



Gut microbiome alterations in the crustacean *Pacifastacus leniusculus* exposed to environmental concentrations of antibiotics and effects on susceptibility to bacteria challenges[☆]

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ABSTRACT

Gut-associated microbiota in crustaceans are recognized as a key element for maintaining homeostasis and health in the animal. Since the richness of these microbial communities is strongly influenced by the local environment, especially in aquatic organisms, it is important to address to what extent environmental variations can affect these communities. In the present study, we used high-throughput 16S rRNA sequencing technology to study the composition of gut-associated microbiota of the crayfish *Pacifastacus leniusculus* after exposure to environmentally-relevant concentrations of an antibiotic, namely sulfamethoxazole. Also, we examined if alterations of microbiota caused by environmentally-relevant concentrations of this antibiotic affected the host susceptibility to bacterial diseases, including *Vibrio* species. As a result, we found high individual variability of bacterial abundance and composition in the intestinal microbiome of crayfish, in both antibiotic-exposed and antibiotic-free crayfish. However, an increase of chitinolytic bacteria including *Vibrio* spp. was detected in some animals exposed to the antibiotic. Moreover, when crayfish susceptibility to bacterial infections was tested, the antibiotic-exposed crayfish survived longer than the control crayfish group. This study represents the first approach for investigating the interplay between crayfish and intestinal bacteria during antibiotic-pollution scenarios. Results herein should be considered by scientists before planning experiments under laboratory conditions, especially to study environmental effects on aquatic animals' intestinal health and immune status.

1. Introduction

Freshwater crayfish play a fundamental role in freshwater ecosystems (Richman et al., 2015). Native crayfish are found in a diversity of habitats all over the world (Rode and Babcock, 2003). Moreover, aquaculture and fisheries of freshwater crayfish are of significant economic importance, particularly at a large scale in Europe, China, and the United States (Reynolds and Souty-Grosset, 2011; Thies and Porche, 2007).

Notwithstanding its importance, it is estimated that about one-third of freshwater crayfish species are threatened with extinction, where

freshwater pollution and parasites such as the crayfish plague, *Aphanomyces astaci*, are considered major causes (Richman et al., 2015). Thus, water pollution with antibiotics is considered an emerging threat to aquatic organisms (Carvalho and Santos, 2016; Hughes et al., 2013), and one of the most frequently detected antibiotics in the freshwater ecosystems worldwide is sulfamethoxazole (SMX), with a frequency of detection in freshwaters worldwide of more than 50%, with median concentrations of about 83 ng/L (Hughes et al., 2013). Monitoring of this antibiotic has become of particular interest since its presence has been reported in different aquatic environments, including rivers and effluents of wastewater treatment plants in Europe, North America, and

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Asia, with detection ranges varying from 1–11,920 ng/L (Hughes et al., 2013).

Despite the importance of this, the exact mechanisms by which pollution of aquatic ecosystems with antibiotics impacts the normal physiology of crayfish species is not yet fully understood.

Recently, studies of gut-microbiota dynamics have been proposed as a strategy for investigating host-environment interplay in aquatic invertebrates, including crustaceans (Holt et al., 2020). The reason is that it is commonly accepted that intestinal microbiota communities contribute to host evolution, development, physiology, behavior, immunity, and defense against pathogens and that dysbiosis or alterations of these communities could be detrimental for host fitness (Blum, 2017; Brune and Dietrich, 2015; Johnson and Foster, 2018; Thursby and Juge, 2017). In the particular case of crustaceans, including freshwater crayfish, the community structure of internal microbiota is defined by ecological filters encompassing geographic to cellular levels, and this relation is considered to be dependent on the environment, where water quality and pollution play an important role (Holt et al., 2020; Skelton et al., 2017).

Previously, we demonstrated that environmentally-relevant concentrations of the antibiotic sulfamethoxazole increased susceptibility of crayfish, *Pacifastacus leniusculus*, to succumb to viral infections (Hernández-Pérez et al., 2020). However, no information is available regarding what effect environmentally-relevant concentrations of these antibiotics have on the intestinal microbiota of freshwater species.

The aim of the present study was first, to analyze the alterations that environmentally-relevant concentrations of SMX cause in the structure of the intestinal bacterial communities, using the freshwater crayfish *Pacifastacus leniusculus*. Then, also test the susceptibility of crayfish to some of the most representative bacteria genus and how they are affected by the antibiotics, including *Vibrio* species will be tested.

2. Materials and methods

2.1. Crayfish acclimatization and antibiotics exposure

Male freshwater crayfish, *Pacifastacus leniusculus* (mean weight: 37 ± 2 g) were obtained from Lake Erken in Sweden. Crayfish were transported to our lab facilities, and kept in aquaria with aerated clean tap running water at 10–12 °C. After acclimatization (~2 weeks), chronic exposure for 21 days to environmentally-relevant concentrations of SMX was started in order to evaluate the effect of this substance upon the intestinal microbiota.

Experimental setup of crayfish exposure to antibiotics was performed as previously reported by Hernández-Pérez et al. (2020). Briefly, fresh stocks of sulfamethoxazole (SMX) (Sigma-Aldrich, CAS: 723-46-6) were prepared following the standard recommendation from the Clinical and Laboratory Standards Institute (CLSI), (2012). Then, test solutions with final concentrations of 100 ng/L and 1 µg/L of SMX, were prepared from the stock solutions.

In all experiments, the water was renewed every second day. For the antibiotic-treated groups, a freshly prepared antibiotic solution was added to the water every time after the water was exchanged. During all experiments, the photoperiod was maintained at 12:12 h light:dark, and animals were fed with carrots and potatoes once a week with 3% of its live weight. Three experimental biological replicates with five crayfish per treatment were performed.

2.2. Intestine sample collection and DNA isolation

After antibiotic exposure, crayfish were euthanized by decapitation and the intestine from midgut to rectum was collected, rinsed with cold CPBS (10 mM Na₂HPO₄, 10 mM KH₂PO₄, 150 mM NaCl, 10 mM CaCl₂, and 10 mM MnCl₂, pH 6.8), and immediately stored at –80 °C until DNA extraction.

DNA was extracted from each individual intestine using the QIAamp

DNA Stool Mini Kit (QIAGEN), and PCR inhibitors were removed from purified DNA using the kit DNeasy PowerClean Pro Cleanup (QIAGEN), following the manufacturer's recommendations. The purity of DNA obtained was determined by an A260/A280 nm ratio using a spectrophotometer and only samples within the expected range (1.8–2) were included for further analysis as follows: a total of nine crayfish from control group (no antibiotic added), eight crayfish from 100 ng/L SMX group, and eight crayfish from 1 µg/L SMX group.

2.3. 16S rRNA sequence analysis, data processing and statistical analysis

For bacterial community composition analyses, the 16S rRNA gene (V3–V4 dual region) was amplified following Vass et al. (2020), using primers 341F and 804NR (Herlemann et al., 2011). The detailed two-step PCR procedure followed is available in the protocols.io repository (Vass and Székely, 2020) (dx.doi.org/10.17504/protocols.io.468gzhw). Amplicons were sequenced at the SciLifeLab SNP&SEQ Technology Platform hosted by Uppsala University using Illumina MiSeq v3 sequencing chemistry.

Raw sequences were processed using the DADA2 package, which was used to resolve the amplicon sequence variants (ASVs) (Callahan et al., 2016). We included the following filter criteria: i) cut of the sequences at 280 and 230 bp, of the sense and antisense reads, respectively; ii) an error threshold of one biased assigned base in sense reads and two in antisense reads, respectively and iii) deletion of sequences with ambiguous bases. From the filtered sequences, error modeling was performed (Callahan et al., 2016, 2017). The paired sequences were merged and filtered to remove chimeric sequences, by using the “removeBimeraDenovo” algorithm with the “consensus” method (Callahan et al., 2016, 2017). Sequences were used to construct merged sequences. Then, the taxonomic assignment was performed with DECIPHER 2.14.0 (Wright, 2016) using the SILVA database version 138 (Quast et al., 2013). Unidentified sequences, those with a relative abundance of less than 1%, and those identified as “Mitochondria” and “Chloroplast”, were removed at the Phylum level (Callahan et al., 2016, 2017). The feature table was standardized to the median sequencing depth. The statistical analyses were performed with the phyloseq (McMurdie and Holmes, 2013), ggplot2 (Ginestet, 2011), and vegan (Oksanen et al., 2008) packages. The core microbiota, or the set of amplicon sequence variants detected in 20–100% (prevalence) of the samples with a relative abundance threshold value above 0.01%, was identified using the core function in the microbiome R package version 1.5.28 (Shetty and Lahti, 2019).

We applied a PERMANOVA test with 1000 permutations to check differences between the microbiota of control and treated samples. The Simpson and Shannon diversity indexes were calculated. Non-metric multidimensional scaling (NMDS) with the unweighted UniFrac distance was applied to data to assess how microbiota of control and antibiotic samples are clustered. All analyses were performed under the R environment (“R Team” <http://www.R-project.org/>), version 3.6.3. The nucleotide sequence data for the 16S rRNA gene can be found in BioProject accession number: PRJNA717320.

2.4. Bacteria challenge after antibiotics exposure

In order to test if the microbiota alterations caused by the antibiotics in the intestine increased crayfish susceptibility to bacterial infections, new sets of animals were exposed to the antibiotic SMX (100 ng/L and 1 µg/L) as mentioned before (section 2.1). Then, the crayfish were challenged with highly pathogenic or non-pathogenic bacteria that were previously isolated from *P. leniusculus* by Jiravanichpaisal et al. (2009) and that are considered to be part of the crayfish normal microbiota: *Citrobacter freundii* and *Aeromonas hydrophila*. Moreover, since microbiome sequencing results showed the presence of *Vibrio* spp., we also performed bacteria challenge with *Vibrio parahaemolyticus* and *Vibrio areninigræ*.

Prior to the bacteria challenge experiments, bacteria growth determination was performed to establish infective doses (data not shown). Table 1 summarizes the infection route and doses (CFU) used. The bacteria, *C. freundii* and *A. hydrophila* were cultured in sterile tryptic soy broth (TSB, G-Biosciences) and were grown at 22 °C with constant shaking (250 revolutions/min). The *Vibrio* species, *V. parahaemolyticus* and *V. areninigræ* were cultured in sterile Marine broth (Mb, Difco) and grown at 37 °C with constant shaking (250 revolutions/min). The bacterial cells were pelleted by centrifugation (2500 × g for 10 min at 4 °C), washed twice using sterile 0.9% or 2.0% NaCl in the case of the *Vibrio* species, and then re-suspended in sterile 0.9% or 2.0% NaCl.

After the end of the 21-day SMX treatments (Section 2.1), crayfish were challenged by immersion or injection with the bacterial strains (Table 1). Immersion challenges were performed individually in 500 mL of sterile 0.9% NaCl plastic boxes containing bacteria with constant aeration. For injection challenge, the bacteria stock was 1:10 diluted to the concentration needed in 0.9% or 2.0% NaCl for the *Vibrio* species and then injected in the base of the fourth pair-leg of the crayfish. Survival rates were recorded during the next days. Neither water renewal nor feeding was performed during the infection/experimental period. This experiment was repeated with a total number of 6 animals for each group. The comparison of survival during the bacteria challenges was analyzed using the Log-rank (Mantel-Cox) Test. GraphPad Prism 8.1.2 software was used to analyze all data and differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of environmentally relevant concentrations of antibiotics on the intestinal microbiome of crayfish

In the present study, we investigated the dynamics of *P. leniusculus* intestinal microbiome after chronic exposure to environmentally relevant concentrations of one antibiotic. Thus, first the crayfish were taken from lake Erken, and five animals in each group were treated in water containing environmentally-relevant concentrations of SMX, or as controls according to section 2.1.

After 21 days of exposure, DNA was extracted from the intestines of each individual animal, and after PCR product cleaning and purification, high-quality products of bacterial 16S rRNA were subjected to Illumina MiSeq sequencing. In total 1,374,136 sequences were obtained. After filtering, we kept 354,619 clean sequences assigned to 3324 different ASVs.

The core microbiome shared among all treatments can be found in Fig. 1 and consists of nine bacterial genera: *Citrobacter*, unknown, *Vibrio*, *Aeromonas*, *Flavobacterium*, *Shewanella*, *Candidatus-Bacilloplasma*, *Firmicutes bacterium ZOR0006*, and *Chryseobacterium*. Differences between the composition and abundance of the core microbiome by treatment showed no significance ($P > 0.05$) (data not shown).

The total and relative abundance of different bacteria identified at genus level are shown individually in Fig. 2 and treatment-grouped in Supplementary Fig. S1. The 16S amplicon sequencing analysis identified a total of 49 bacterial genera in the samples (Fig. 2).

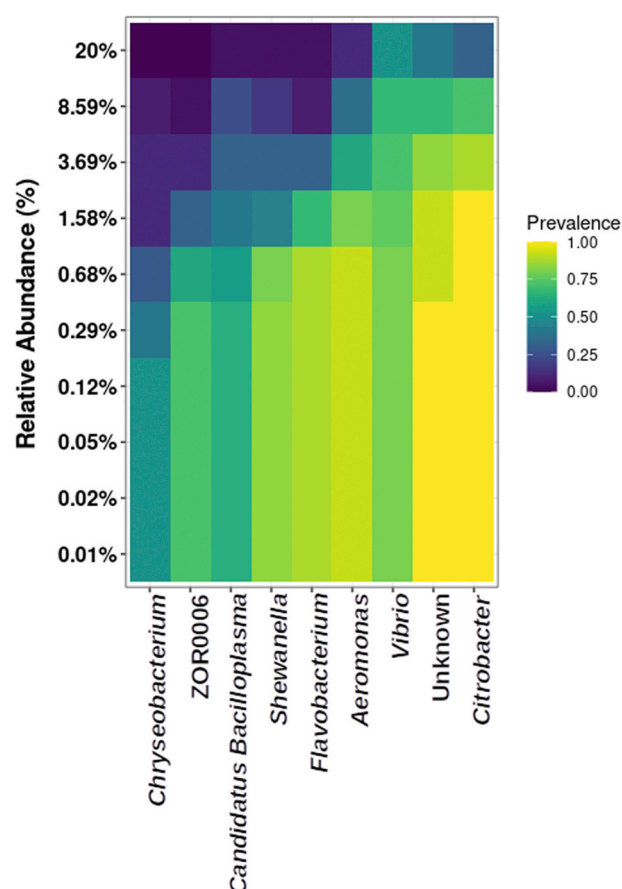


Fig. 1. Treatment-grouped core microbiome analysis in crayfish *P. leniusculus*. The core microbiome is displayed by a heat map that identifies the nine most prevalent and abundant genera. The unknown category includes members of the family Enterobacteriaceae that was not resolved to genus species. The x-axis represents the prevalence level of the bacteria genus across the detection threshold (relative abundance) range on the y-axis. The prevalence of each genus is indicated by a gradient of color from yellow (most prevalent) to blue (less prevalent).

A calculation of the alpha diversity measure, Shannon and Simpson diversity indexes (meaning a measure of the diversity of different species or genera in each group), didn't show any differences in gut microbiota diversity in the antibiotic treated groups relative to the control group ($P > 0.05$) (Fig. 3). Likewise, the weighted UniFrac Principal Component Analysis (PCoA) plot didn't show any clear separation in the distribution of ASVs between the gut-microbiota of antibiotic exposed and non-antibiotic exposed group of crayfish (Fig. 4).

Although not significant, a notable increment in the number of sequences assigned as *Vibrio* species was detected in animals treated with SMX at 1 µg/L (Fig. 2 and Supplementary Fig. S1), which are considered chitinolytic bacteria. We could also note an increase in *Pseudomonas*

Table 1

Bacterial strains, route, and dose of infection used to challenge crayfish after exposure to environmentally-relevant concentrations of SMX. The median survival time of crayfish obtained is shown. Data analyzed by Log-rank (Mantel-Cox) and significant differences were considered in contrast with control at $**P < 0.01$ and $***P < 0.001$, dpi = days post-infection, hpi = hours post-infection, NM = no mortality.

Bacterial strain	Pathogenicity	Route of infection	Doses	Median survival (dpi, or hpi)		
				Control	SMX 100 ng/L	SMX 1 µg/L
<i>Citrobacter freundii</i>	Non-pathogenic	Injection	10 ⁸ CFU	NM	NM	NM
<i>Aeromonas hydrophila</i>	Highly pathogenic	Immersion	10 ¹¹ CFU/mL	1.5 dpi	2 dpi	3.5 dpi**
<i>Aeromonas hydrophila</i>	Highly pathogenic	Injection	10 ⁶ CFU	1 dpi	1.5 dpi	2.5 dpi**
<i>Vibrio parahaemolyticus</i>	Highly pathogenic	Injection	10 ⁸ CFU	4.5 hpi	5 hpi	5.5 hpi
<i>Vibrio areninigræ</i>	Highly pathogenic	Injection	10 ⁸ CFU	1 dpi	2 dpi***	3 dpi***

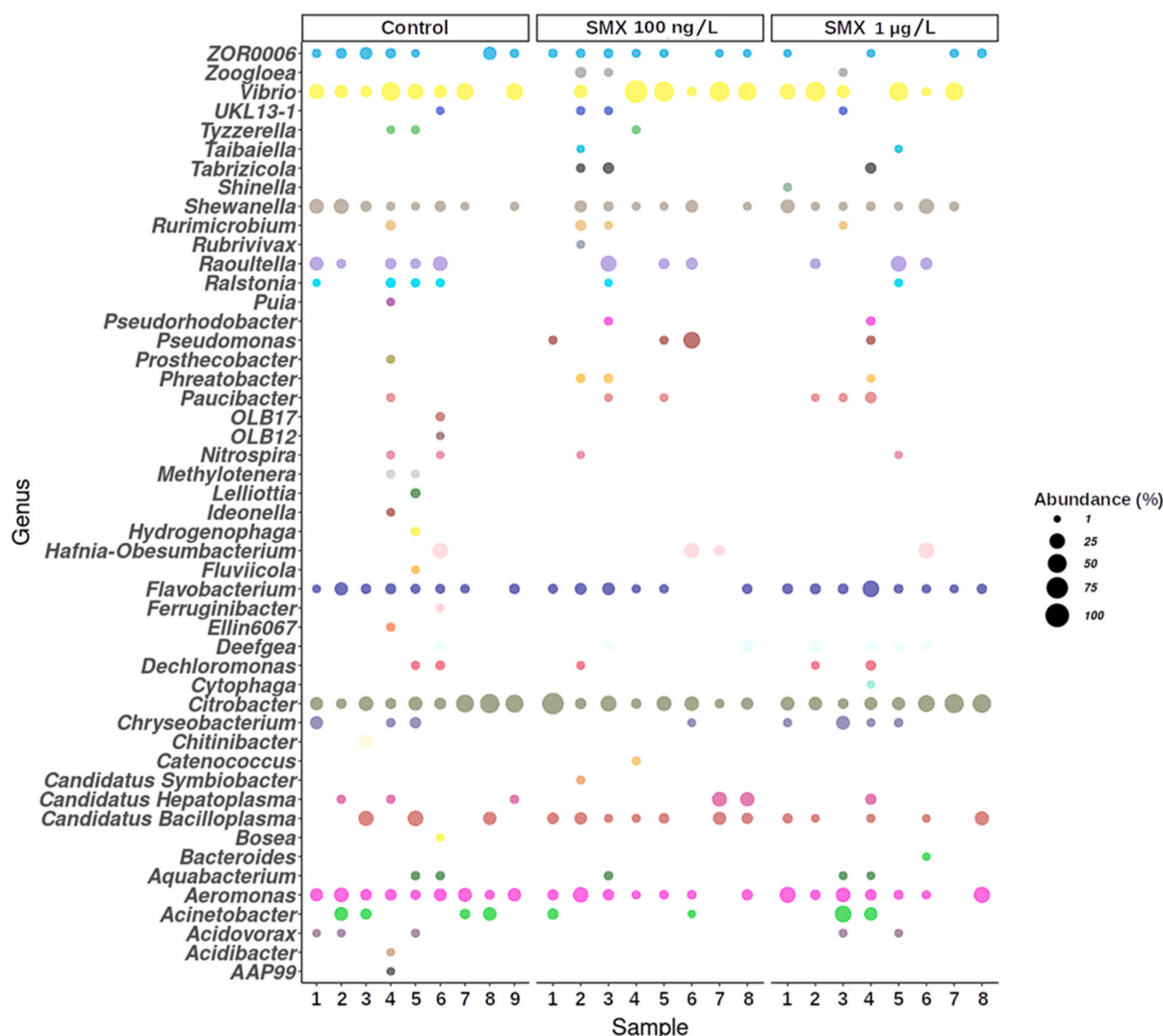


Fig. 2. Bubble plot showing the relative abundance of ASVs belonging to different bacterial genera, shown as the proportion of the total bacterial community, in individual intestine samples of crayfish *P. leniusculus* in control animals and after exposure to SMX at 100 ng/L or 1 µg/L for 21 days. No significant differences were found between groups ($P > 0.05$).

species in this group, which was absent in the control group. Interestingly, in these individuals, *Citrobacter*, which was the most well-represented genus in the control group, as well as *Acinetobacter*, were reduced. In contrast, such a similar effect was not observed in the animals treated with SMX 100 ng/L, except for the notable increase in *Flavobacterium* (Fig. 2 and Supplementary Fig. 1). For detailed information about the number of sequences found for each genus per sample see Supplementary Table S1.

As shown in Figs. 1 and 2 and Supplementary Fig. S1, we could observe a high proportion of *Vibrio* sequences in all the crayfish groups, with an increase of this genus in the 1 µg/L SMX treated animals. Therefore, we decided to investigate the distribution of *Vibrio* species using the SILVA132 database; nonetheless, annotation to species level was not possible (Supplementary Fig. S2). The lack of resolution at species level of the 16S rRNA marker obeys to different factors including shortness of the reads, which difficult separation to level species (Escobar-Zepeda et al., 2018), as well as biological characteristics of complex genus including the Enterobacteriaceae family (Pei et al., 2010).

3.2. Survival of freshwater crayfish to bacterial challenge after SMX exposure

In order to test the effect of antibiotic exposure and crayfish susceptibility to bacteria diseases, crayfish groups were exposed to SMX, as mentioned in the 2.1 section, followed by challenges with pathogenic and non-pathogenic bacteria previously isolated from *P. leniusculus* (Jiravanichpaisal et al., 2009) and with two *Vibrio* species. All these genera were found in the microbiome and infection routes and doses were defined following previously established conditions (Hernández-Pérez et al., 2021; Jiravanichpaisal et al., 2009; Korkut et al., 2018; Zhang et al., 2010).

The median survival of crayfish is shown in Table 1. None of the crayfish groups challenged with the non-pathogenic bacterium *Citrobacter freundii* showed any mortality. Survival of crayfish after immersion in highly pathogenic *A. hydrophila* and injection challenges was significantly longer between the group exposed to 1 µg SMX/L (median survival of 3.5 and 2.5 days, respectively) when compared with the control group (median survival of 1.5 and 1 days, respectively) ($P < 0.01$).

For crayfish challenged by injection of pathogenic *V. parahaemolyticus*, the control group survived for 4.5 h, SMX 100 ng/L

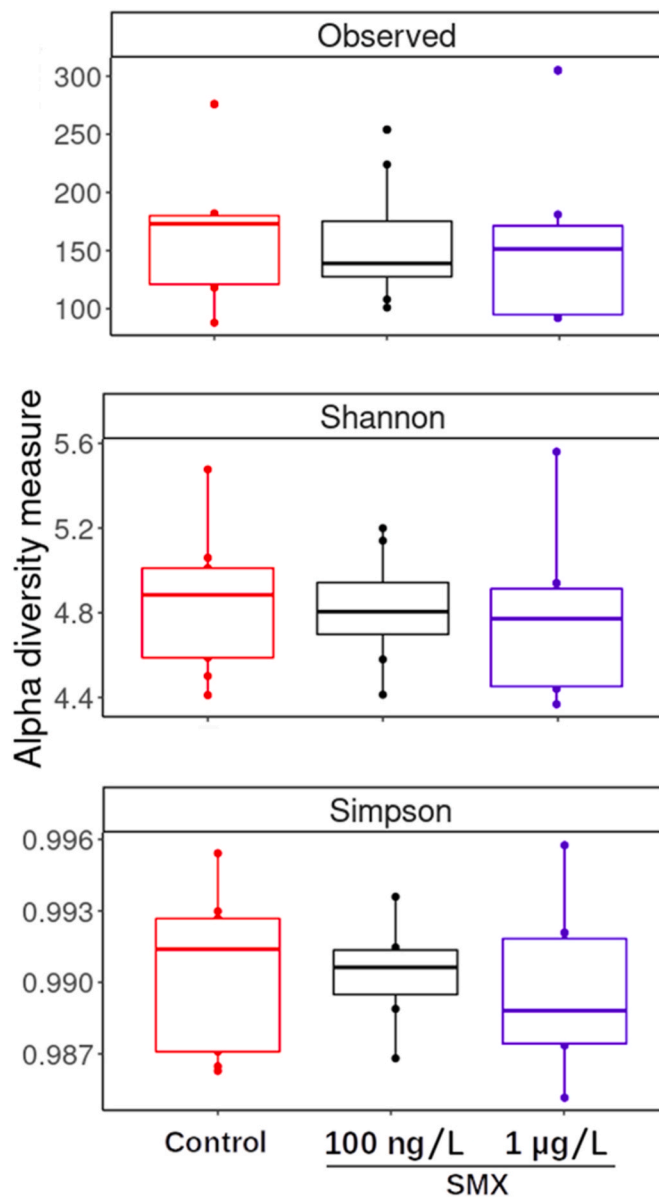


Fig. 3. Alpha diversity between the intestinal microbiota in control crayfish and after exposure to SMX at 100 ng/L or 1 µg/L for 21 days. From top to bottom: Observed diversity, Shannon diversity index, and Simpson diversity index. The line inside the box represents the mean, while the whiskers represent the lowest and highest values. Statistical tests didn't show differences between different groups ($P > 0.05$).

treated group survived 5 h, and 1 µg SMX/L treated group survived 5.5 h. No significant differences were found between the susceptibility to *V. parahaemolyticus* in these groups ($P > 0.05$).

Results obtained with crayfish injected with pathogenic *V. areninigras* showed a median survival of 1 day for the control group, 2 days for the group exposed to 100 ng SMX/L, and 3 days for the group exposed to 1 µg SMX/L. Significant differences were found between the survival of control in comparison with 100 ng SMX/L ($P < 0.001$) and 1 µg SMX/L groups ($P < 0.001$).

4. Discussion

The present study aimed to first characterize the composition of gut-associated microbiota of the freshwater signal-crayfish *Pacifastacus leniusculus* and, then to evaluate the impact of environmentally relevant

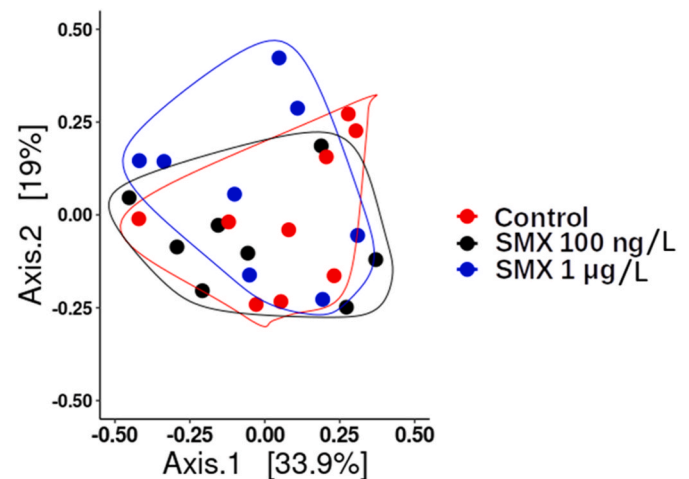


Fig. 4. Weighted UniFrac principal coordinate analysis (PCoA) plot illustrating no separation between the samples of each condition.

concentrations of antibiotics on these bacteria communities. Moreover, we studied if changes in the dynamics of the bacteria communities have any effect on the susceptibility to bacterial infections.

A small core microbiome was found in crayfish at this life stage (Fig. 1) and consists of nine genera: *Citrobacter*, unknown, *Vibrio*, *Aeromonas*, *Flavobacterium*, *Shewanella*, *Candidatus-Bacilloplasma*, *Firmicutes bacterium ZOR0006*, and *Chryseobacterium*. Notably, the second most prevalent sequences were categorized as unknown. Unknown taxa, commonly refer as “microbial dark matter”, usually represents an important proportion of intestinal microbiome (Zamkovaya et al., 2021), in agreement with our findings. Future characterization of this genera will help to elucidate the relationship between crayfish intestinal microbiota and the environment.

Furthermore, we also found that the genus *Citrobacter* was the most well-represented in control and SMX exposed groups. In addition, although not statistically significant, the abundance of *Citrobacter* showed an overall reduction after chronic exposure to 1 µg/L of SMX (Supplementary Fig. S1). Recent studies conducted in red swamp crayfish *Procambarus clarkii* revealed that exposure to nitrite and sulphide contaminants in the water led to a decrease of this genus (Guo et al., 2020). *Citrobacter* species are cellulose-degrading bacteria abundantly found in the intestines of herbivorous and omnivorous aquatic organisms (Liu et al., 2016), and it is considered to promote colonization resistance against pathogenic bacteria in the intestine of animals (Buffie and Pamer, 2013). The *Citrobacter* genus is also considered to have pathogenic potential to humans and rodents (Mundy et al., 2005), as well as to crayfish when environmental conditions are favorable for this bacterium (Boemare and Vey, 1977; Edgerton et al., 2002). However, when crayfish exposed to antibiotics were challenged with injection of *Citrobacter freundii*, no mortality was observed. These results show that *Citrobacter* has an important role in maintaining a stable gut-microbiota community in crayfish and that its abundance is negatively affected by SMX exposure. Nonetheless, this doesn't represent a threat in terms of mortality to some of the tested bacterial species during aquatic pollution scenarios, but of course, such change could influence crayfish health and growth in the long-term by mechanisms not investigated here.

Moreover, results obtained after exposure to the antibiotic within the same crayfish group in each individual animal (1 µg/L of SMX), showed an increase of *Vibrio* spp. proportion. The *Vibrio* genus is considered ubiquitous in marine and estuarine ecosystems, and it represents up to 70% of the total sequences isolated from intestinal microbiota in marine shrimps (Holt et al., 2020). Its presence has also been found in freshwater ecosystems (Mishra et al., 2010) and species that inhabit such ecosystems, including crayfish, where the *Vibrio* genus represents ~30% of the total sequences of the gut-microbiota (Foyssal et al., 2019). Despite

contributing to the digestion of crustaceans food (Itoi et al., 2006), it is also known that some *Vibrio* species can cause severe diseases in crayfish (Dong et al., 2016; Hernández-Pérez et al., 2021; Martin et al., 2004; Thune et al., 1991), as in other crustaceans species (Holt et al., 2020). In the particular case of Sweden, *Vibrio* spp. increase in aquatic ecosystems has been correlated with the increase in temperatures during summer, where peaks of high temperatures usually are present in late July (Collin and Rehnstam-Holm, 2011). However, since *Vibrio* spp. can also be found in water temperature of 5 °C and low-salinity (2–4‰), *Vibrio* pathogens are now considered a health hazard (Eiler et al., 2006). Due to this, we decided to test the potential of SMX exposure as a risk factor for vibriosis in crayfish. Our results showed that when crayfish exposed to environmentally-relevant concentrations of SMX were challenged with *V. parahaemolyticus* or *V. areninigræ*, mortality was not significantly accelerated. An interesting observation is the higher survival potential of *P. leniusculus* exposed to *V. areninigræ* after SMX treatment since this genus showed a trend to increase after exposure. This indicates that environmental concentrations of SMX used in the present study may lead to an accumulation of *Vibrio* spp. in the animals and an increased risk for crayfish health problems later on, or to increased spread of these potential pathogens through predators such as fish, birds and mammals to other ecosystems. Moreover, *Vibrio* spp. were also insensitive to SMX at these low concentrations when tested using the disk diffusion method (data not shown). Therefore, we consider that the presence of SMX can pose a risk for increasing *Vibrio* populations in freshwater environments. This may be of high importance due to the fact that SMX is one of the most frequently detected antibiotics in aquatic ecosystems worldwide (Hughes et al., 2013; Kümmerer, 2009), and also in Sweden where waste sewage plants are well developed, SMX was found to be present in the largest lakes and their water courses (Malnes et al., 2020). Our present study is the first regarding *Vibrio* genus abundance in the crayfish *P. leniusculus*, so further studies should be conducted in order to elucidate *Vibrio* spp. dynamics in freshwater crustaceans.

Likewise, the genus *Pseudomonas*, which was absent in individuals from the control group, accounted for some of the individuals that were exposed to SMX environmental concentrations. This is of interest since we had previously demonstrated that low-pathogenic bacteria like *Pseudomonas*, can cause deaths in crayfish when environmental conditions are adverse for the organisms (Korkut et al., 2018). This makes it important to characterize potential pathogenicity of low-pathogenic bacteria.

It is also important to mention that, although we analyzed microbiome data considering high-quality standards (Holt et al., 2020; Pollock et al., 2018), the results obtained showed strong variability between their microbiomes. It is known that variations in the environment and food intake determine gut-microbiota communities (Cornejo-Granados et al., 2018; Hammer et al., 2019; Skelton et al., 2017), and since tap water in Sweden is considered free of chemical contaminants and nearly aseptic (Heibati et al., 2017; Sveriges geologiska undersökning, 2020), we hypothesized that the low-intake of bacteria from the environment could be the cause of variability in the animals' microbiota in lab-maintained crayfish. This means, a random selection depending on the relative presence of the species that the individual has upon entering the laboratory environment can cause this large individual variation. Furthermore, high inter-individual variability due to environmental input is considered a sign that a core gut-microbiota in some invertebrate species does not exist (Hammer et al., 2019). However, to the best of our knowledge, none of published articles on crayfish-microbiome reports results from individual animals, and instead, records summaries of total sequences from several animals per treatment(s) (Foyssal et al., 2019; Guo et al., 2020; Skelton et al., 2017; Zhang et al., 2020; Zhang et al., 2020). Therefore, further studies at an individual level should be conducted to elucidate accurately the dynamics that both transient and resident microbiota have upon crayfish and crustacean intestinal health. Ideally, the microbiota should be analyzed before and after an exposure at individual level, which

unfortunately not is possible.

5. Conclusions

The present study represents the first survey of the gut-microbiome composition of *P. leniusculus* as well as the first evaluation of the changes in the microbiome occurring after antibiotics pollution. Gut-associated microbiota from crayfish exposed to environmental concentrations of SMX tends to increase the *Vibrio* genus. Gut-associated microbiota from crayfish showed an important individual variation. Such alterations, however, do not represent a risk factor in crayfish for bacteria-diseases susceptibility. Data provided herein could be included in further risks-factors assessment studies related to polluted aquatic ecosystems, and to study disease presentation under pollution scenarios.

Author contribution statement

Methodology, Formal analysis, Investigation AHP and IS, Conceptualization AHP,KS and IS, Writing - original draft AHP, Writing - review & editing AHP,KS,IS and JAZB, Bioinformatic analysis JAZB, Project administration, KS and IS. Funding acquisition, IS.

Declaration of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.dci.2021.104181>.

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