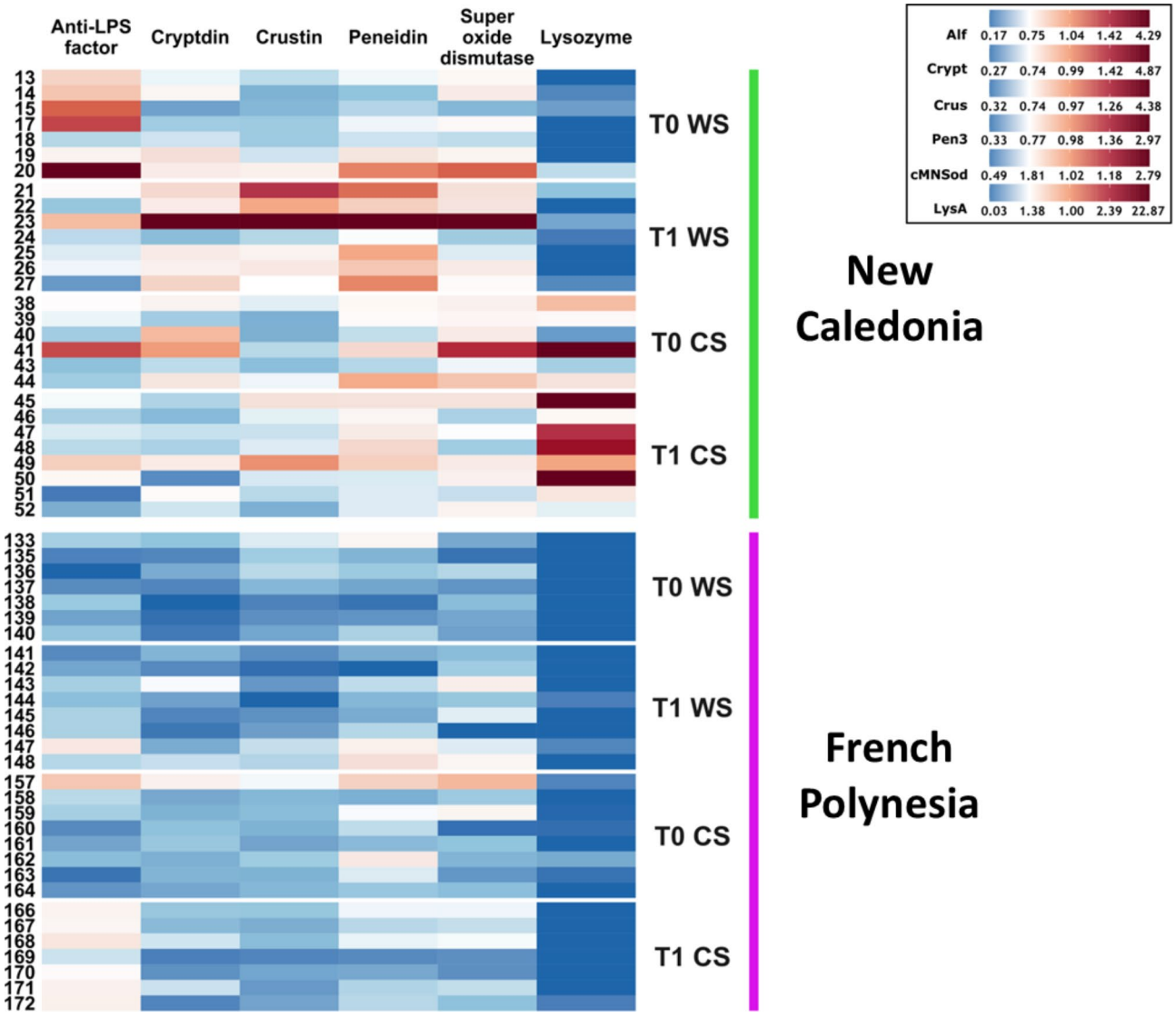

Fig. 8 Relative expression of six immune effectors in shrimp hemocytes. Heatmap of the relative expression of each immune effector per individual shrimp sample. Four peptides: anti-LPS factor (Alf), cryptdin (Crypt), crustin (Crus), peneidin (Pen3), and two enzymes: cytosolic manganese superoxide dismutase (cMnSOD) and lysozyme (LysA). The heatmap was obtained with a 3-colors scale. The minimal, midpoint and maximal color values were set at the percentiles 5, 25 and 95 for each effector independently

Table 3 Explicative percent of the dissimilarity of the effector relative expressions in hemocytes samples

Impacting variables[a]	NC hemocytes	FP hemocytes
Time	7.69%	8.90%
Season	33.54%	10.75%
Molt stage	0%	27.60%
Time and volume	0%	8.35%
Sum Variance Explained	**41.23%**	**55.60%**

*a*The variables with no significative impact based on their dissimilarity were not shown

Caledonia (NC) and in French Polynesia (FP) where this specie has been reared since the 70s. To identify potential mechanisms of shrimps' acclimation to these two rearing sites, we focused on analyzing both the active hemolymph microbiota and the expression of immune effectors in shrimp hemocytes (circulating immunocompetent cells of hemolymph). The hemolymphatic compartment was chosen because it is a semi-open circulatory system with both hemolymph microbiota and shrimp immune cells (hemocytes) [9] in interaction. Thus, hemolymph is at the interface between the microbiota known to have an important role on their host health and to be influenced by environmental factors [19, 20, 57–59] and circulating immune cells known to involve in the animal immunity and homeostasis through the expression of several immune effectors [17, 28]. Interestingly, the two sites studied present specific seasonal variabilities in terms of abiotic pressures (more contrasting seasons in NC

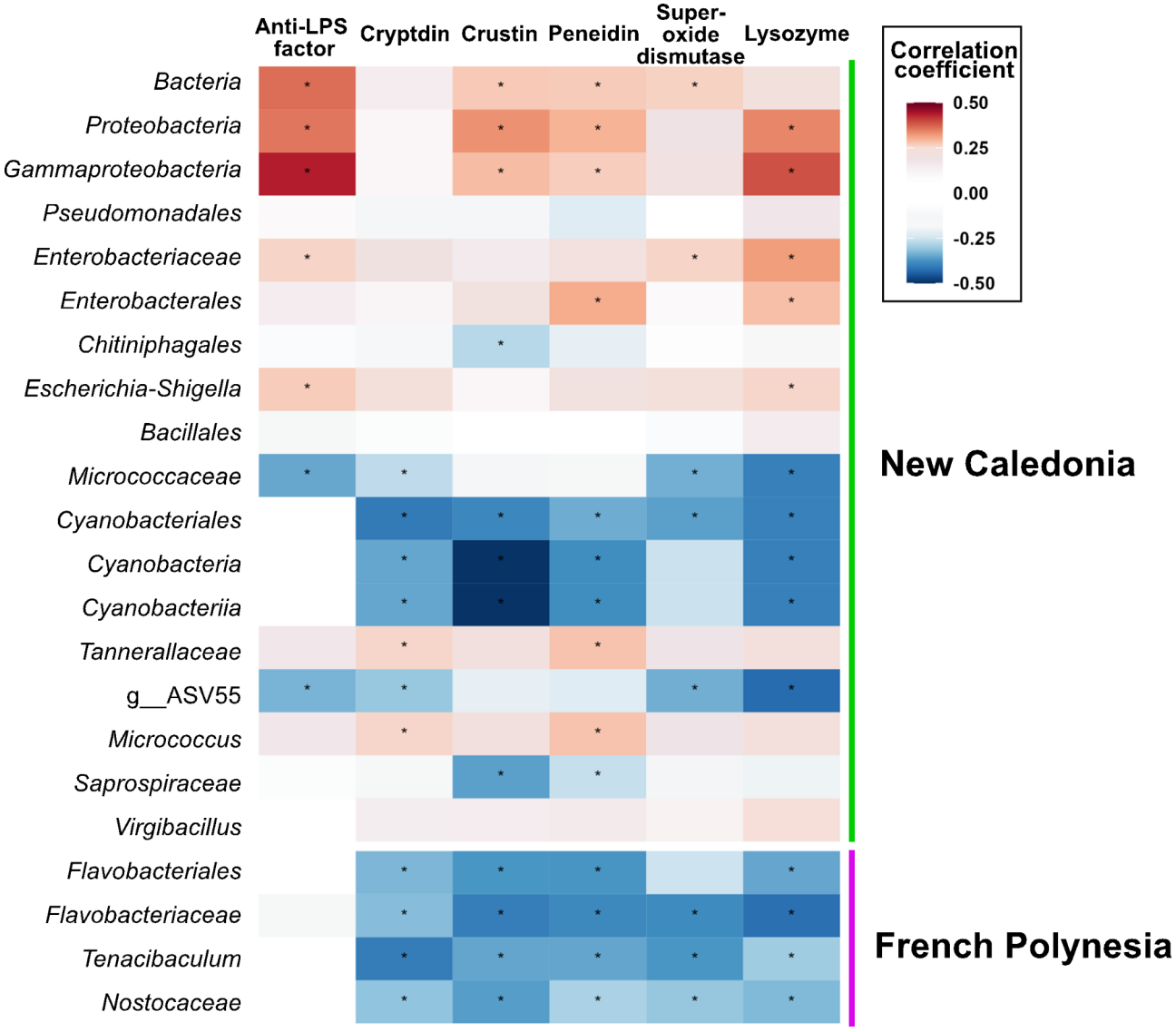

Fig. 9 Correlogram based on the biomarkers and the relative expressions of the immune effectors. Heatmap of the spearman correlation coefficient comparing the relative expressions of each immune effector and the biomarkers identified in the Fig. 5. The calculations were performed for each site independantly. Four peptides: anti-LPS factor, cryptdin, crustin, peneidin, and two enzymes: cytosolic manganese superoxide dismutase and lysozyme. The heatmap was obtained with a 3-colors scale with the minimal, midpoint and maximal color values set at -0.50, 0, and 0.50. Asterix indicated significant correlations

compared to FP) and biotic pressures (seasonal vibriosis in NC [60–62]). To account for these seasonal differences and associated variabilities between sites and in each site, we collected shrimps and seawater in each site during the two tropical seasons with two collection times for each season with one-month interval.

That allowed us to show that the microbiota was different between seawater and hemolymph samples and interestingly between the hemolymph samples in NC and in FP. Moreover, the relative expressions of 6 immune effectors of the shrimps varied between NC and FP (Fig. 8). Our results revealed a restrained core microbiota despite the inter-site variability. Finally, the correlations between the microbiota biomarkers and the level of expression of immune effectors suggested a possible rearing site adaptation and acclimation of the shrimps and their microbiota to their rearing site.

In the light of our results, we have identified a restrained active core microbiota composed of 6 ASVs affiliated to the genera *Blautia*, *Collinsella*, *Enterococcus*, *Escherichia-Shigella*, *Peptoclostridium*, and *Streptococcus* (Fig. 4C). The same trend was observed when OTUs were used (using FROGS method, data not shown) [54]. In this case we found a shared core microbiota between NC and FP composed by 9 OTUs. That proved that the core microbiota shared by the breeder shrimp female at

the intermolt stage is narrowed, as observed at the ASV level. At the writing time, except *Collinsella* and *Peptoclostridium*, the other genera have been found in the gut of marine and freshwater shrimps, but not in the hemolymph. For example, members of the *Blautia* genus have been detected in the gut of *Macrobrachium rosenbergii* [63]. *Enterococcus* species are often used as probiotic in shrimp rearings [64, 65] and are usually found in the gut of *Penaeus vannamei* [64]. The same result is observed with *Streptococcus* that is used as probiotic in *P. monodon* against vibriosis [66], and members of this genus are found in the gut of *P. vannamei* and *P. monodon* [67]. *Escherichia-Shigella*, have been detected in the intestine of *P. vannamei* in their nursery phase [68], in the intestine of adults *P. vannamei* [69] as well as in the hemolymph of mud crab and oysters [70]. However, *Escherichia-Shigella* was often found in the "kitome" being a polymerase contamination [71], so care must be taken considering this taxon. The narrow core microbiota of ASVs that were common between one rearing site in NC and one rearing site in FP, could be explained by the microbiota variability at intra-site level (Table 2). Indeed, within each site, microbial variability appeared to be influenced by different factors (Table 2). In NC, the samples variance based on the ASVs phylogeny and abundance (UniFrac and Weighted UniFrac) was influenced by the rearing conditions (sampling time, season), while in FP the samples variance based on the presence, abundance and phylogeny of the ASVs (Bray-Cutis, Jaccard, UniFrac and Weighted UniFrac) was influenced by the rearing conditions and by the molt stages (Table 2). Thus, one can also assume that the food diet that differ between NC and FP (we have followed rearing protocols classically used in each site since several years), may influence the microbiota inhabiting the shrimps' hemolymph. Despite that, the explanatory percentages appeared surprisingly low, which could be explained by a high intra-individual variability that may mask the influence of the environmental factors. To validate this, a potential approach could involve analyzing a larger sample size. Another possible explanation could be that the microbial variability was primarily driven by factors that were not quantified in our study. Identifying these additional factors could contribute to a better understanding of the hemolymph microbiota. Beside this intra-site variability, we have identified different biomarkers by rearing condition in each site with no common biomarker to both sites (Fig. 5A-B). Thus, there were specificities of the microbiota in each site that could contribute to the small core microbiota.

Beyond the intra-site variability, the rearing site (NC and FP) was the first explanatory factor that has driven the microbiota variability within the shrimps' hemolymphs according to PERMANOVA analysis (Table 1). Comparing both microbial communities (NC and FP), based on the alpha diversity indexes, we showed lower microbial diversity in NC than in FP with or without weighting the rare and abundant ASVs (Fig. 2B and Fig. S2). The Pielou alpha diversity index displayed that the ASVs distribution was less homogeneous in NC compared to FP (Fig. 2C). Moreover, inter-site variability of microbiota associated with a lower diversity (alpha diversity) and homogeneity (Pielou index) in NC compared to FP could also be visualized with the relative abundance of the 20 most abundant bacterial orders in the hemolymph samples (Fig. 3).

In order to identify the potential interactions within microbiota, we have analyzed them with co-occurrence networks for each site separately. The co-occurrence network in NC appeared more complex compared to FP, with higher number of implicated orders and interactions (Fig. 6). In addition, in both networks, all the interactions were negative, with higher negative co-occurrences in NC than in FP. The higher number of negative co-occurrences suggested that the taxa have more contrasting niche preferences where they can interact with their surrounding environment in NC than in FP [72, 73] and may also indicate more bacterial competition for the same resources or/and antagonism in NC than in FP [74]. The latest hypothesis could be supported by the putative enriched functions associated with bacterial competition in NC while in FP enriched functions were associated with metabolism (Fig. 7). The main nodes in networks underlined taxa that influence the microbial community inhabiting the hemolymph [75] and that might also have a key role in the circulatory system. In the NC network, the main node was the *Francisellales*, an order that includes the *Francisella* genus. Even if several species of this genus are known to be pathogens to human, rodents, fishes and even bivalves [76–79]; members of the *Francisellales* order were also found in healthy Manila clam digestive glands [80] and in the superficial sediment of a recent artificial reef area in the Beibu Gulf [81]. In this study the shrimp were healthy (i.e., without signs of vibriosis disease), we can then suggest that this order had a potential role in the immunostimulation of the host. In FP network, the main node was the *Bifidobacteriales*, that include species known to be probiotics for humans [7] or shrimps such as *Penaeus monodon* [82]. The *Bifidobacteria* may also be implicated in the fermentation of fructooligosaccharides which seemed to promote the growth and health of *P. vannamei* [22]. Here, we hypothesis that members of the *Bifidobacteriales* could have enhance the fitness of *P. stylirostris*. Functional validations *via* metatranscriptomic analysis will help to decipher the role of these key lineages in the shrimps' hemolymph.

To explore the interactions between microbiota and shrimp immunity according to the rearing sites (NC vs.

FP), the relative expression of antimicrobial peptides (AMPs) or enzymes in shrimp's hemocytes (circulating immune cells) were quantified and then correlated with the identified bacterial biomarkers per site. These AMPs were selected because they interact directly with microorganisms through different antimicrobial activities against Gram-positive and Gram-negative bacteria including pathogenic *Vibrio* and filamentous fungi [84–89], and can therefore modulate the microbiota of the hemolymph. In addition, cMnSod (cytosolic manganese superoxide dismutase) was selected for its antioxidant activity which is involved in the modulation of the immune response in shrimps and through microbicidal activity [90, 91]. In this study, the relative expressions of all the immune related molecules discriminate according to the site, with higher expression levels in NC compared to FP (Fig. 9). The over expressions of these immune related molecules in shrimps is described to be associated with an immunostimulation by non-pathogenic bacteria [92], LPS [93] or pathogenic bacteria such as *Vibrio* species: *V. parahaemolyticus* [94–97], *V. harveyi* [98, 99] or *V. alginolyticus* [100, 101]. With the season as the first explanatory factor for the variability of expression of immune molecules in NC (Table 3), the greater seasonal climatic variability in NC seemed to influence the expression of immune molecules of the shrimps. The influence of abiotic factors such as temperature and salinity on the expression of immune molecules like AMPs (crustin and peneidin) is already described in arthropods and in shrimp [102–104] and could contribute to the higher expression of immune related genes in NC compared to FP.

Although, no signs of shrimp disease were observed during the experiment, it is important to consider the presence only in NC of mortality events affecting hatcheries (larvae stages) and farms (juvenile and adult stages) since decades and that not occurred in FP which could participate to the immunostimulation observed in NC. Important larval mortalities occurred in all the hatcheries in NC [56] and the larvae might have undergone the stimulation of immune-related genes as previously shown for the embryos of *Penaeus vannamei* [105] as well as for the early stages of farmed shrimps [106]. In addition, all farms in NC were affected by acute mortality outbreaks since decades and associated to two seasonal vibriosis. One of these vibriosis occurred in winter and is caused by *V. penaecida* ("Syndrome 93" [107]); while the other one occurred in summer and is due to *V. nigripulchritudo* ("summer syndrome" [60]). During our experiment, no signs of disease were observed, which could be due to low-shrimp density rearing that impede pond eutrophication due to lower input of food and therefore limit vibriosis outbreak [108] while classical farms have higher shrimp density. However, we cannot rule out the possibility that the shrimp sampled met pathogenic or opportunistic *Vibrios* during rearing in tanks up to their reproductive size, the stage used in these experiments. In addition, these breeders are potentially the result of larval rearing affected by recurrent mortalities, which could have had a shaping and immune priming effect on the shrimp sampled as part of this experiment. Thus, the potential contact with pathogenic or opportunistic *Vibrios* of shrimps used and/or their past generations, coupled with higher seasonal variations of abiotic factors, could have induce the immunostimulation observed in NC compared to FP. Therefore, it is necessary to determine whether this immunostimulation observed in NC is induced during shrimp development or whether these higher expression levels of immune molecules are a heritable characteristic of genotypes raised in NC, which would have adapted to the rearing conditions of NC by genetic [109] and/or epigenetic mechanisms [110, 111].

In another hand, Spearman correlations made between the gene expression levels of immune effectors and the microbial biomarkers identified in each site, evidenced positive, negative or no significant correlations in NC, while only negative correlations were found in FP (Fig. 9). With the 22 identified biomarkers, between 7 and 14 correlations per effectors (positives or negatives) were identified, indicating complex host-microbiota interactions in shrimps' hemolymph. These correlations (positives or negatives) between microbial families and immune gene expressions were already described in a nonmammalian vertebrate and could participate to cross talk between the gut microbiota and the immune status of the host in healthy animals [112, 113]. Positive correlations identified only in NC seemed more important than negative correlations, as they involved biomarkers with higher LDA scores. These positive correlations could indicate an adaptation of given bacterial biomarkers to certain shrimp immune effectors only in NC. Bacterial adaptation to host immunity was already described for some beneficial or pathogenic bacteria, which have developed mechanisms to escape or resist to the host immunity and particularly to their antimicrobial effectors. Several mechanisms of bacterial resistance (pathogens or/and symbiotes) to the host immunity were evidenced in invertebrate and vertebrate hosts like proteolytic degradation of AMPs by extracellular enzymes, biofilms formation to increase physical and coordinate protection to AMPs, surface modifications to decrease the affinity of AMPs to bacterial cell envelope or the elimination of AMPs already attached to bacterial cell envelope by dedicated efflux pumps [114, 115]. Interestingly, seven biomarkers (*Proteobacteria*, *Gammaproteobacteria*, *Enterobactriaceae*, *Enterobacterales*, *Escherichia-shigella*, *Tannerellaceae* and ASV 55) were positively correlated to several immune molecules (between 2 and 4 among the 6

tested), which could indicate an adaptation of these bacterial biomarkers to different antimicrobial effectors of shrimps. This adaptation to multiple antimicrobial effectors seemed to indicate a long-term association between shrimps and their microbiota in NC [30, 116]. However, mechanisms used by these biomarkers positively correlated with elevated levels of immune molecules expression, to resist and/or escape shrimp immunity, remain to be elucidated. In contrast, several biomarkers in NC and FP were negatively correlated with the antimicrobial effectors tested (Fig. 9). In NC, the 7 biomarkers negatively correlated were *Chitinophagale*, *Micrococcaceae*, *Cyanobacteriales*, *Cyanobacteria*, *Cyanobacteriia*, *Micrococcus* and *Saprospiraceae*, while in FP the 4 biomarkers negatively correlated were *Flavobacteriales*, *Flavobacteriaceae*, *Tenacibaculum* and *Nostocaceae*. Moreover, the higher levels of immune effectors expression in NC compared to FP were associated to a less diverse and less homogenous microbiota according to the alpha diversity indexes in NC compared to FP (Fig. 2B-C). In addition, correlations made between the putative functions of the microbiota identified as biomarkers and the relative expression of the immune effectors (Fig. S6) highlighted that in NC, all the biomarkers at the function level interplayed positively with the lysozyme. Then, it is not surprising that lysozyme was correlated with functions such as: infection disease, replication and repair, signal transduction or two component system as the lysozyme is an antibacterial enzyme [117]. Therefore, microbial community of the hemolymph could have to repair their membrane to overcome the lysozyme action. In FP, putative function biomarkers were strongly positively correlated with the superoxide dismutase and the anti-LPS factor and less with the peneidin and crustin (Fig. S6). In crustacean, superoxide dismutase is among the main pathway against oxidative stress that damages cells, nucleic acids proteins and lipids. It then makes sense that metabolism related to amino acid, energy, glycine - serine and threonine, histidine or nitrogen metabolisms were positively correlated with this effector in FP to help microorganisms to repair damages caused by oxidative stress [91, 118]. Anti-LPS factor, peneidins and crustin are three antimicrobial peptides, which can act by disrupting bacterial membranes, interfering intracellular communication or by hampering bacterial growth [89, 119]. As for the superoxide dismutase, that can explain the positive correlations in FP with the functions related to certain metabolisms (amino acid, energy, histidine, …). Activities of the peneidins target mostly Gram-positive bacteria through inhibition mechanisms [120] which explained their positive correlation in FP, with the *Gammproteobacteria* (Gram-negative) that might not be affected by this effector. Also, cryptdin, an antimicrobial peptide, had no correlation with the functional biomarkers in any sites (Fig. S6) and was neither strongly correlated with any putative microbial function biomarkers (Fig. 9). Thus, even if shotgun sequencing and meta-transcriptomic are needed to prove these relations, that reinforce the site adaptation of the holobiont.

Negative impact of an immunostimulation on microbial diversity was already evidenced by immune challenges with lipopolysaccharide or peptidoglycan in gut microbiota of several animal models like ants, laying hens or piglets [121–124], but not for microbiota of other animal organs such as human gut or in pig vagina [125, 126]. Thus, in NC the higher basal levels of expression immune effectors seemed to decrease bacterial diversity in favor of some adapted microbial biomarkers. This potential higher basal immunity in shrimp sampled in NC seemed to induce more complex interactions with positive and negative correlations between microbiota and the host immunity, with an overall negative impact on bacterial diversity [127]. Nevertheless, this present work was only a correlative study between the level of expression of immune effectors and microbial biomarkers evidenced, which does not allow us to conclude on a direct link between immunity and microbiota. Indeed, both immunity and microbiota were known to be influenced by several environmental factors, which were variable between the two sites, like water temperature, salinity or food intake and we cannot exclude the influence of these factors on our results [21].

Thus, to really infer causation between immunity and microbiota, we need further functional validations based on gene silencing of some of immune effectors and/or the supply of bacterial biomarkers to the shrimps, associated to host and microbiota analysis. However, the data presented here rise new questions to solve such as deciphering the effects of the basal immunity on the microbiota and vice versa or understanding the mechanisms inducing both the immune and microbial site acclimation and adaptation. Ultimately this will allow to determine the weight of basal immunity in the shaping of the microbiota of the hemolymph and inversely the weight of the microbiota inhabiting the hemolymph to modulate the basal immunity of shrimps. Yet, it is important to notice that these results were obtained from 2 rearing sites (one in each territory) and might be not the glint of all the farms on each island. Validation of such results should be done by considering other farms.

Conclusion

Taken together, our results provide first-time evidence of intra- and inter-site variability of both the microbiota and the immune effectors expressions in *P. stylirostris* shrimps rearing in 2 production sites, one in New Caledonia and one in French Polynesia. Our results highlight a narrowed active core microbiota, biomarkers enriched

in each site as well as putative functions orientated towards bacterial competition in NC and towards metabolism in FP. All our results suggest a site-acclimation of the shrimps regarding the shrimp as an holobiont, the expression of their immune effectors and the microbiota inhabiting the hemolymph. Indeed, the active microbiota of the hemolymph is highly variable, and its composition is notably driven by geographical locations. Differential composition across sites is most likely linked to local difference in environmental pressure leading to differential mortality events. Biotic factors with the microbiota inhabiting the hemolymph and abiotic factors (seasonal effect: temperature, terrestrial runoff in the lagoon, salinity drop when high rain etc.), seemed to influence the immunostimulation of the shrimps in NC. It is also highly probable that both larval mortality and long history of vibriosis outbreak in NC partially shaped the microbiota of the hemolymph as well as the basal level expression of the measured immune effector. Therefore, how this differential mortality might have driven selection at the genotype and microbiota levels remains to be examined. Thus, further analyses are needed to validate such hypothesis such as metatranscriptomic analysis of the holobiont (shrimp and its hemolymph microbiota) as well as an RNAi approach to turn off the expression of specific immune effector and monitor the impact on the microbiota.

Methods

Experimental design and sample collection

The experiments were performed in two sites: New Caledonia (later named NC) and French Polynesia (later named FP), two Pacific islands 4.715 km apart (Fig. 1A). The shrimps were supplied locally by the experimental shrimp hatchery located at the Station Aquacole de Saint Vincent (SASV, Boulouparis, New Caledonia) and by the territorial hatchery located at Vairao (Tahiti, French Polynesia). The shrimps were produced both in New-Caledonia and in French Polynesia following the same rearing protocol as described by Pham, et al. [50]. Briefly, the post-larvae from the nurseries were reared at low density in earthen pond at a density of (5 post larvae per m^2, when reach adult size, shrimp density in pond were reduced to 0.5 to 1 shrimp per m^2, to allow them to grow to the breeder stage after 6 to 8 months of rearing. Through the whole rearing from post larvae to breeders with a seawater renewal set at 15% per day, during eight-months until the shrimps grown into breeders' stage (Fig. 1B). During the rearing, shrimps were fed twice a day with commercial shrimp feed (SICA grower 40 in NC and Aliment Ridley Prawn MR implemented with astaxanthin in FP). At the age of nine-month, eight females' shrimps were randomly selected for the experiment (T0). Then, one month later (later named T1), eight shrimps were randomly sampled from the same earthen ponds, according to the same protocol as the initial sampling. The experiments were realized both in warm and cold season (respectively named WS and CS, Table S8). The one-month interval and the two season experiments were done in order to identify the seasonal and time variability or stability of the microbiota. During the sampling campaigns, eight females were collected and kept in a 50 L tanks filled with seawater aerated with intensive air bubbling, for less than 1 h until both molt stage identification and weight measurement (Table S8). After the determination of these parameters, hemolymph was sampled by direct punction in the anterior ventral artery of the shrimps and mix to Alsever anticoagulant volume to volume (27 mM sodium citrate, 336 mM sodium chloride, 9 mM ethylenediaminetetraacetic acid, 115 mM glucose, pH 7). The volume of hemolymph sampled was recorded (Table S8). Microorganisms from hemolymph were isolated by two successive centrifugations. Hemolymph samples (later named Hem) were centrifuged in a first time (800 g, 10 min, 4 °C) to separate the pellet (hemocytes and some microorganisms) from the supernatant (the rest of the microorganisms) that was mostly transferred in a new tube. The pellets were suspended and vortexed in 500 µL of Trizol™, while the supernatant was centrifuged a second time (10,000 g, 10 min, 4 °C) and the new pellet with the microorganisms was also vortexed in 500 µL of Trizol™. The two tubes were incubated under agitation (2 h, 4 °C) then merged together and centrifuged (12,000 g, 10 min, 4 °C). The new supernatant was kept in a new microtube and stored at -80 °C until the RNA extractions.

Besides, during each sampling campaign, 1 L of seawater was collected, filtered on sterile 0.22 µm filter (S-Pak, Millipore) and stored at -80 °C until RNA extractions. Overall, 8 seawater samples and 64 hemolymph samples were collected through eight conditions (2 sites x 2 seasons x 2 sampling times), with total of 72 samples to analyze.

RNAs extractions and sequencing

Due to the 2 kind of samples (filtered seawater and hemolymphs), two RNA extractions protocols were carried out using the Direct-Zol Mini-Prep kit (Zymo Research) for the hemolymph samples and using the RNeasy PowerWater kit (Qiagen) for the seawater samples, both following the manufacturer protocol. All RNAs were reverse-transcripted into complementary DNA (cDNA) as previously describe by Giraud, et al. [14]. All cDNAs were send to MrDNA (Shallowater, TX, United States) where the V4 region of the 16 S rRNA were amplified and sequenced using 515 F/806R primers [128]. The HiSeq Illumina sequencing was performed using a 2 × 150 paired end run

with an average sequencing depth of 20k raw reads per sample.

Sequences pre-processing

Raw sequences were pre-processed using the Divisive Amplicon Denoising Algorithm (DADA), adapted to Illumina amplicons (*version 1.16*) [129], with the package *dada2* in RStudio (*version 1.24.0*) following the default parameters (maxN = 0, truncQ = 2, rm.phix = TRUE). The sequences were filtered using a maximum expected error rate of 2 (maxEE = 2) and dereplication were calculated from filtered sequences. These measures were implemented within the function *dada()* to detect sample inference based on pooled samples (pool = TRUE), and to identify Amplicon Sequence Variants (ASVs) as described by Callahan et al. [129, 130]. Paired-end reads were merged with a minimum overlap of 20 bases (minOverlap = 20) and the chimeras were removed using the consensus method (method = "consensus", see supplementary results for proportion of chimera identified with consensus or polled method). Taxonomy was assigned downstream up to the genus level using the Silva database (nr99 *version 138.1*) [131], this is due to the low species accuracy (below 20%, against 50% for the genus) for the short V4 fragment used along with the database coverage and error rates generating false positives [132]. Prokaryotic sequences identified as *Archaea* and *Bacteria* were kept, whereas *Chloroplasts*, *Mitochondria*, *Eukarya* and unassign reads at the Kingdom level were removed from the dataset. All 16 S rRNA raw data were made available in the European Nucleotide Archive (ENA), under the accession number PRJEB59838.

Microbiota analysis

Statistical analyses were performed using R in Rstudio as describe in the R markdown script available at [https://gitlab.ifremer.fr/ressac/comistyl]. Briefly, the random nu mber needed for computational purposes was fixed with the function *set.seed()* at 428,131,020 and kept for all statistical analysis. The alpha diversity indices were obtained with the package *phyloseq* [133]. Then, the ASV table was normalized using the Cumulative Sum Scaling (CSS) using the *metagenomeSeq* package [134] and then used for all the following microbiota analysis. A phylogenetic tree was generated with the *DECIPHER* package [135].

The beta diversity was measured with Bray-Curtis, Jaccard, UniFrac and Weighted UniFrac indices using the package *vegan* (*version 2.5-7*) [136]. The variability between samples was established on the whole ASV table using both Kruskal-Wallis and pairwise Wilcoxon tests with Benjamini & Hochberg correction (BH) with *stats*, a built-in R base package.

The permutational analysis of variance (PERMANOVA), and Principal Coordinate Analysis (PCoA) were measured using the package *vegan* [136] as describe in the Riffomonas project [137]. The four beta diversity indexes were used to ordinate the samples with the PCoA and to identify variables correlations with the PERMANOVA based on 10,000 permutations. The PCoA and histograms were drawn with the function *ggplot()* [138]. PCoA ellipses were drawn based on the centroid at a radius of 0.8. Histograms were obtained with the relative abundance from ASV regrouped at the order rank.

Venn diagrams and tables were constructed with the packages *ggvenn* and *gplots* [139, 140]. The core microbiota and ASVs specific of a given condition or site were established on presence of at least 75% of all hemolymph samples from each group, i.e., a minimum of 6 samples per condition and 24 samples per site. The Venn diagrams built with the seawater samples were based on the presence of the ASVs on all 4 samples in each site. The linear discriminant analysis effect size (LEfSe) were performed using the package *microbiomeMarker* (*version 1.2.2*) with an LDA cutoff set at 4 by condition and by site (Fig. 5) [141]. The co-occurrence networks were obtained with the R package *cooccur* [142]. Finally, all functional analysis were performed using the package *Tax4Fun*, the KEGG Orthology (KO) reference data (Tax4Fun2_KEGG), the prokaryote database FAPROTAX and the Silva KO references (SILVA123) as described in the *microeco* package [55, 143]. The results were obtained at the level 2 of the KO ranks.

Immune effector quantification in the shrimps hemocytes

The RNAs extracted from the shrimp hemocytes, after reverse transcription, were also used to quantify the relative expression of six shrimp's immune effectors using quantitative PCR (qPCR) and specific primers (Table S9). A reference gene expression based on the relative expression of the Elongation Factor (EF-1α) was also quantified, along with four peptides: the peneidin (Pen3), the anti-LPS factor (Alf), the cryptdin (Crypt) and the crustin (Crus), and two enzymes: the cytosolic manganese superoxide dismutase (cMnSOD) and the lysozyme (LysA). For each sample, the total RNA (150ng) was reverse transcribed using random hexamer primers and the Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) in 20 µl, according to manufacturer's instructions (Invitrogen). For gene expression analyses, qPCR assays were carried out on the Light-Cycler 480 System (Roche Diagnostics GmbH) in qPHD-Montpellier GenomiX platform (Montpellier University, France). The final volume of each qPCR reaction, 1.5 µL, consisted of 1 µL of 1 X Light-Cycler 480 master mix containing 0.3 µM of each specific primer and 0.5 µl of cDNA diluted at 1/8 (equivalent to 9.38 ng of total RNA) in DNAse-RNAse free water. The primer pair efficiencies (E) were calculated using amplification from five serial dilutions

of pooled cDNA (all samples from hemocytes of NC and FP) ranging from 1/4 to 1/64 in Dnase-Rnase free water in triplicate. Primer pair efficiencies were calculated from the given slopes in LightCycler software according to the equation: $E = 10^{-1/slope}$. The qPCR assays were submitted to an initial denaturation step of 3 min at 95 °C followed by an amplification of the target cDNA (40 cycles: denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s and extension time at 72 °C for 25 s) and fluorescence detection. Relative expression of immune related genes was calculated using the $2^{-\Delta\Delta Cq}$ method [144], i.e. using the mean of the measured threshold cycle values of the housekeeping gene (EF-1α) to normalize the measured threshold cycle values of target genes.

Kruskal Wallis, pairwise Wilcoxon, PERMANOVA, and Spearman correlations were performed using RStudio with the same packages as those used for the microbial analysis. The heatmap was obtained with *ggplot()* with the 3-colors scale. The minimal, midpoint and maximal values were set at the percentiles 5, 50 and 95 for each effector independently.

Statistical analysis coupling the microbiota biomarkers and the immune effectors

The *stats*, built-in R base package, was used to perform Spearman correlation analysis using the functions *cor()* with BH correction and *cor.test()*. Then, the correlogram heatmap were drawn with the function *ggplot()* [138].

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

V.B., J.d.L., D.P., J.L.L., D.S., N.W. and N.C. conceived and designed the experiment. V.B., D.P., D.A., G.P., V.Ba., J-S.L., O.R., J.L.L., C.F., C.B., S.F., M.M., M.A.L., C.L., D.S., N.W. and N.C. performed the experiment and acquired the data. V.P., V.B., J.d.L., D.P., G.P., N.W. and N.C. analyzed the data. V.P. prepared the figures and drafted the manuscript. V.P., V.B., J.d.L, D.P., M.A.L., D.S., N.W. and N.C reviewed and edited the draft. All authors approved the final version of the manuscript.

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Data availability

The raw 16S rRNA data are available in the European Nucleotide Archive (ENA) under the accession number PRJEB59838. All R scripts used for statistical analysis are available in a GitLab directory at [https://gitlab.ifremer.fr/ressac-public/comistyl]. Additional data from this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests

The authors declare no competing interests.

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