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PRIMARY RESEARCH PAPER

Environment and host-related factors modulate gut and carapace bacterial diversity of the invasive red swamp crayfish (*Procambarus clarkii*)

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Abstract The invasive red swamp crayfish *Procambarus clarkii* is present in most of the Portuguese rivers with well-known economic and environmental impacts, being also an important reservoir for many fungal and bacterial pathogens that can affect native aquatic fauna. Here the bacterial microbiota of the gut and carapace of the red swamp crayfish from three distinct localities in Portugal was characterized, using a metataxonomic approach. We tested whether biological measurements (sex, cephalotorax lenght, body condition and stress through glucose measurements) and sampling locality can be used as predictors of alpha and beta bacterial diversity. Results showed that the carapace microbiota are more responsive to differing environmental conditions than the gut microbiota, which are likely more affected by host factors, such as sex. Additionally, we have also found several potential pathogens in the microbiota of the analyzed crayfish. Our data provide a relevant baseline of the red swamp crayfish microbiota but highlight the need for further research, namely to fully characterize its role as a vector for bacterial diseases in freshwater ecosystems.

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Introduction

The rate of introduction of invasive species throughout the world has been accelerated by human population growth, increasing transport capacity and economic globalisation (Sakai et al., 2001). The rapid proliferation of these species, often at the expense of native species, has numerous negative impacts on biodiversity (e.g. increased predation pressure, habitat disruption; Sala et al., 2000), and can cause severe economic damages (e.g. as agricultural-based pests; Vilcinskas, 2015; Souty-Grosset et al., 2016). Additionally, invasive species may facilitate the introduction of novel pathogens into new regions with potentially catastrophic effects for native species (e.g. Vilcinskas, 2015).

Bacteria play a pivotal role at all biological scales from the individual point of view to the entire ecosystem perspective (Gibbons and Gilbert, 2015; Delgado-Baquerizo et al., 2016; McFall-Ngai et al., 2013). Commensal bacterial consortia can be affected by biotic (e.g. host age or moult stage; Zhang et al., 2020a; Middlemiss et al., 2015) and abiotic factors (e.g. environmental conditions; Xavier et al., 2020; Guo et al., 2020). Commensal microbes may be beneficial to hosts in many ways, including dietary supplementation (microbes supplement their hosts with nutrients that are limited or absent in their diet or that they cannot produce on their own), they can boost or depress host immune system, and influence social interactions (Behar et al., 2008; Ridley et al., 2012). Furthermore, recent research has demonstrated how bacterial microbiomes can have pivotal roles in invasion ecology. For example, changes in the microbiome of native species, such as in ghost ants competing with invasive fire ants, can help buffer the adverse effects of competition with the invasive species by modulating changes in behavior and diet (Cheng et al., 2019). On the other hand, microbiomes of many successful invasive species are able to improve their growth or confer adaptive advantages in newly invaded areas in relation to native counterparts (e.g. Hayward et al., 2015; Fontaine & Kohl, 2020). Therefore, a greater understanding of the microbial landscape of invasive species may be determinant to predict how these can threaten vulnerable local ecosystems.

In Portugal, the relative isolation of most freshwater ecosystems (Sousa-Santos et al., 2019) allowed the evolution of numerous endemisms that are particularly vulnerable to biological invasions, which might seriously compromise the regional biodiversity (Barbosa et al., 2009; Cabral et al., 2005). A great example of a successful invasive species is the case of the red swamp crayfish *Procambarus clarkii* (Girard 1852), which is used for human consumption and is now present in most of the Portuguese rivers with well-known economic and environmental impacts, for example negatively affecting rice production and local macroinvertebrate, amphibian or macrophyte communities (Anastácio et al., 2000; Anastácio et al., 2005; Souty-Grosset et al., 2016). The red swamp crayfish has been identified as an important reservoir for many fungal or fungus-like pathogens that can affect native aquatic animals (e.g. the chytrid fungus *Batrachochytrium dendrobatidis* Longcore, Pessier & Nichols 1999; *Trichosporon jirovecii* Fragner 1969; *Aphanomyces astaci* (Schikora 1906), referred in Filipová et al., 2013; Abdallah et al., 2018; Oficialdegui et al., 2019) and also many pathogenic bacteria that can affect other aquatic animals (e.g. *Vibrio* spp.; Sherry et al., 2016; *Citrobacter* spp.; Chen et al., 2017a) and cause human disease (e.g. *Citrobacter freundii* (Braak 1928) Werkman & Gillen 1932; *Francisella tularensis* (McCoy & Chapin 1912) Dorofeyev 1947 in the works by Anda et al., 2001 and Chen et al., 2017b). Despite the large body of literature on specific pathogens and other microorganisms of the red swamp crayfish (e.g. see Evans & Edgerton, 2002 for pathogens in general, and Garzoli et al., 2014 for microfungi), only a few very recent studies characterized this species' entire bacterial communities, by using culture-independent methods and high-throughput sequencing. These studies focused on gut microbiomes of crayfish from China and identified development stage, environment, season and diet as factors modulating gut bacteria (Zhang et al., 2020a; Shui et al., 2020; Liu et al., 2020). Importantly, the exposure to toxic pollutants, such as nitrites and cadmium, was found to induce imbalance in the gut microbiota of the red swamp crayfish and favour the abundance of potential pathogens, which

may have negative effects in crayfish health (Zhang et al., 2020b; Guo et al., 2020).

Although knowledge on the red swamp crayfish microbiome has advanced considerably in recent years, there is still a general lack of knowledge regarding the microbiota of introduced populations in Europe (but see Rossi et al., 2001; Garzoli et al., 2014). Here we aimed to characterise the bacterial microbiota of the gut and carapace of the red swamp crayfish occurring in southern Portugal using high-throughput sequencing of the 16S rRNA V4 hypervariable region. Analyzed crayfish were collected at three distinct localities: a less polluted site at the Alqueva Reservoir (the largest reservoir in the Iberian Peninsula; Potes et al., 2012), and two other localities with reportedly higher pollution levels, a rice paddy and a locality in Xarrama River. Additionally, we collected biological data on the analysed crayfish, including measures of individual baseline stress levels (using hemolymph glucose levels as proxy) as a marker for metabolic expenditure. Finally, we tested whether biological measurements and sampling locality could be used as predictors for bacterial diversity. We expected that different environments and/or stress factors could modulate carapace and gut microbiomes of the red swamp crayfish.

Materials and methods

Study sites

We chose three distinct sites with differing environmental conditions: (a) the Alqueva Reservoir (38°13′28.41″N; 7°32′4.18″W), (b) a rice field near Salvaterra de Magos (39°2′9.80″N; 8°44′16.65″W), and (c) the Xarrama River (38°21′37.41″N; 8°4′3.33″W) (Supplementary Figs. 1 and 2). The Alqueva Reservoir integrates the catchment of the Guadiana River Basin, being an important water supply for human and agricultural consumption in the Alentejo region. Nonetheless, because its water quality varies spatially and seasonally, we chose a less polluted sampling locality, closer to the dam and mainly surrounded by Montado, a human-made ecosystem, characteristic of Alentejo, with forests of holm oaks, cork oaks and chestnut trees (Pinto-Correia et al., 2011). Our second chosen sampling site was at an experimental rice research station (COTArroz — Centro Operativo e Tecnológico do Arroz), within Paul de Magos (in Salvaterra de Magos), which is a rice production area of 700 ha, characterized by rice paddies that are continuously flooded from spring to summer (Correia, 1995). Adjacent drainage channels capture overflow and drainage water from the rice paddies. Finally, the third sampling site was at the Xarrama River, which is included in a system that is susceptible to recurrent droughts and under expansion as an adaptation to climate change. Indeed, this specific sampling locality was set at one permanent pool ~ 27 km downstream from a wastewater treatment plant (Nunes et al., 2017) and immediately downstream of an intensive milk production farm. Based on the data retrieved from the National Information System on Hydric Resources (SNIRH, Supplementary Fig. 2), Alqueva Reservoir is the least polluted of the three sampled localities.

Sampling and preparation

All crayfish were captured during 2019 using Swedish type traps, baited with sardines (*Sardina pilchardus* Walbaum 1792). Twenty crayfish were collected: six in the Alqueva reservoir (3rd July 2019); seven in the Xarrama River (15th of July 2019); seven in Salvaterra de Magos (29th of May 2019). Carapace bacterial samples were taken using sterile swabs (Medical Wire & Equipment, UK). Each specimen was kept in individual ziploc bags, stored in cold temperature conditions inside a cool box, from capture to laboratorial processing, which took between 1 and 1 h 30 m. Individuals were measured (total weight and cephalothorax width) and sexed. Fulton's condition index (K) was estimated for each individual with the formula: $K = W/L^3$, where W—wet weight (g); L—cephalothorax length (mm) (Anastácio & Marques, 1998; Ricker, 1975) (Supplementary Table 1). After being measured, individuals were placed at − 20°C for 10 min. Individuals were then taken from the freezer and decapitated. Specimens were dissected and the entire guts were removed. Bleach was used to sterilize all the material between dissections. Swabs and tissues were kept at − 80°C until processing. DNA was extracted using the PowerSoil DNA Isolation Kit (QIAGEN, Netherlands), DNA concentration and quality were measured in a NanoDropTM 2000 Spectrophotometer (ThermoFisher Scientific, USA). DNA extractions were shipped on dry ice to the

University of Michigan Medical School (USA) for amplification and sequencing according to the protocol of Kozich et al. (2013). Each sample was amplified for the V4 hyper-variable region of the 16S rRNA gene ($\sim$ 250 bp). PCR amplification failed for five gut samples probably due to high concentration of PCR inhibitors. All amplicon libraries were pooled and sequenced in a single run of the Illumina MiSeq sequencing platform. Raw sequences were deposited in the National Center of Biotechnology Information's (NCBI) Sequence Read Archive (SRA) under the BioProject ID PRJNA684689.

For glucose measurements, hemolymph was collected by inserting a syringe between the cephalothorax and the abdomen (Lee et al., 2000), and collecting 1 mL of hemolymph from the heart or pericardial cavity. Glucose determination was done according to Ribeiro et al. (2015), and in reference to the method described for crayfish by Lee et al. (2000). Hemolymph samples were centrifuged at 2500 $\times$ *g* for 10 min at 4°C and the supernatant was collected and analyzed for glucose concentrations using the Glucose Assay kit from QCA (Spain), based on the sequential reactions from glucose oxidase (GOD) and peroxidase (POD). Briefly, 10 µl of supernatant was added to the microplate with 200 µl of kit reaction buffer and read at 505 nm in a microplate spectrophotometer reader (Multiskan Go). Saline solution (0.9% NaCl) was used to dilute hemolymph samples if needed. Glucose concentration in samples was inferred from the standard curve made of known glucose standards (25–100 mg/dL).

Microbiome data processing and statistical analysis

Raw FASTQ files were denoised using the DADA2 pipeline in R with the parameters for filtering and trimming being trimLeft = 20, truncLen = c(220,200), maxN = 0, maxEE = c(2,2), truncQ = 2 (Callahan et al., 2016). A total of 831,257 16S rRNA sequences were retrieved. A table containing amplicon sequence variants (ASVs) was constructed and taxonomic inferences made against the SILVA (138 release) reference database (Quast et al., 2013). ASVs that were not identified as bacteria were removed. Samples with less than 1,000 bacterial sequences were eliminated from downstream analyses, which meant eliminating three gut samples (plus the five that did not amplify). For these reasons only 12 out of the initial 20 gut samples were kept in subsequent analyses. Bacterial sequences retrieved from the carapace varied between 17,848 and 46,426 per sample, and sequences retrieved from the gut varied between 2,673 and 22,324. ASV abundances were normalized using the negative binomial distribution (McMurdie et al., 2014), which accounts for library size differences and biological variability. After normalization and removal of non-bacterial reads, 729,509 sequences and 5,458 ASVs were retrieved from the 20 carapace samples; and 89,940 sequences and 925 ASVs were retrieved from the 12 gut samples. Venn diagrams were built to depict the number of ASVs shared between body sites in each locality, and the number of ASVs present in the gut and carapace that were shared across localities.

Bacterial composition (alpha-diversity) was calculated for each sample using Shannon and Faith's phylogenetic diversity (PD) indices as implemented in the R package phyloseq (McMurdie et al., 2013). Bacterial community structure (beta-diversity) was estimated inter-sample using taxon-based Bray–Curtis dissimilarity distance (quantitative), and phylogenetic-informed weighted (quantitative) and unweighted (qualitative) UniFrac distance (Parks et al., 2012). Linear mixed effect models (using the lmer R package; Gałecki et al., 2013) were used to assess differences between bacterial microbiota from carapace and gut, using individuals as a random factor. PERMANOVA (using the adonis function with 10,000 permutations and implemented in the vegan R package; Oksanen et al., 2020) was used to assess the differences in bacterial microbiota structure between carapace and gut microbiota, with individuals in strata option.

Regarding crayfish biological measures, weight and cephalothorax length were correlated (Pearson's correlation: $r = 0.82$, $P < 0.001$). For this reason, only cephalothorax length was used in the statistical models. Kruskal–Wallis tests were used to assess the differences in crayfish biological measures (i.e. weight, cephalothorax length, Fulton's condition index and glucose) between localities and sex (Supplementary Table 1). Pairwise comparisons were performed using the Wilcoxon test with Benjamini–Hochberg correction.

Cephalothorax length, Fulton's condition index and glucose were standardised to a mean of zero

Table 1 Summary of results obtained from the linear and mixed effects models for alpha-diversity (*F*-statistics and respective *P* values), and from permutational multivariate analysis of variance (adonis function) for beta-diversity estimates (R^2 and respective *P* values)

Tissue	Alpha and beta diversity	Locality	Glucose	Cephalothorax length	Fulton's index	Sex
Gut	PD	0.61 (0.578)	0.11 (0.751)	0.02 (0.881)	0.03 (0.858)	0.00 (0.967)
	Shannon	0.29 (0.756)	0.43 (0.541)	0.38 (0.565)	0.31 (0.599)	0.03 (0.860)
	UniFrac Unweighted	0.20 (0.196)	0.08 (0.913)	0.08 (0.875)	0.08 (0.795)	0.08 (0.892)
	UniFrac Weighted	0.19 (0.412)	0.05 (0.848)	0.08 (0.450)	0.08 (0.516)	0.13 (0.166)
	Bray–Curtis	**0.22 (0.003)**	0.07 (0.765)	0.10 (0.072)	0.09 (0.172)	0.10 (0.040)
Carapace	PD	0.60 (0.560)	0.03 (0.855)	0.07 (0.789)	2.64 (0.128)	0.33 (0.572)
	Shannon	0.58 (0.574)	0.67 (0.426)	0.26 (0.621)	0.24 (0.663)	0.09 (0.763)
	UniFc Unweighted	**0.30 (0.000)**	0.04 (0.488)	0.04 (0.486)	0.03 (0.824)	0.05 (0.118)
	UniFac Weighted	**0.45 (0.000)**	0.02 (0.689)	0.02 (0.787)	0.05 (0.177)	0.03 (0.333)
	Bray–Curtis	**0.50 (0.000)**	0.03 (0.356)	0.02 (0.661)	0.02 (0.655)	0.045 (0.119)

PD stands for Faith's phylogenetic diversity. Significant associations are depicted in bold

("centering") and standard deviation of one ("scaling"). Due to the significant differences between microbiota of gut and carapace, the effects of locality and measured biological factors on microbiota composition and structure were assessed independently for each tissue using linear models (alpha-diversity $\sim$ locality + glucose + fulton condition index + cephalotorax length + sex) and PERMANOVA with 10,000 permutations (beta-diversity $\sim$ locality + glucose + Fulton condition index + cephalotorax length + sex). Variation in the mean proportions of the most abundant taxa ($\geq$ 5% of all reads after normalization for phyla level and $\geq$ 1% for genus level) in each tissue between localities was assessed by linear models.

Results

Significant differences were found in glucose (Kruskall-Wallis test = 19.66, $P <$ 0.001) and cephalothorax length (Kruskall-Wallis test = 6.40, $P = 0.040$) of crayfish between sampling localities. Pairwise comparisons showed cephalothorax length was significantly lower at Salvaterra de Magos relative to Alqueva ($P = 0.024$). Levels of glucose (our proxy for stress levels) were significantly lower in Alqueva, than at Salvaterra de Magos and Xarrama. Conversely, sex had no significant effect on crayfish biological measurements.

Significant differences were found in alpha-diversity indices between the carapace and gut microbiota ($F = 122.8$ and $P < 0.001$ for Shannon; $F = 315.1$ and $P < 0.001$ for PD; Fig. 1), with higher diversity found in the carapace. Significant differences between beta-diversity of the carapace and gut microbiota were also found when using both UniFrac and Bray–Curtis distances (PERMANOVA, $R^2 = 0.14$ and $p < 0.001$ for unweighted UniFrac; $R^2 = 0.06$ and $P = 0.036$ for weighted UniFrac; and $R^2 = 0.17$ and $P < 0.001$ for Bray–Curtis). The results from linear models revealed no effects of tested variables, i.e. locality and biological measurements, in alpha-diversity of the gut and carapace microbiota (Table 1). Results from PERMANOVA showed that locality had a significant effect in all metrics used to measure microbiota structure of the carapace (see Table 1 for statistics and *P*-values; and Fig. 2 for a graphical representation). For gut microbiota, a significant effect of locality and sex also was detected for the Bray–Curtis distance (Table 1 and Fig. 2).

Taxa from Proteobacteria (40%), Bacteroidota (26%), Verrucomicrobiota (9.1%) and Planctomycetota (7.9%) were highly abundant ($\geq$ 5% of all sequences) in the carapace microbiomes. From these, the proportion of Proteobacteria ($F = 12.0$, $P < 0.001$), Verrucomicrobiota ($F = 4.6$, $P = 0.025$) and Planctomycetota ($F = 9.1$, $P = 0.002$) varied significantly between localities. Proteobacteria (43%), Firmicutes (32%) and Planctomycetota

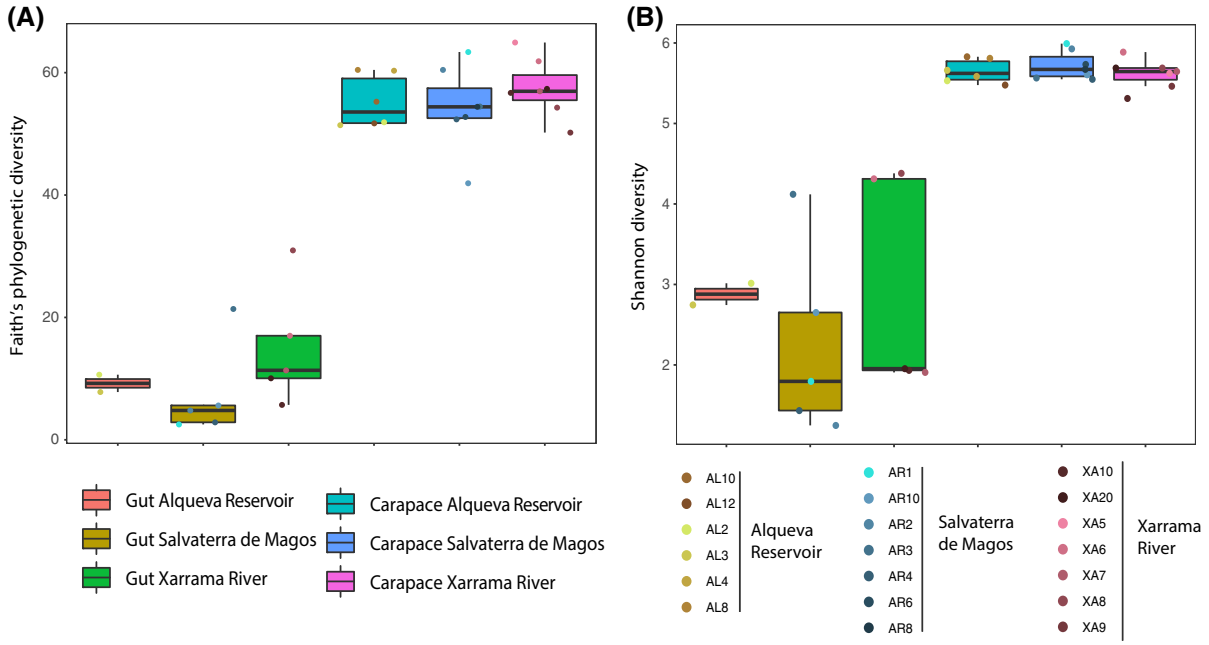

Fig. 1 Alpha-diversity measures estimated for carapace and gut bacterial microbiota at each sampled locality: **A** Faith's phylogenetic diversity; **B** Shannon diversity. Localities, sampled tissues and individuals are color coded

(5.5%) were highly abundant (≥ 5% of all sequences) in the gut, and their proportions did not vary significantly between localities.

More than 50% of the ASVs found in the gut of the analyzed crayfish were shared with the carapace across localities (Supplementary Fig. 3). Only 359 of the ASVs present in the carapace microbiota of analyzed crayfish were shared between localities (Fig. 3), and 12 were present in all individuals. Twenty-three genera were represented with ≥ 1% of the reads for the carapace samples after normalization (Fig. 4), with the relative proportion of about half of these varying significantly between localities: *Cyanobium* PCC-6307 ($F = 9.6$, $P = 0.002$), an unclassified genus from Spirosomaceae ($F = 8.1$, $P = 0.003$), an unclassified genus from Bacteroidota ($F = 5.4$, $P = 0.015$), an unclassified genus from Methylomonadaceae ($F = 19.6$, $P < 0.001$), *Crenothrix* Cohn 1870 ($F = 48.2$, $P < 0.001$), C39 ($F = 54.7$ $P < 0.001$), *Hydrogenophaga* Willems et al., 1989 ($F = 9.1$, $P = 0.002$), an unclassified genus from Comamonadaceae ($F = 26.9$, $P < 0.001$), *Rhodobacter* Imhoff et al., 1984 emend. Wang et al., 2014 ($F = 41.5$ $P < 0.001$), *Novosphingobium* Takeuchi et al., 2001 ($F = 48.7$, $P < 0.001$), an unclassified genus from Verrucomicrobiaceae ($F = 8.6$, $P = 0.003$) and SH-PL14 ($F = 9.7$ $P = 0.002$).

The gut of the crayfish analyzed shared 10 ASVs across localities (Fig. 3). No single ASV was present in the gut of all the individuals. Nineteen genera were represented in ≥ 1% of the reads for the gut samples after normalization (Fig. 5), from which the relative proportion of three significantly varied between localities: an unclassified Enterobacterales ($F = 78.8$, $P < 0.001$), *Hafnia-Obesumbacterium* ($F = 5.8$, 0.024) and *Terrimicrobium* Qiu et al., 2014 ($F = 7.0$, $P = 0.014$).

Discussion

In the present study, the bacterial microbiota of the carapace and gut of red swamp crayfish (*Procambarus clarkii*) from three different localities in Southern Portugal was characterized, by sequencing the v4 region of 16S rRNA of bacterial communities. Analyses of alpha- and beta-diversity indexes showed that bacterial diversity was higher in the carapace than in the gut. A similar pattern has been identified in other crustaceans (e.g. fiddler crabs [*Uca* spp. Leach, 1814] in the work by Cuellar-Gempeler & Leibold, 2018),

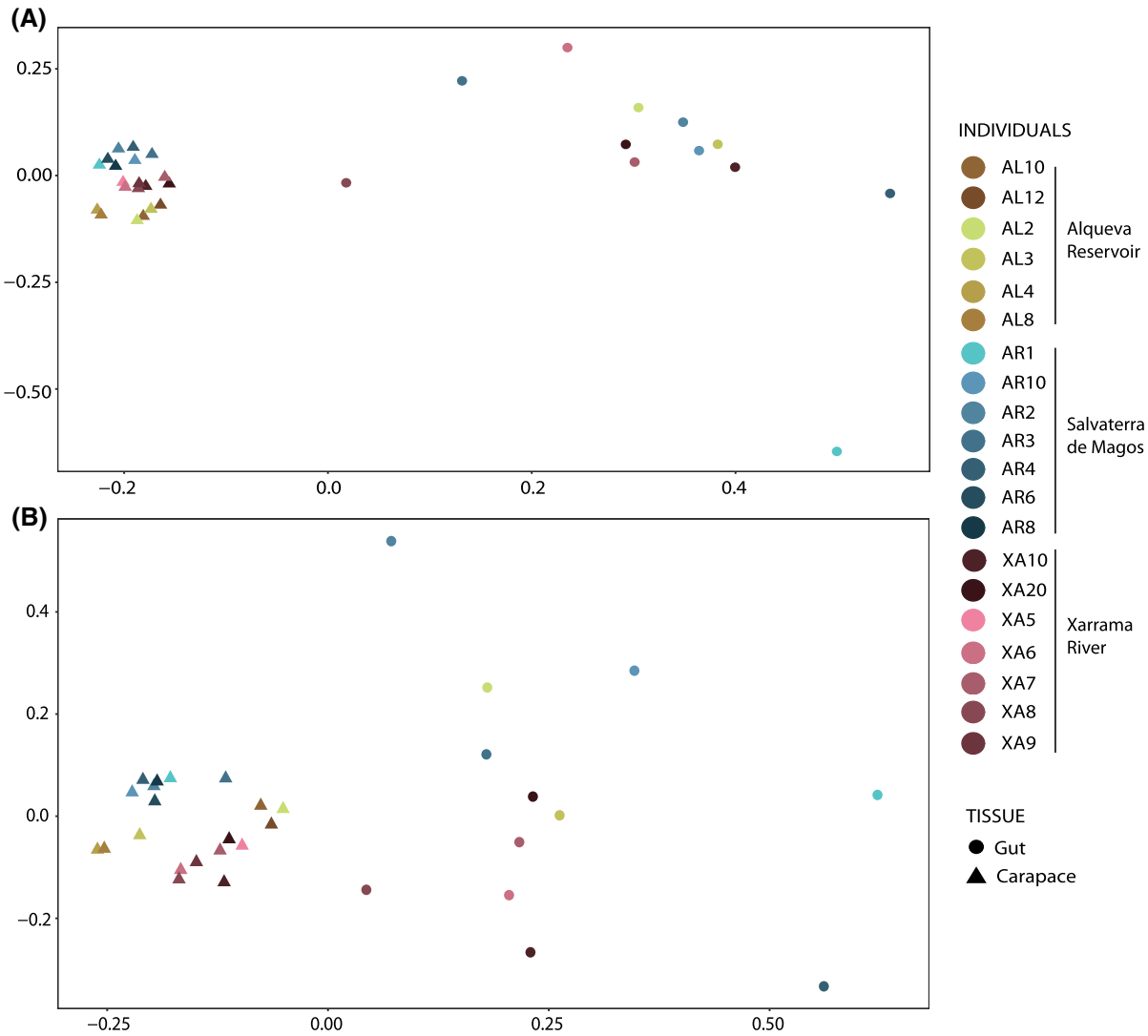

Fig. 2 Non-metric multidimensional scaling analysis of bacterial beta diversity of the carapace and gut at each sampled locality using **A** UniFrac (unweighted) and **B** Bray–Curtis distances. Sampled tissues and individuals are color coded

however this trend is not universal among crustaceans and was found inverted in some species (e.g. in the Atlantic horseshoe crab [*Limulus polyphemus* (Linnaeus, 1758)]; Friel et al., 2020). Not surprisingly, a large proportion of the most abundant bacteria identified to genus level and found in the carapace are known to be able to form biofilms (e.g. Stoecker et al., 2006; Inaba et al., 2018; Wilkinson et al., 2011). Interestingly, some of the most abundant bacterial genera that were found in the gut and carapace have also been previously found in the gut microbiomes of red swamp crayfish collected in China (*Tyzerella* Yutin and Galperin 2013, effective name; *Candidatus Bacilloplasma* Kostanjsek et al., 2007, effective name; *Clostridium* Prazmowski 1880 (Approved Lists 1980) emend. Lawson and Rainey 2016, nom. approb.; *Acinetobacter* Brisou and Prevot 1954; reported by Shui et al., 2020; Liu et al., 2020; Zhang et al., 2020a) and in Egypt (*Flavobacterium* Bergey et al., 1923 (Approved Lists 1980) emend. Kuo et al., 2013, nom. approb.; reported by Khalil et al., 2009), and also in the heamolymph of red swamp crayfish collected in the U.S.A. (*Flavobacterium*; Scott & Thune 1986). The fact that the same bacterial genera may be shared globally among red swamp crayfish indicates there might be a core microbiome (i.e. present in the

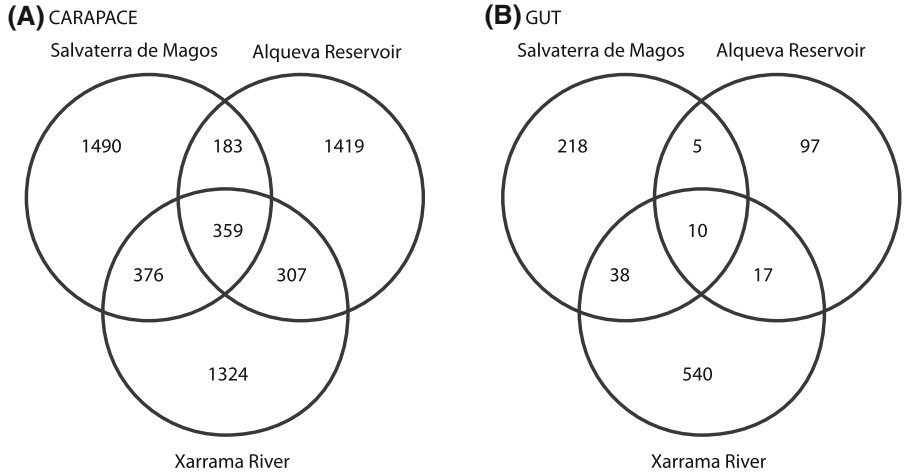

Fig. 3 Venn diagrams depicting the number of shared and unique amplicon sequence variants (ASVs) in the **A** carapace and **B** gut across localities

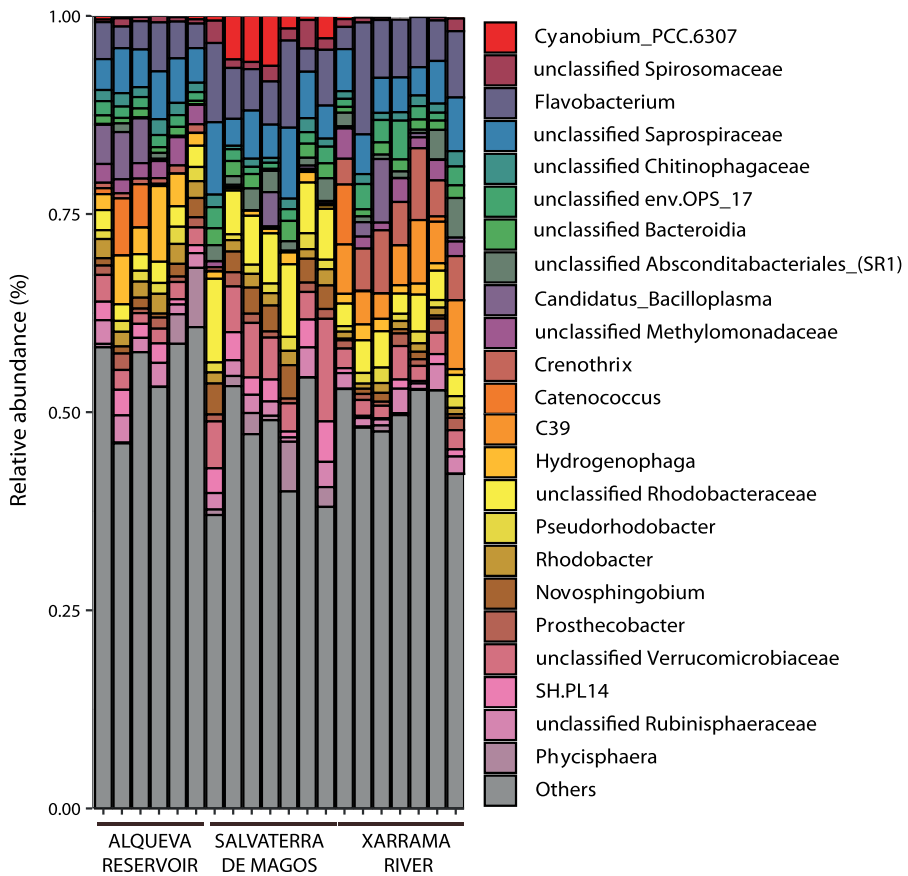

Fig. 4 Summary of the taxonomic composition of crayfish carapace microbiota at genus level

majority of the specimens) for this species. The results from the current study show that on average about 4% and 15% of the bacterial taxa (i.e. ASVs) recovered from the gut and carapace of crayfish, respectively, were present at the three sampled localities. However, no single ASV was recovered from the gut of all

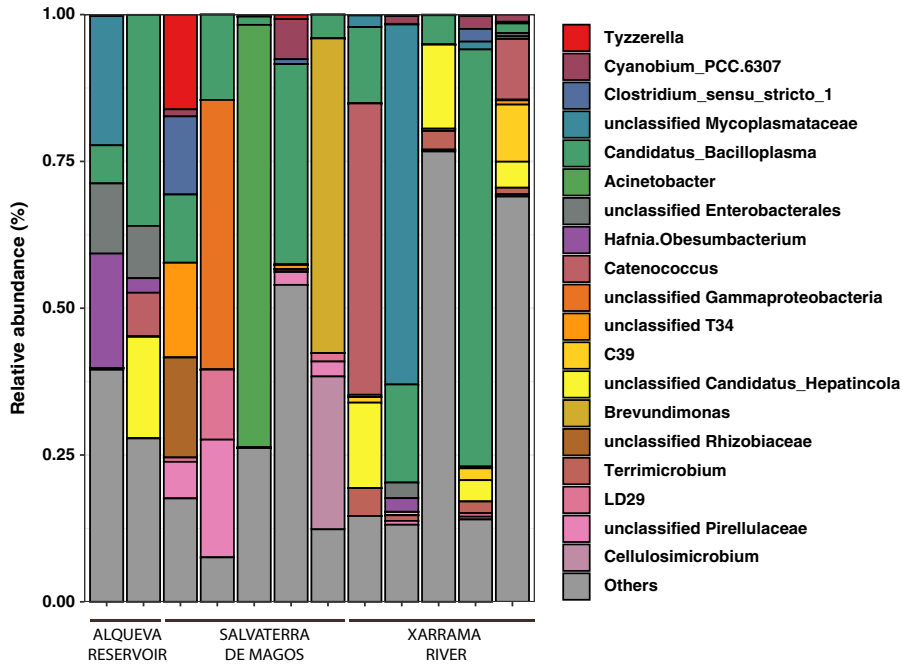

Fig. 5 Summary of the taxonomic composition of crayfish gut microbiota at genus level

individuals analyzed, and only 12 ASVs (0.2% of the ASVs present in the carapace) were present in the carapace of all the individuals analyzed. Additionally, it is worth noticing that a high proportion (> 50%) of the ASVs found in the gut were also found in the carapace. Sharing of bacterial taxa between tissues of the same host species is not unusual and has been described before for many animals (e.g. Rosado et al., 2019; Hoffman et al., 2017).

Importantly, several of the most abundant bacterial genera found in the carapace and gut of the red swamp crayfish in the present study are known to harbor bacterial pathogens that can be harmful to aquatic animals, including other crustaceans and fish, and even to humans (e.g. taxa belonging to genera *Cellulosimicrobium* Schumann et al., 2001 emend. Yoon et al., 2007; *Hafnia-Obesumbacterium*; *Clostridium* sensu stricto 1; *Candidatus Bacilloplasma*; and *Catenococcus* Sorokin 1994 [see for example Janda & Abbott, 2006; Felföldi et al., 2010; Guibert et al., 2020; Hou et al., 2018; and Rath et al., 2018]). Given that previous studies have also identified the red swamp crayfish as reservoirs of a multitude of pathogens (e.g. Barkate 1967; Evans & Edgerton 2002; Khalil et al., 2009), further studies are warranted to determine whether crayfish established in continental Portugal are carriers of such pathogens and the potential risk of their transmission to native fauna, and also humans through consumption.

Association between the studied microbiomes and environmental conditions, host factors and metabolic expenditure (aka stress levels)

Marked differences were reported between the environmental conditions at each of the sampling sites, especially between Alqueva, the less polluted site, and the other two localities (Supplementary Fig. 2). Such differences are likely to be translated in different bacterioplankton communities (e.g. Yan et al., 2020; Wang et al., 2020), and thus affect the pool of bacteria available to colonize crayfish. For these reasons, it is not surprising that significant differences in microbiome structure were found in the carapace of the red swamp crayfish between the three sampling localities. The gut microbiomes also showed structure difference between localities, albeit only when using the Bray–Curtis distance. The results presented here are in line with those of a previous study on gut microbiota of farmed red swamp crayfish from China, which showed that geography alone did not explain differences in community structure, which were instead shaped by host-intrinsic factors, such as development stage, and supplied diet (Zhang et al., 2020a). On the contrary,

the carapace microbiome of another crayfish, *Cambarus sciotensis* Rhoades, 1944 sampled in the New River drainage (Virginia, U.S.A.), was found to be highly susceptible to different local conditions, with carapace microbial communities closely related to those found in local environments (Skelton et al., 2017).

There was no significant effect of cephalothorax length, body condition or glucose levels (aka stress levels) in the bacterial diversity patterns of the carapace and gut. Interestingly, sex had a significant, albeit very moderate, effect in the gut bacterial community when Bray–Curtis distances were analysed. Sex was seen to affect gut bacteria richness in fiddler crabs (*Uca thayeri* Rathbun 1900 in the work by Cuellar-Gempeler & Munguia, 2013), with such differences later attributed to distinct physiology and behaviour (Cuellar-Gempeler & Leibold, 2018). The present results may be an indication of a distinct behavioural response between females and males, putatively associated with reproduction. In this species, females are more prone to occupy burrows than males (Ilhéu et al., 2005), and often spend the reproductive period secluded inside them while raising their broods (Anastácio et al., 1999; Correia & Ferreira, 1995; Hasiotis, 1995). Burrows have distinct conditions from the local aquatic environment and induce metabolic changes in crayfish (McMahon & Stuart, 1995, 1999). Additionally, prey availability and quality also differ during burrow confinement, and diet alone can alter gut microbiome in other freshwater crayfish (*Cherax cainii* Austin 2002 which studied by Foysal et al., 2020). Results suggest that the gut microbiomes of the red swamp crayfish may be linked to host factors, including sex, but other variables such as body size and condition or stress levels may have lower effect on them. While significantly changing between sites (particularly between Alqueva and Xarrama), changes in glucose levels may also result from short term alterations (of social or environmental conditions) and thus being mostly local drivers of behavioural and metabolic change, and perhaps less as modulators of microbial assemblages.

Conclusions

Our results show that the carapace microbiomes of the red swamp crayfish are shaped by environmental conditions. In turn, gut microbiota seem to be more stable and linked with host factors, as was previously hypothesized (Zhang et al., 2020a). Nevertheless, results suggest gut bacterial composition and structure may be unrelated to glucose levels, size or body condition. Finally, we have also found several taxa which could correspond to potential pathogens in the microbiota of crayfish. These findings emphasize the need to continue research to fully characterize the role of red swamp crayfish as vectors for bacterial diseases in freshwater ecosystems, as well as their potential to affect human health through crayfish manipulation and consumption.

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Data Availability Raw sequences were deposited in the National Center of Biotechnology Information's (NCBI) Sequence Read Archive (SRA) under the BioProject ID PRJNA684689 (BioSample accessions SAMN17065835-74).

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