Dysbiosis of the *Biomphalaria glabrata* vector snail microbiota following infection by *Schistosoma* parasites.

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Abstract:

The importance of the microbiota in host-parasite interactions has taken a deeper interest recently. Microbiota has to be considered as a third partner playing a central role in these interactions. *Biomphalaria glabrata* is the vector snail of the trematode *Schistosoma mansoni*, the agent of human schistosomiasis causing hundreds of thousands of deaths every year. How snail microbiota is affected in such host-parasite interaction following infection remains unstudied. Here, we analyse for the first time how the snail bacterial microbiota is affected or modified by schistosoma infections. We used a massive 16s DNA sequencing approach (MiSeq) to characterize the *Biomphalaria* bacterial microbiota at the individual level in naïve and infected snails. Sympatric and allopatric strains of parasites were used following infections and re-infections to analyse the modification or dysbiosis of snail microbiota populations in different host-parasite co-evolutionary contexts. Concomitantly, using RNAseq data, we investigate the link between bacterial microbiota dysbiosis and snail anti-microbial immune response. These investigations would have a central role in the understanding of snail/schistosome interaction, and would have critical consequences in terms of snail control strategies for fighting schistosomiasis disease in the field.

Key words: microbiota; bacteria; Biomphalaria snail; *Schistosoma* infection; immune response; dysbiosis

Introduction:

Biomphalaria glabrata is a freshwater snail (Lophotrochozoan, Planorbidae), living in inter-tropical regions more precisely in Latin America. It is found in rivers, ponds, waterways or other freshwater impoundments. *B. glabrata* snails have important medical and epidemiological impacts due to their role as the main vector of *Schistosoma mansoni* (Lophotrochozoan, Platyhelminth, trematoda), the agent of intestinal schistosomiasis. Schistosomiasis is the second most widespread human parasitic disease after malaria affecting over 200 million people worldwide and causing 200 000 deaths annually (WHO 2010). The *Schistosoma* adult parasites mate in the human host venous system. Female worms produced eggs that came across endothelial mesenteric vessels and intestinal epithelium to reach faeces and finally the aquatic environment. Into fresh water, eggs hatched and released miracida, the free-living snail-infective parasite stage. At this step of its life cycle the miracidium needs to infect the fresh water snail *B. glabrata*. Into snail tissues, intensive asexual multiplication leads to the continuous production of hundreds of generations of cercariae, the free-living human-infective parasite stage.

Nowadays, there is no effective vaccine and the treatment for schistosomiasis relies on a single chemotherapeutic treatment, the Praziquantel (Doenhoff, Hagan et al. 2009), against which resistances have been observed (Fallon and Doenhoff 1994). Molluscicides have been also used to impaired Schistosoma transmission in the field. However, the dramatic effects of molluscicides on natural environments are prompting us to seek new ways to prevent and / or control the disease in the field (Tennessen, Théron et al. 2015). To meet this goal, we considered that a better understanding of the interaction between *Biomphalaria glabrata* snails and *Schistosoma mansoni* and the abiotic or biotic factors affecting such interaction might constitute an interesting approach to find alternative control strategies.

In this context, we have conducted numerous studies on immunological interactions between the snail and the parasite (Mitta, Galinier et al. 2005; Bouchut, Roger et al. 2008; Moné, Ribou et al. 2011; Coustau, Gourbal et al. 2015; Galinier, Roger et al. 2017; Mitta, Gourbal et al. 2017). We have demonstrated that the nature of the snail immune response could be dependent to the type of interactions and influenced by both the host and parasite intrinsic capacities. In compatible or incompatible interactions, parasites developed normally in snail tissue or were encapsulated by hemocytes, the snail immune cells (Mone, Gourbal et

al. 2010; Mitta, Adema et al. 2012; Coustau, Gourbal et al. 2015) (Fig. 1). During a sympatric interaction, an immunosuppression is observed while an activation of the immune response is set up during an allopatric interaction (Portet, Pinaud et al. 2018). Following primo-infection and successive challenges, a shift from a cellular immune response toward a humoral immune response have been described (Pinaud, Portela et al. 2016) (fig. 1). Moreover, abiotic environmental factors can modify the snail immune response and change the interaction issue. For example, an increase in temperature leads to a modification of the snail susceptibility towards the *S. mansoni* parasite (Ittiprasert and Knight 2012; Knight, Elhelu et al. 2015).

Finally concerning biotic factors, several studies have shown that microbiota can influence the host / pathogen interaction and/or the host immunity and this within several respects.

The host microbiota can play a role in pre-conditioning immunity. Indeed, in *Aedes sp.,* the midgut microbiota elicits a basal immune activity of mosquito immunity (Ramirez, Souza-Neto et al. 2012), by activating mostly, the Toll pathways (Xi, Ramirez et al. 2008). In *Anopheles gambiae*, the microbiota is know to naturally limit malaria transmission, inducing a wide antimicrobial immune response (Gendrin 2017). Moreover, the bacteria *Enterobacter* which is part of the microbiota was shown to produce reactive oxygen species (ROS) against pathogens (Cirimotich, Dong et al. 2011). Again in *Anopheles sp*., the endosymbiotic bacteria *Wolbachia* leads to an induction of immune genes, like TEP1 (Thioester-containing protein 1), LRIM1 (Leucine-Rich Immune Molecule 1) or defensin 1 (Joshi, Pan et al. 2017). In drosophila, the bacterial microbiota is necessary to produce the Pvf2 factor, during stimulations (Sansone, Cohen et al. 2015). In the tick *Ixodes scapularis* the secreted protein, PIXR, inhibits the bacterial biofilm formation and protect the gut microbiota composition, its inactivation impairs the microbiota homeostasis and increases and facilitates the colonization by *Borrelia burgdorferi*, the Lyme disease agent (Narasimhan, Schuijt et al. 2017). The microbiota may also play a role in host immune shaping. For example, in *Anopheles gambiae*, an unspecific priming based on hemocyte differentiation, naturally induced by the gut microbiota is observed when *Plasmodium* disrupts the barriers that normally prevent the gut microbiota from coming in direct contact with epithelial cells (Rodrigues, Brayner et al. 2010). Similarly, the gut microbiota is necessary for immune priming in *Tribolium castaneum* and efficient response against *Bacillus thuringiensis bv. tenebrionis* (Futo, Armitage et al. 2015). Finally, the host immunity can influence the

tolerance or control of its microbiota. In drosophila, the intestinal homeobox gene Caudal regulates the commensal-gut mutualism by repressing nuclear factor kappa B-dependent antimicrobial peptide genes (Ryu, Kim et al. 2008). In Hydra, the innate immune sensors and effectors protect not only against pathogens but also control microbiota homeostasis (Augustin, Fraune et al. 2010). Moreover, host specific antimicrobial peptides (Fraune, Augustin et al. 2010; Franzenburg, Fraune et al. 2012) and a rich repertoire of pattern recognition receptors (Bosch 2013) are involved in maintaining homoeostasis between animal and the resident beneficial microbiota. Recently, it was also demonstrated that a bactericidal permeability-increasing proteins (BPIs) of the squid *Euprymna scolopes* play different defensive roles to modulate interactions with the squid's symbiont, *Vibrio fischeri*, which colonizes light organ crypt epithelia (Chen, Krasity et al. 2017).

However, to our knowledge no studies have been carried out to analyse the tripartite interaction between the host, its microbiota and the parasite in *Biomphalaria glabrata* / *Schistosoma mansoni* interactions.

Until now few studies have been conducted on *Biomphalaria glabrata* snails to characterize their bacterial microbiota (Ducklow, Boyle et al. 1979; Silva, Melo et al. 2013). The aerobic heterotrophic cultivable flora of 200 snails has been investigated on Agar-Petri-dishes (Ducklow, Boyle et al. 1979). Five predominant bacteria genera including *Pseudomonas*, *Acinetobacter, Aeromonas, Vibrios* and *Enterobacteria* have been identified. In a more recent study, the *Biomphalaria glabrata* bacterial microbiota has been characterized using 16S rRNA sequencing (Silva, Melo et al. 2013) but only on predominant taxa grown in culture. In addition to the 5 genera previously identified, 6 additional bacteria genera were found: *Citrobacter, Cupriavidus, Rhizobium, Stenotrophomonas, Klebsiella* and *Sphingomonas*. In addition, resistant and susceptible phenotypes of *Biomphalaria* snails have been shown to influence diversity and composition of bacterial microbiota. The relative abundances of *Gemmatimonas aurantiaca* and *Micavibrio aeruginosavorus* seemed to be affected. This observation supports the hypothesis that resistance against *Schistosoma* parasite main influence snail microbial communities (Allan, Tennessen et al. 2018).

However, no studies have depicted changes in bacterial microbiota communities in *Biomphalaria* snails following Schistosoma infections. The aim of the present study was thus to investigate the inter-individual structure of microbiota in snail population as well as investigating the influence of various immune stimulations on the host microbiota

composition and dynamic following Schistosoma infection. We realised a global characterization of the bacterial microbiota of naive *Biomphalaria glabrata* snails using 16S rDNA metabarcoding. Then, we analysed changes in microbiota composition following parasite infections with sympatric and allopatric *S. mansoni* isolates, or following primo-infection and challenge with the same two parasite isolates. Knowing that snail immune response is totally different in these various snail/parasite interactions, we concomitantly analysed, using a massive sequencing transcriptomic (RNAseq) approach, the snail immune response that can potentially be involved in the observed microbiota modifications.

Materials and Methods:

Ethical statements

Our laboratory holds permit # A66040 for experiments on animals, which was obtained from the French Ministry of Agriculture and Fisheries and the French Ministry of National Education, Research, and Technology. The housing, breeding, and care of the utilized animals followed the ethical requirements of our country. The experimenter possesses an official certificate for animal experimentation from both of the above-listed French ministries (Decree # 87–848, October 19, 1987). The various protocols used in this study have been approved by the French veterinary agency of the DRAAF Languedoc-Roussillon (Direction Régionale de l'Alimentation, de l'Agriculture et de la Forêt), Montpellier, France (authorization # 007083).

Biological Materials

In this study, we used a single strain for the host snail, the albino Brazilian strain of *Biomphalaria glabrata* (BgBRE) and two strains for the trematode parasite *Schistosoma mansoni*: a Brazilian strain (SmBRE, for sympatric infection) and a Venezuelan strain (SmVEN, for allopatric infection). The Brazilian strains of snail and parasite originate from the locality of Recife; the Venezuelan parasite strain was recovered from the locality of Guacara. All host and parasite strains were maintained in the laboratory on their sympatric snail hosts. The snails were reared in constant temperature at 26°C and fed only with washed salad every 3 days. During experiments, snails were not fed 24 hours before the DNA extraction for metabarcoding analysis.

Experimental Infections (fig. 1)

In order to decipher the inter-individual structure of snail microbiota as well as investigate the influence of different snail immune stimulations on its microbiota structure and dynamics we conducted a complex experimental infection protocol (fig. 1); using naive snail BgBRE and infected snail BgBRE by SmBRE or SmVEN parasites.

Briefly, snails were primo-infected with two parasite strains and either they are individually recovered at 1, 4 and 25 days after infection or challenged with these two same parasite strains and individually recovered at 1, 4 days after challenge. For all experimental infections, the snails were individually exposed for 12h to 10 miracidia in 5mL of pond water, after that they are put in the water aquarium back and separately according to the parasite strain infection.

- *Microbiota analysis*

We performed a primo-infection of 63 BgBRE snails with either SmBRE (sympatric, 49 snails) or SmVEN (allopatric, 14 snails) strains. Then, 25 days after the primo-infection, we re-infected a subset (28 snails) of SmBRE-infected snails with either SmBRE (14 snails) or SmVEN (14 snails).

To examine the effect of primo-infection on the snail microbiota, we took 7 whole-snails for each infection samples on days 1 (groups BB1 and BV1; the second letter refers to the Sm strain origin used for prim-infection), 4 (BB4 and BV4), and 25 (BB25, no SmVEN-infected snails were sampled on day 25) after the infection. Similarly, we sampled 7 whole-snails on days 1 (BBB1 and BBV1; the third letter refers to the Sm strain origin used for challenge) and 4 (BBB4 and BBV4) after the challenge. In addition, we used 6 naive snails collected at the beginning of the experiment (B0) and 6 naive snails collected at the time of challenge (i.e. day 25, B25) as controls. These samples were used to establish if the bacterial communities of naive BgBRE snails were stable in breeding and rearing laboratory conditions.

- *Host antimicrobial immune response*

We performed a primo-infection of 180 BgBRE snails with either SmBRE (sympatric, 140 snails) or SmVEN (allopatric, 40 snails). Then, 25 days after infection, we re-infected a subset (80 snails) of SmBRE-infected snails with either SmBRE (40 snails) or SmVEN (40 snails).

To study the primo-infection transcriptomic immune response of the snail, we took 20 snails pooled for each infection samples on days 1 (BB1 and BV1), 4 (BB4 and BV4), 25 (BB25). After the challenge, we sampled 20 snails in each condition (1 and 4 days), equimolar

amounts for each experimental groups were joined together into a single sample (BBB and BBV). In addition, we used 2 pools of 30 naive snails (B0.1 and B0.2) to establish the basal snail transcriptomic activity.

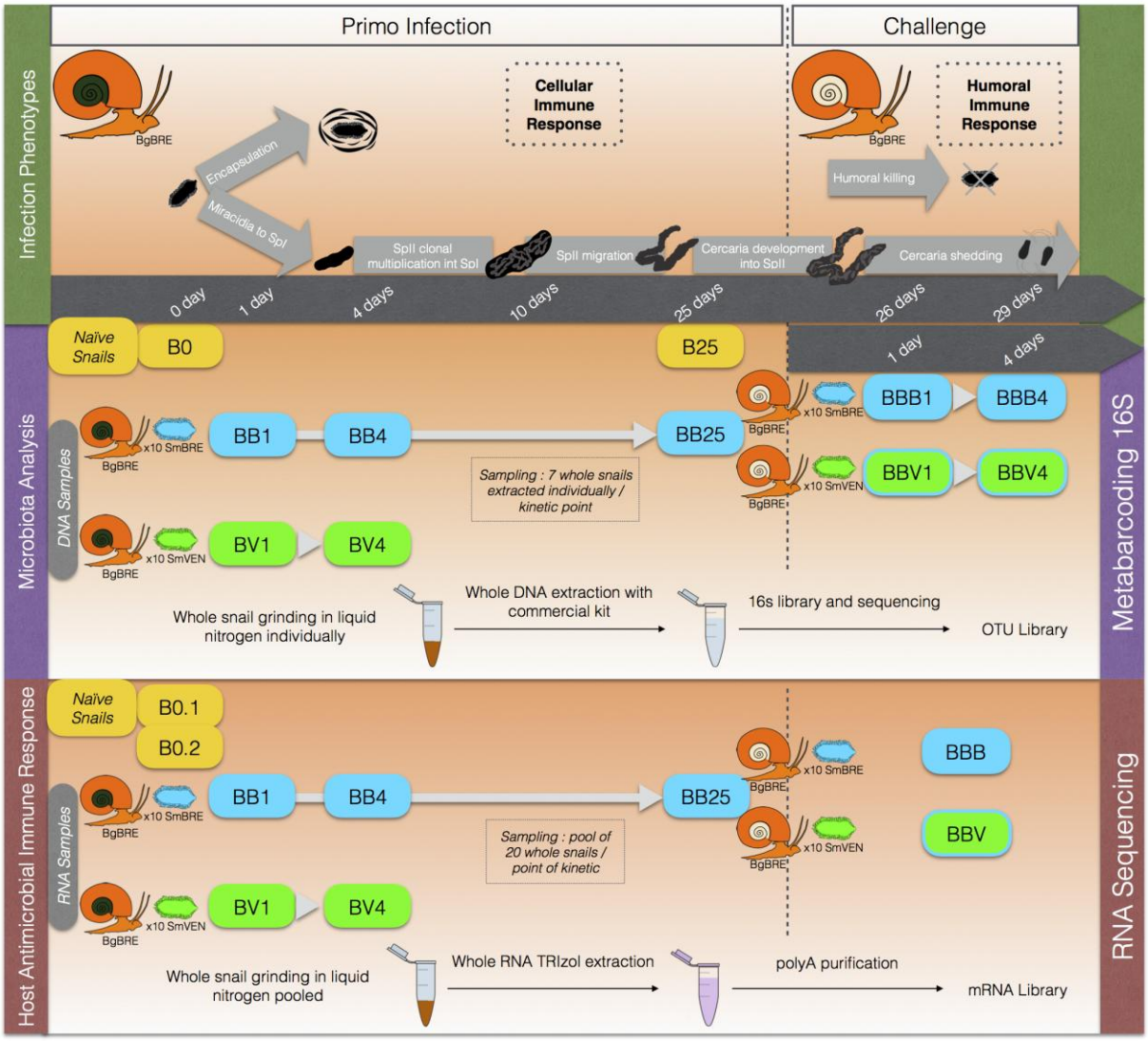

Fig. 1: Overview of experimental procedures

An identification of the bacterial community structure and dynamic and the immune response influence were realised according to *Biomphalaria glabrata* infections. For primo-infection Brazilian *Biomphalaria glabrata* (BgBRE) snails were individually exposed to 10 miracidia of either their sympatric Brazilian *Schistosoma mansoni* trematode parasite (SmBRE – BB - blue) or Venezuelian *Schistosoma mansoni* (SmVEN – BV - green). Following the infection some of miracidia were encapsulated by the hemocytes or developed into primary sporocysts (first intra-molluscan stage). Intra-molluscan parasite stages include two generations of sporocysts (primary sporocyst (SPI) and secondary spororcyst (SpII)) and the production of cercariae. Twenty-five days after primo infection, the snails primo infected with SmBRE were challenged with either 10 SmBRE (BBB) or 10 SmVEN (BBV). In this case all miracidia degenerated into snail tissues, demonstrating the activation of a

humoral immune response (Pinaud et al. 2016). The bacterial community in naive snails (see fig. 2) and its dynamic following infection (see fig. 3,4,5) were analysed by metabarcoding 16S sequencing. The antimicrobial immune response was analysed concomitantly (see fig. 6).

DNA Extraction

Immediately after sampling, shell snails were cleaned with alcohol and the hemolymph was extracted from the head-foot according to standard procedures (Sminia and Barendsen 1980) and individually recovered in Eppendorf tube. Shell snails were removed and total snails were individually with its hemolymph frozen in liquid nitrogen and ground. The total DNA was extracted with DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. The DNA quantification was performed by Qubit 2.0 Fluorometer, using dsRNA BR Assay kit. Individual 16S rDNA amplicon libraries were generated using the 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) primers targeting the variable V3-V4 loops (Klindworth et al., 2012). Paired-end sequencing with 250 bp read length was performed on the Illumina MiSeq sequencing system (GenomeQuébec, Montréal, Québec, Canada) using the v2 chemistry according to the manufacturer’s protocol.

RNA Extraction

Immediately after sampling, snail shells were cleaned with alcohol and removed, then snails are pooled by infection conditions. Total RNA was extracted using TRIZOL (Sigma Life Science, USA) according to the manufacturer’s instructions. Sequencing was performed in paired-end 72-bp read lengths, using an Illumina Genome Analyzer II (MGX-Montpellier GenomiX, Montpellier, France).

Microbiota Analysis

- Data analysis of 16S sequences

The FROGS pipeline (Find Rapidly OTU with Galaxy Solution) implemented on a galaxy instance (http://sigenae-workbench.toulouse.inra.fr/galaxy/) was used for data processing (Escudié, Auer et al. 2017) Briefly, paired reads were merged using FLASH (Magoč & Salzberg, 2011). After denoising and primer/adapters removal with cutadapt (Martin, 2011), de novo clustering was done using SWARM, which uses a local clustering threshold, with aggregation distance d=3 after denoising. Chimeras were removed using VSEARCH (Rognes,

Flouri, Nichols, Quince, & Mahé, 2016) . We filtered out the singletons and performed taxonomic assignment using Blast+ against the Silva database (release 128, sept 2016).

All statistical analyses were done using R v3.3.1 (R: a language and environment for statistical computing, 2008; R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria [http://www.R-project.org]). We used the phyloseq R package for community composition analysis (McMurdie & Holmes, 2013) to infer alpha diversity metrics at the OTU level, as well as beta diversity (between sample distance) from the OTU table. Community similarity was assessed by Principal Coordinate Analysis (PCoA) using the Bray-Curtis distance matrices.

We performed a Mann Withney U test ($p<0.05$) to compare alpha diversity and one-way PERMANOVA with a Benjamini & Hochberg post-hoc to compare beta diversity metrics between groups of samples. For all analyses, the threshold significance level was set at 0.05.

- *Analysis of core-microbiota*

We defined the core-microbiota as the set of bacterial groups that were present in all naive snails. To determine which families belong to core-microbiota, we made a pivot table from the OTUs and the taxa ones, and we conserved only which were present in 100% of the naive individuals excluding unknown or multi-affiliation affiliations at lower taxonomic ranks (supplementary table 1). Once the OTUs selected we recovered the abundance of each of these families to establish the composition of the core-microbiota.

To check if the core-microbiota is affected by infection, we compared the abundance of core families between infected snails and naive conditions with a one-way PERMANOVA with a Benjamini & Hochberg post-hoc. Conditions with significant differences ($p<0.05$) were noted. Moreover, a frequency test was realised to determine which specific families were affected during infection. Families with significant differences ($p<0.05$) were noted. The number of significantly differentially represented families at each sampling day (1, 4 and 25 days primo infected and 1, 4 days challenged) was calculated to examine the temporal variability during the course of infection.

Transcriptome analysis of antimicrobial immune response

- *Antimicrobial response transcriptome*

An antimicrobial transcriptome was constituted with transcripts known to be involved in *Biomphalaria* immune response against bacteria (see supplementary table 2 for transcript

details). These transcripts were recovered from GenBank and the *Biomphalaria* genome (Adema, Hillier et al. 2017) as full-length sequences. This antimicrobial transcriptome was then additionned with concatenate datasets tools on a Galaxy server with the *Biomphalaria* transcriptome de novo assembled in the laboratory (see (Dheilly, Duval et al. 2015; Pinaud, Portela et al. 2016; Galinier, Roger et al. 2017) for details). Before concatenation a Blastn (70 % identity and 90% coverage) was conducted to identify the similar transcripts between both transcriptomes, them discarded from the *de-novo* assembled transcriptome to avoid mapping error and bias during Bowtie2 read counting.

- *Differential expression analysis*

High-quality reads (Phred score >29) were aligned to the concatenated transcriptome using Bowtie2 (v.2.0.2), which was run locally on a Galaxy server. The DESeq2 (v2.12) R package was used to identify differential expression levels between uninfected (B0.1 and B0.2) and infected conditions (p value < 0.05).

Results:

Biomphalaria microbiota characterization

To examine the stability of naive snail microbiota, we first inspected the microbiota diversity and composition in the control snails. We found no significant differences between the B0 and B25 snails in any of the alpha diversity indices (supplementary table 3).

At the phylum level, individual naive snails at B0 and B25 time points show little inter-individual variability and a stable composition over time (fig. 2B), with Proteobacteria, Bacteroidetes, Cyanobacteria and Planctomycetes phyla were the most represented. Therefore, the bacterial microbiota of BgBRE seems to have a considerable temporal and inter-individual stability in our laboratory rearing conditions (fig. 2A).

In terms of composition, we observed that 67 (69%) and 86 (89%) of the 97 OTUs families were shared by B0 and B25 naive snails respectively (fig. 2B). We determined the core microbiota as the families the most abundant and present in 100% of naive snails. We highlighted 62 on 97 OTUs families present in all naive individuals, which represent the *B. glabrata* core-microbiota (table 1), and correspond to 92.5% and 69.6% of OTUs families in B0 and B25 respectively.

Fig. 2: *Biomphalaria* microbiota characterization

Characterisation of the *Biomphalaria* bacterial microbiota of six naive snails recovered at the start of experimentation (B0.1; B0.2; B0.3; B0.4; B0.5 and B0.6) and 6 naive snails recovered 25 days after the start of experimentation (B25.1; B25.2; B25.3; B25.4; B25.5 and B25.6). A. Phylum level composition of the 20 most abundant OTUs phyla among the 12 naive snails. B. The Venn diagram represents the number of the 97 OTUs families which shared between the 6 naive snails at B0 (left Venn diagram), and between the 6 naive snails at B25 (right Venn diagram).

Microbiota dynamics during *Biomphalaria* infection by *S. mansoni*

After establishing the stability of naive *Biomphalaria* bacterial microbiota, we next investigated whether *Schistosoma mansoni* infections affected the microbiota composition, structure and dynamic.

To investigate the influence of parasite infections on the bacterial microbiota communities, we then analysed microbiota dynamic during sympatric and allopatric primo-infections and challenge (fig. 1).

We did not observe significant changes in alpha diversity during the course of primo-infection compared to naive snails, except a decrease of Shannon diversity four days after sympatric infection (figure 3, supplementary table 3, Mann-Whitney U test: supplementary table 4). Conversely, all indices were affected following the immune secondary challenge compared to naive snails (figure 3, supplementary table 3, supplementary table 4). Indeed, the observed species richness (Mann-Whitney U test, p=0.002), the Chao 1 richness index (Mann-Whitney U test, p=0.006), Shannon diversity index (p=0.0004) and Pielou evenness index (Mann-Whitney U test, p=0.009) were significantly reduced in challenged compared to naive and primo-infected snails (figure 3, supplementary table 3, supplementary table 4). However, 4 days after allopatric challenge the alpha diversity of microbiota was not affected, this phenomenon may highlight a return to normal microbiota composition (figure 3, supplementary table 4). Generally, to conclude the alpha diversity seemed to be affected by the type of infection (sympatric or allopatric primo-infection, primo-infection or challenge,) and decreased following challenge infection.

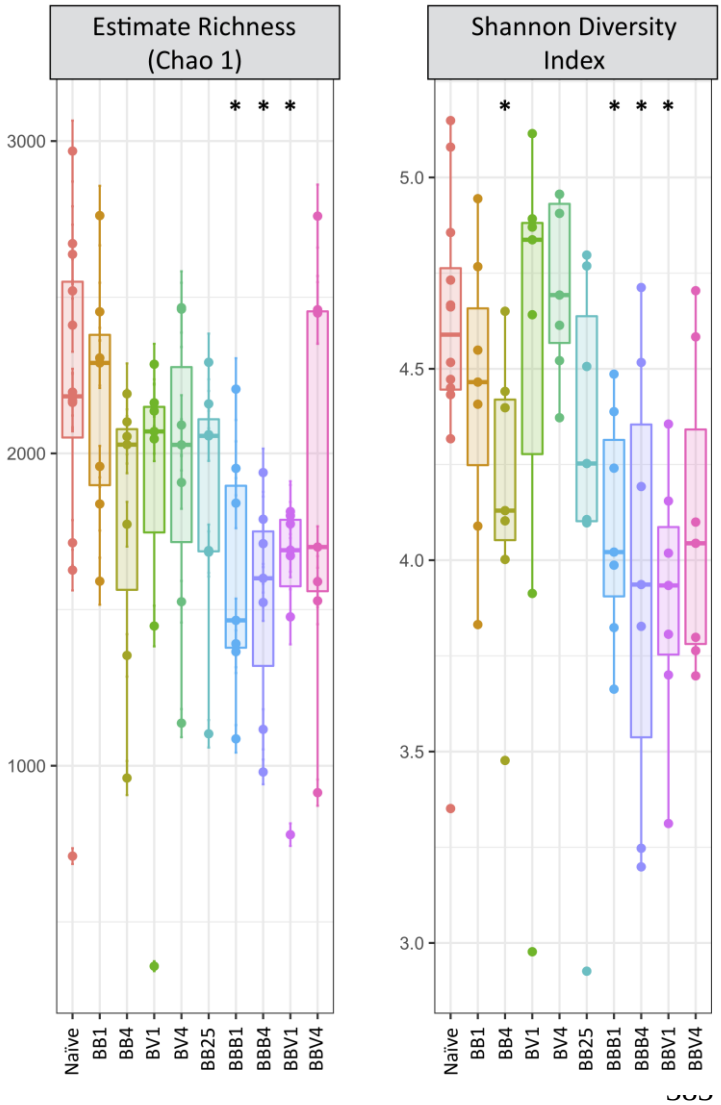

Fig. 3: Alpha Diversity

Boxplots of Chao1 and Shannon indices for all samples. For the Naive condition, B0 and B25 snails were pooled; BB: primo infection of BgBRE by SmBRE; BV: primo infection of BgBRE by SmVEN; BBB: primo infection of BgBRE by SmBRE and challenge by SmBRE; BBV: primo infection of BgBRE by SmBRE and challenge by SmVEN. The time point is mentioned with 1, 4 or 25 corresponding to the day after primo-infection or challenge. The differences between naive and infected conditions were tested with a Mann-Whitney U test and significant differences mentioned with "*".

Regarding beta diversity, Principal Coordinate Analysis (PCoA) on Bray-Curtis dissimilarity between samples revealed grouping of the BB25 samples with the naive snail samples (B0 and B25) separately from the other infected-snail samples (fig. 4A, supplementary table 5). Although the BB25 group was still significantly different from the naive snails, it suggests that the snail microbiota is resilient to infection, with a tendency to recover between 4 and 25 days after the primo-infection.

Moreover, the samples from day 1 and day 4 after infection were separated along the first axis (fig. 4A). This axis separated the type of infection (primo-infection and challenge) as well (fig. 4A). These results indicate that the response of the snail microbiota to the infection is linked to the infection type (primo-infection vs challenge) and the stage of infection (early vs late) rather than by the identity of the parasite strain (sympatric or allopatric). Analysis of Bray-Curtis dissimilarity index revealed a significant difference between naive and primo-

infected samples (p=0.001) and also between naive and challenged samples (p=0.001) (supplementary table 5).

Moreover, we observed the OTUs phyla dynamic following infections, some phyla decrease (Bacteroidetes or Tenericutes) or increase ((Planctomycetes) consistently following the infection, quite similarly following primary or secondary (fig. 4B).

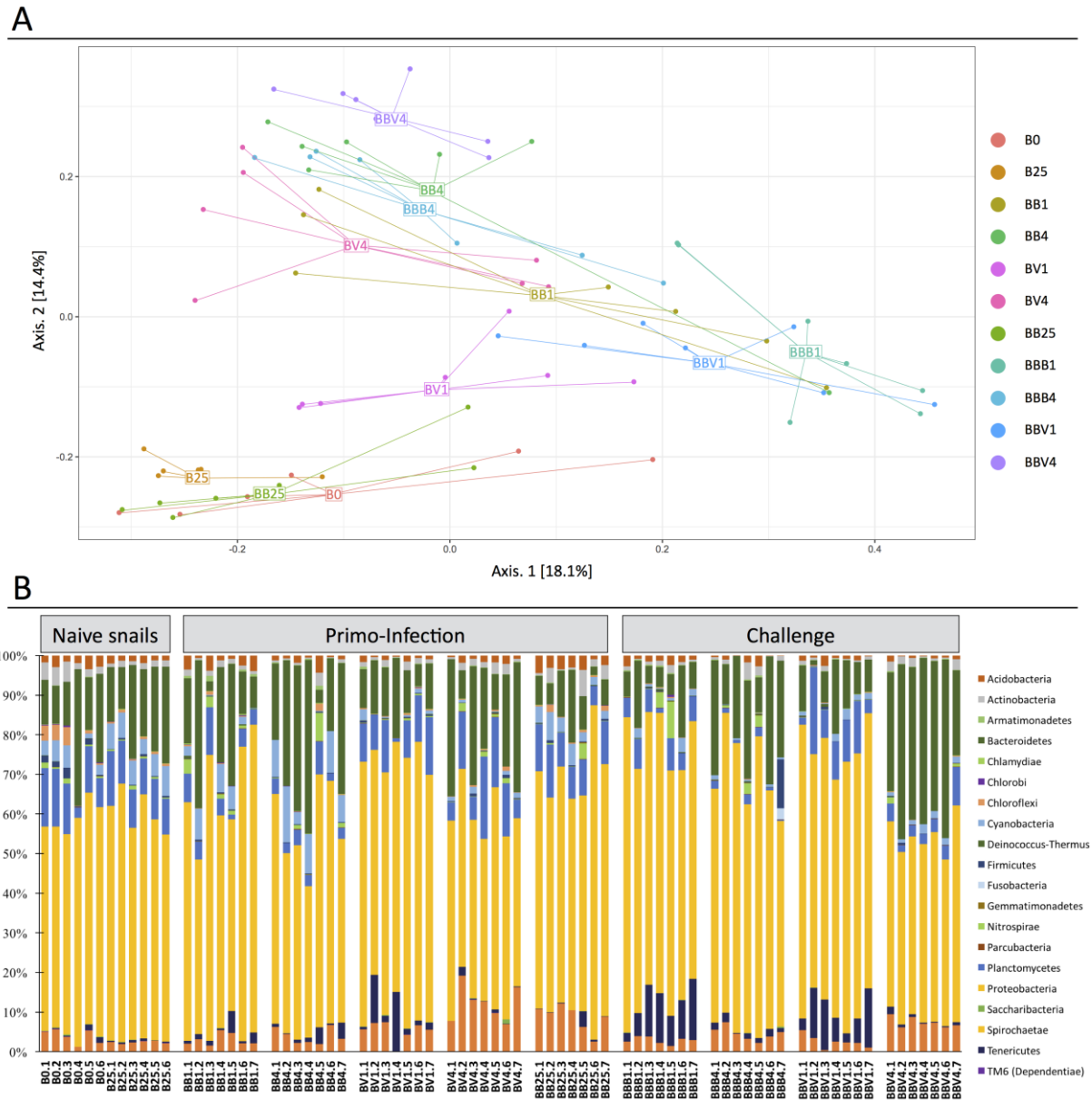

Fig. 4: Beta diversity and bacterial communities following *Biomphalaria* infection

Dynamics of the bacterial microbiota of *Biomphalaria glabrata* following *Schistosoma* primo-infection and challenge. A. Functional diversity comparisons of *Biomphalaria* microbiota along infection. Principal coordinate analysis of pairwise Bray-Curtis distances between all infection type and time samples. Axes represent the two synthetic variables explaining the greatest proportion of variation in the data set. The sample name indicated

in the figure corresponds to the centroid of all the biological replicates points of the respective experimental sample. B. Phylum level composition of the 20 most abundant OTUs among all points of the kinetic. In this representation, the replicate naive snails were pooled for more readability.

Finally, we then focused on the core microbiota dynamics during infections. In contrary to the entire microbiota, the core-microbiota composed of 62 families was affected by the type of infection (naive vs primo-infection (p=0.003) and naive *vs* challenge (p=0.003)), by the time of infection (early vs late) (p=0.006) and also by the strain of parasite used for infection (SmBRE vs SmVEN) (p=0.016) (supplementary table 6).

We observed that the 69.4% (43 families) of the core microbiota was significantly affected by infections, among them 6.5% (4 families) were affected in all conditions (fig. 5A, supplementary table 7). Further 34.9% (15 families) of the core-microbiota responded solely to the primo-infection and 4.7% (2 families) to the challenge (fig. 5B, supplementary table 7). In the same way 20.9% (9 families) of the core-microbiota was affected early after infection (1 day) and 7% later (3 families) (4 days). Finally, 23.3% (10 families) are affected by the SmBRE infection and 9.3% (4 families) by the SmVEN infection (fig. 5A, supplementary table 7).

Among the most abundant core-microbiota families, two are significantly affected in all infection conditions, the *Xanthomonadeceae* and *Rhodopirillales Incertae Sedis* (supplementary table 7). The OTUs number of these families has a tendency to decrease following infections.

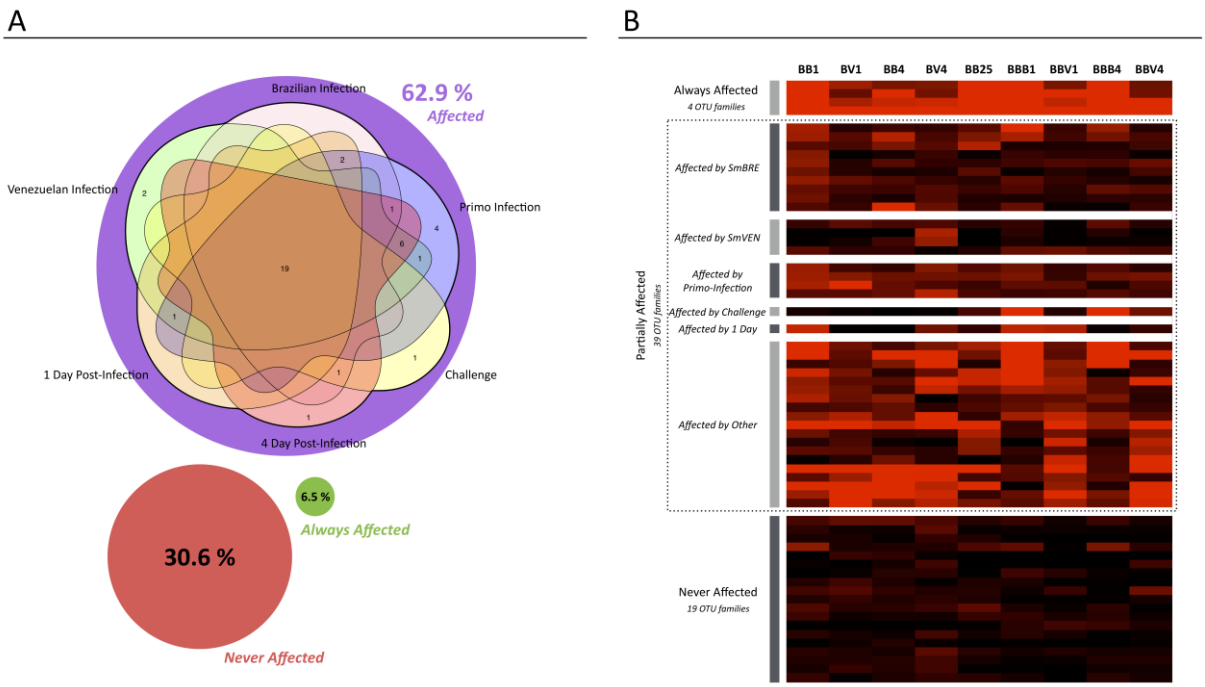

Fig. 5: Core Microbiota analysis

Modifications of the bacterial core microbiota of *Biomphalaria glabrata* following *Schistosoma* primo-infection and challenge. After a one-way Permanova with a Benjamini & Hochberg post-hoc, a frequency test was realised to determine which specific families were affected during infection. A. Proportion of OTU Families affected by infection are represented by colour circles. The Venn diagram shows more precisely the specific impact of infections on the OTU families partially influenced. B. Heatmap corresponding to all core microbiota with clustering on Familiy modification patterns between conditions. The scale of red correspond to ratio between naive condition and each point of infection.

<u>Link between microbiota dynamics and antimicrobial immune response of *Biomphalaria*</u>

We followed the gene expression of the antimicrobial protein type, the LBP/PBI (Lipopolysaccharide-binding protein / Bactericidal Permeability-Increasing protein), and two antimicrobial peptide families, the biomphamacins and achacins, after primo-infection with the two strains as well as immune challenge with the sympatric strain (fig. 6). The LBP/BPI the 3.1 and 3.2 were over-expressed until 25 days after infection, while all other were under-expressed in all conditions (fig. 6A). Concerning the biomphamacins, the biomphamacin 1, 4, 5 and 6 were over-expressed throughout infections, while biomphamacin 3 was under-expressed (fig. 6B). Finally, the achacin was under-expressed in sympatric primo-infection and following challenges and not differentially expressed in allopatric primo-infection (fig. 6B).

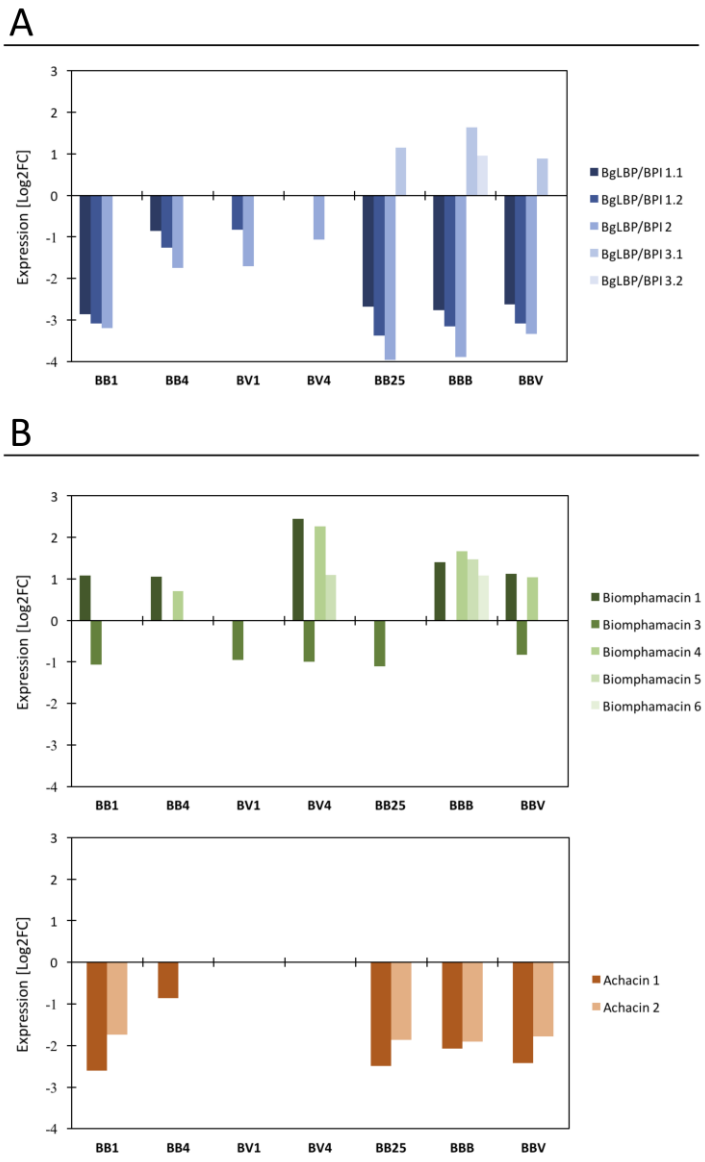

Fig. 6: Differential gene expression for 3 antimicrobial peptide families of *Biomphalaria*

Log2FC (fold change) of antimicrobial immune transcripts between naive and infected snails inferred from previous RNAseq analysis on the same experiment. A positive Log2 fold-change indicates over-expression in infected snails compared to the naive snails. A. Antimicrobial protein family LBP/BPIs (*Lipopolysaccharide-binding protein / Bactericidal Permeability-Increasing protein*) consisting of 5 genes (in blue) B. Antimicrobial peptide families included 6 Biomphamacins (type of macin) consisting of 6 genes (in green), and achacins consisting of 2 genes (in orange).

The LBP/BPI and achacin expression levels seemed not to be correlated directly with the microbiota community modifications; for all time points of all infection kinetics all transcripts were under-expressed even when the microbiota community was modified. The biomphamacins, except the 6, were over-expressed after infection, from day 1 in BB infection and day 4 in BV infection and in challenge (BBB and BBV infections). These results suggest that the 3 *Biomphalaria* antimicrobial protein families were not involved in the same immune response; and only the biomphamacin family can potentially directly affect the bacterial communities.

Discussion:

Microbiota communities would be expected to be associated mainly with tegument, gut and even, in some specific cases, with hemolymphatic compartment. In *Biomphalaria glabrata*, no installed hemolymphatic microbiota community was identified (personal observation). Thus, for the first characterisation of non-cultivable bacterial microbiota in this species, we studied the whole snail without *a priori* using a 16S rDNA metabarcoding approach.

The first step of this analysis was to characterize the microbiota community structure in *Biomphalaria glabrata* healthy snails. The bacterial microbiota of BgBRE has a temporal and inter-individual stability (figure 2). We were thus able to identify numerous taxa constituting a core microbiota shared by all individuals (table 1).

An intimate interaction between the microbiota, the host immunity and pathogens has been revealed in many vertebrate and invertebrate models. Following an infection by a pathogen, the host displays a specific or adapted immune response against the intruder. This activation of the immune response may affect the microbiota community structure. Two hypotheses have been proposed to explain this intricate dialogue between the host and its microbiota though the immune system. First, it has been proposed that the host immune system ensures constant pressure on the microbiota and thus constrains its composition permanently (Hooper, Littman et al. 2012). The microbiota may also have in some organisms an immune-shaping capacity, the resident bacterial microbiota profoundly shape the host immunity conversely the host immune system play a role to maintaining the homeostasis with resident microbial communities (Hooper, Littman et al. 2012). Second, the antigenic discontinuity theory proposes that the immune system reacts to a strong variation of antigens detected, for example following an infection, but would be tolerant for weak and continuous immune stimulations experienced during a lifespan (Pradeu and Eric 2014). In this case, no immune response of the host toward a installed and stable microbiota would be expected.

Most of these theories were proposed based on work on vertebrate models and especially mammals. Herein we investigated the interaction between the host, the microbiota and the immune system in an invertebrate model. We chose the model involving the gastropod snail *Biomphalaria glabrata and its* trematode parasite *Schistosoma mansoni*. *S. mansoni* parasite was used herein as a biotic stress for the host that could result in different issues. Depending

on the past evolutionary history between snail and schistosome, different immune responses against *S. mansoni* were observed. We showed recently that in a sympatric interaction, the parasite was adapted to its host and induced a strong immunosuppression, whereas in an allopatric interaction the host immune response is activated (Portet, Pinaud et al. 2018) . Moreover, in innate immune memory, a cellular immune response was observed following primo-infection, and a humoral immune response was observed following secondary challenge (Pinaud, Portela et al. 2016).

Thus using *S. mansoni* infections, we were able in the present study to induce different immune stresses (i.e. immunosuppression with sympatric parasite strain, immune cellular response with allopatric parasite strain, humoral response following challenge with a similar or different parasite strain) and analyse the impacts of these stresses, experienced by the host, on microbial community modifications or dynamics. We showed that the alpha diversity does not change following primo-infection whatever was the parasite strains used or the time point of infection (figure 3, supplementary table 3). A difference on the alpha diversity is observed exclusively following challenge infection, highlighted by a diversity and richness decrease (figure 3, supplementary table 3). However, primo-infection and challenge affected the bacterial OTUs composition whereas the allopatric and sympatric parasite strains did not appear to affect differently their relative proportions (figure 4, supplementary table 5). Interestingly, all these OTUs affected rapidly following infection, returned to proportion values similar to naïve snails at 25 days after infection, this suggesting a resilience of the bacterial community following a first stress of infection (figure 4). However the microbiota seems to be affected in a similar way whichever the type of infection. This was totally unexpected, as the immune response following each of these experimental infections was totally different, but microbiota dysbiosis remained similar.

Then we paid a particular attention to the core-microbiota. Given the various ways to define core microbiota, we considered exclusively the permanent members of the bacterial community in *Biomphalaria glabrata* (Astudillo-García, Bell James et al. 2017). Part of the bacterial family composition is disrupted by the primo-infection and also by the challenge (figure 5, supplementary table 6). We observed difference in this disruption depending on the early or late time of infection. Moreover, unlike whole microbiota, the core-microbiota composition is also affected by the strain of the parasite used for infections (figure 5, supplementary table 7). A stronger disruption of the OTU families was observed following

SmBRE infection in early time (figure 5, supplementary table 7). In this point the snail immune response against the parasite is strongly immunosuppressed. This immunosuppression seems thus to affect greatly the core microbiota. One can proposed that the immune system was no longer able to maintained the core microbiota homeostasis that results in changes in microbiota composition and structure.

Given that infections affect the microbiota structure, we explored the antimicrobial immune response following infection to find potential explanations of the observed microbiota dysbiosis (figure 6). We analysed more specifically antimicrobial peptides and proteins and we demonstrated that only the biomphamacin family could potentially affect directly the microbiota communities.

It has been yet shown in some models that the immune system is a key determinant of host-associated bacterial communities, and more precisely that species-specific antimicrobial peptides can shape species-specific bacterial associations in *Hydra* (Franzenburg, Walter et al. 2013). Other immune pathways have been also demonstrated to regulate or control the microbiota communities, like the intestinal homeobox gene Caudal in *Drosophila* (Ryu, Kim et al. 2008), or neuropeptides with antibacterial activity which are secreted to shape the microbiome on the body surface of *Hydra* (Augustin, Schröder et al. 2017).

Thus, if a biotic stress (i.e. an infection) modifies the expression of the antimicrobial peptides or other immune pathways, an effect on the microbiota community may be expected. Our first results seem to indicate that we have in *B. glabrata* a regulation of the bacterial communities by the immune system. Based on the hypothesis proposed by Hooper (Hooper and Macpherson 2010; Hooper, Littman et al. 2012) the immune system maintain permanently the endosymbiotic microbial communities in a physiological threshold compatible with their role of symbionts. Thus following an infection, the immune system is diverted from its function of managing the microbiota and bacterial communities change profoundly. When the immune response stopped and returned to a basal level the microbiota is again under-control and resilience can be observed (Hooper and Macpherson 2010; Hooper, Littman et al. 2012). In this context, the microbiota dysbiosis would be a consequence of the infection and of the activation of the immune system against an intruder or a modification of the host physiological status. Herein, totally different immune responses resulted in similar modifications of the microbiota composition. This reveals that more than the nature or type of the immune response, the microbiota seems to be affected by the

overall stress experienced by the host snail.

Finally, microbiota dysbiosis would have consequently strong effects on the host homeostasis. In this specific interaction between the snail *Biomphalaria glabrata* and the *Schistosoma* agent of the Schistosomiasis disease, it would be now essential to determine if microbiota bacterial communities may have an impact on the parasite transmission or compatibility. This consideration would be of central importance in terms of disease transmission and in finding new ways to control the expansion of this widespread human parasitic disease in the field.

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