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What shapes a microbiome? Differences in bacterial communities associated with helminth-amphipod interactions

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ABSTRACT

The fast technological advances of molecular tools have enabled us to uncover a new dimension hidden within parasites and their hosts: their microbiomes. Increasingly, parasitologists characterise host microbiome changes in the face of parasitic infections, revealing the potential of these microscopic fast-evolving entities to influence host-parasite interactions. However, most of the changes in host microbiomes seem to depend on the host and parasite species in question. Furthermore, we should understand the relative role of parasitic infections as microbiome modulators when compared with other microbiome-impacting factors (e.g., host size, age, sex). Here, we characterised the microbiome of a single intermediate host species infected by two parasites belonging to different phyla: the acanthocephalan *Plagiorhynchus allisonae* and a dilepidid cestode, both infecting *Transorchestia serrulata* amphipods collected simultaneously from the same locality. We used the v4 hypervariable region of the 16S rRNA prokaryotic gene to identify the hemolymph bacterial community of uninfected, acanthocephalan-infected, and cestode-infected amphipods, as well as the bacteria in the amphipods' immediate environment and in the parasites infecting them. Our results show that parasitic infections were more strongly associated with differences in host bacterial community richness than amphipod size, presence of amphipod eggs in female amphipods, and even parasite load. Amphipods infected by acanthocephalans had the most divergent bacterial community, with a marked decrease in alpha diversity compared with cestode-infected and uninfected hosts. In accordance with the species-specific nature of microbiome changes in parasitic infections, we found unique microbial taxa associating with hosts infected by each parasite species, as well as taxa only shared between a parasite species and their infected hosts. However, there were some bacterial taxa detected in all parasitised amphipods (regardless of the parasite species), but not in uninfected amphipods or the environment. We propose that shared bacteria associated with all hosts parasitised by distantly related helminths may be important either in helping host defences or parasites' success, and could thus interact with host-parasite evolution.

1. Introduction

Microbes are ubiquitous, found in all natural environments and associated with all multicellular organisms. The entire microbial community (and their genes) associated with an organism form its microbiome. In animals, microbiomes significantly contribute to the physiology (Brealey et al., 2022; Midha et al., 2022), behaviour (Cryan and Dinan, 2012; De Palma et al., 2015), phenotype (Li et al., 2008; Brealey et al., 2022), and may even influence and regulate gene expression in the organism to which they are linked (Hayes et al., 2010; Afrin et al., 2019). The microbiome has also been proposed to confer new genetic functions to its host, and since microbes have very short generation times, they have been hypothesised to contribute to fast adaptation of their hosts to new and changing environments (Zhang et al., 2019; Chan et al., 2021). All these findings have called into question the traditional notion of the organism as a singular entity and, although controversial, the hologenome concept has been put forth to describe the evolutionary unit formed by the genome of an organism and its microbiome (Zilber-Rosenberg and Rosenberg, 2008; Bordenstein and Theis, 2015; Moran and Sloan, 2015; Douglas and Werren, 2016; Theis et al., 2016; Roughgarden et al., 2017).

Given the importance of the microbiome to all organisms, many studies have assessed what factors impact the composition, abundance, and diversity of the microbial communities that make up the host microbiome. In brief, these studies have found evidence that the microbiome can be affected by the host's diet and feeding

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ecology (Wong and Rawls, 2012; Hacquard et al., 2015; Smith et al., 2015; Zhang et al., 2016; Qiao et al., 2017; Zhang et al., 2019; Chan et al., 2022), physiology, age, general state of health, trophic level and phylogeny (Sullam et al., 2012; Ye et al., 2014; Zhang et al., 2016; Chan et al., 2021), as well as environmental factors such as salinity (Wong and Rawls, 2012; Qiao et al., 2017), pH, pollution (Suo et al., 2017), water depth, and temperature (Wong and Rawls, 2012). Understanding the factors determining microbiome composition in various organisms is useful not only for identifying symbiotic relationships between specific hosts and microbes, but in a broader context it can also help to determine the potential of microbiomes to influence their hosts' evolution.

Host-parasite relationships are renowned models for studying evolution (Paterson and Piertney, 2011; Lighten et al., 2017; Buckingham and Ashby, 2022; Smith and Ashby, 2023). Hosts are expected to evolve resistance to parasites, while parasites evolve various mechanisms to by-pass such resistance (Anderson and May 1982; Papkou et al., 2019), but the role of the microbiome in this dynamics is only starting to be explored (Biron et al., 2015; Dheilly et al., 2015, 2017, 2019b; Poulin et al., 2023; Salloum et al., 2023a). There is increasing evidence that parasitic infections lead to changes in the parasitised host's microbiome (Biron et al., 2015; Dheilly et al., 2015; Hahn and Dheilly, 2016; Afrin et al., 2019; Gaulke et al., 2019; Hahn et al., 2022). However, these changes are not fixed, and seem to be species-specific, dependent not only on the host species, but also on the parasite species involved (Ling et al., 2020; Hahn et al., 2022; Salloum et al., 2023b). Parasites can influence their host's microbiome in many ways. They are both a vector and a reservoir for numerous microbes that can interact with the host's internal environment during parasitic infection (Dheilly, 2014; Dheilly et al., 2015; Poulin et al., 2023). Parasites can also modulate the host's immune system during infection (Maizels et al., 2004; Maizels and McSorley, 2016). In addition, the host's microbiota may compete with its parasite or with the parasite's microbiota for resources (Dheilly et al., 2019a). Integrating the microbiome dimension in the host-parasite dynamics is then necessary for a full understanding of the evolution of host-parasite interactions, and may contribute valuable additional information to the hologenome controversy.

Here, we explored the interaction among parasites and their hosts' microbiomes, assessing the effect of parasitic infection as a microbiome modulator in comparison with other potential microbiome-impacting factors. Specifically, we characterised the bacterial community of individual amphipods of the same species (*Transorchestia serrulata*) infected by either of two distantly related parasites, the acanthocephalan *Plagiorhynchus allisonae* and a dilepidid cestode. We expected to find the amphipod's bacterial community more strongly associated with parasitic infection than with other factors (namely, amphipod size and presence of eggs in females). We also expected that parasite load would influence the bacterial community of their amphipod host. In addition, because these are different parasite phyla with differences in life cycle, we expected to find little overlap among the bacterial community of acanthocephalan-infected and cestode-infected amphipods, in line with previous evidence of species-specific host microbiome changes induced by parasites. All amphipods included in this study were collected at the same time and place, so we assume no changes in the bacterial community in response to diet, season, or environmental factors (e.g., temperature, pH, toxic compounds). Our findings therefore provide a strong test of the association between helminth infection and the composition of the host's bacterial community, and in particular of parasite-specific effects on the host's bacterial community.

2. Materials and methods

2.1. Collection, dissection, and DNA library preparation

In March 2023, amphipods were collected by manual gathering at Lower Portobello Bay, Dunedin, New Zealand (45° 49′ 48″ S, 170° 40′ 12″ E), from an area no greater than 20 m^2. Microbiota from the substrate's surface (a mix of sand and fine gravel) was sampled with swabs (representing the environment surrounding the amphipods), transported on ice, and then stored at −70 °C until further processing. Collected amphipods were placed in several plastic containers (500 mL) and kept alive. Containers had 200 mg of substrate from the collection site, kept moist with sea water collected at the same time and place. One hundred of the collected amphipods were arbitrarily sampled from random containers and photographed using a Microscope Digital Camera MU1003B (10MP aptina colour CMOS, ulta-fine colour engine, AMSCOPE, USA) mounted on an SZ61 model SZ2-ILST dissecting microscope (Olympus, Japan), with the same camera positioning and the same scale. Photos were used to size the amphipods in Photoshop, by setting a custom transformation of pixels to an area (in cm^2) using the scale on each photo, and then selecting the amphipod with the 'quick selection tool' and measuring with the 'Image –> Analysis –> Record Measurements' feature. Presence/absence of eggs was recorded, as some amphipod females were incubating eggs in their marsupium. The morphology of gnathopods (larger in males) was previously used to determine sex (Lagrue et al., 2016), but since immature males could be confounded with females, sex was not considered as a variable in this study.

All material used for dissections was UV-sterilised, dissecting kits and solutions were autoclaved, and Petri dishes were sterilised by soaking in 1:100 dilutions of TriGene (In Vitro, New Zealand). During dissections, forceps were sterilised by sequential immersion in 1:100 TriGene, 70% ethanol and distilled water. Immediately after being photographed, amphipods were cold euthanised and dissected under a UV-sterilised laminar flow. To reduce body surface contamination, dead amphipods were washed by stirring in a beaker containing 70% ethanol, dried with a paper tissue, and placed in a sterile Petri dish for dissection. A scalpel was used to remove the amphipod's head, following which the dorsal part of the body (around the third pleon) was opened and hemolymph was collected with a sterile cotton swab, which was then stored at −70 °C until further processing. It is worth mentioning that although all hemolymph samples were taken using the same protocol, no negative control was taken from cotton swabs. Each amphipod was then carefully pulled apart and any endoparasites found were counted, isolated, washed three times in PBS and stored. A sample (100 ul) of the PBS stock solution was taken as a negative control. For amphipods infected with acanthocephalans, parasite load was recorded (i.e., number of acanthocephalans infecting a single amphipod). For cestodes, a single parasite was found in all instances of infection. The few amphipods simultaneously harbouring parasites of both species were discarded from further analyses (seven amphipods in total).

DNA extractions from amphipod hemolymph swabs, environmental substrate, whole parasites, and controls were done using the Power Soil Kit (QIAGEN Ltd, New Zealand), following the manufacturer's recommendations (with a 20 min bead beating step, and for parasites we used an additional overnight incubation at 60 °C with 20 ul of proteinase K (20 mg/mL), A&A Biotechnology, Poland). Libraries were prepared as in Jorge et al. (2020), with the primers 515F- 806R (Apprill et al., 2015; Parada et al., 2016), targeting the V4 hypervariable region of the bacterial 16S ssrRNA

gene. Two negative controls for the DNA extraction and two for the PCRs were included, in addition to two samples of the ZymoBIOMICS (Zymo Research, USA) microbial community standard (MCS). Library purification was done with a 0.8:1 ratio of Omega MagBind ® (Omega Bio-Tek, USA) beads to PCR product. Amplicons were normalised to the lowest concentration (based on Qubit quantifications), multiplexed, and sequenced using an Illumina MiSeq platform and v3 reagent cartridge (250 bp, paired- end) at the Otago Genomics & Bioinformatics Facility, New Zealand. Raw sequencing reads were deposited in the GenBank Sequence Read Archive (BioProject PRJNA1070336).

2.2. Bioinformatics and analyses

FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and MultiQC v1.14 (Ewels et al., 2016) were used to check the quality of the demultiplexed sequences. Primers and adaptors were removed using cutadapt (Martin, 2011) as implemented in QIIME2 v2021.4 (Bolyen et al., 2019), with 0 error rate and a minimum sequence length of 190 bp. Sequences were forward- and reverse- trimmed by 13 bp, forward-truncated at 170 bp and reverse-truncated at 190 bp to keep an overall Phred score above 20, and denoised using dada2 in QIIME2 (Callahan et al., 2016). The Naïve Bayes classifier in QIIME2 was used to train the SILVA database SSURef_NR99 version 138.1 on our dataset (Quast et al., 2013), with sequences' minimum length of 900 bp for Archaea, 1200 bp for Bacteria and 1400 bp for Eukaryota, default uniq mode for dereplication, forward primer sequence GTGYC AGC MGC CGC GGTAA and reverse primer sequence GGACT ACN VGG GTW TCTAAT. Taxonomy was assigned to our data based on this trained SILVA database, using the feature-classifier plugin with sklearn mode in QIIME2; amplicon sequence variants (ASVs) were aligned with MAFFT (Katoh and Standley, 2013) using the phylogeny plugin in QIIME2 and rooted and unrooted phylogenetic trees were built with FastTree2 (Price et al., 2010). Trees were visualised with the EMPress plugin in Qiime2 (Cantrell et al., 2021, Supplementary Fig. S1).

Resulting feature tables were filtered to remove mitochondria, chloroplasts, eukaryotes, and features without a phylum assignment. To evaluate data quality, QIIME2 was used to compare the observed and expected MCS composition. The R package decontam v1.18.0 (Davis et al., 2018) was used to identify features that were likely to be contaminants, based on the five negative controls included and using a frequency threshold of 0.1 and a prevalence threshold of 0.5. Identified contaminants were removed from the dataset. Alpha diversity metrics (Shannon Diversity and Observed Richness) were used to build rarefaction curves with the Qiime2 function *qiime diversity alpha-rarefaction* and determine a depth filter by taking the asymptote of the rarefaction curve but keeping as many samples as possible in the dataset. This resulted in a dataset with samples that had at least 500 features, and features that were found at least twice (minimum frequency of 2). The final dataset has 71 amphipods and two environmental samples, in addition to 23 acanthocephalans and nine cestodes. This dataset was split into one containing amphipods and environmental samples (main dataset used in all analyses of this manuscript) and one containing amphipods, parasites and environmental samples (used here only for investigations of shared taxa between hosts and their parasites). The Qiime2 plugin Evident (Rahman et al., 2022) was used to estimate effect sizes for alpha diversity (Faiths' PD and Shannon diversity) and beta diversity (Bray Curtis and Jaccard distances) comparisons among acanthocephalan-infected, cestode-infected and uninfected amphipods in the main dataset.

The main dataset was loaded into R v4.1.3 (R-Core-Team, 2022) with the packages qiime2R v0.99.6 (https://github.com/jbisanz/qiime2R) and file2meco v0.6.0 (Liu et al., 2021). ASVs were grouped into higher taxonomic ranks using Phyloseq v1.42.0 (McMurdie and Holmes, 2013), and the package microeco v1.1.0 (Liu et al., 2021) was used for all analyses, unless otherwise stated, with the False Discovery Rate (FDR) correction for multiple tests (for 0.05 significance).

The bacterial community of uninfected amphipods was compared with that of acanthocephalan-infected and cestode-infected amphipods, in addition to a comparison among the bacterial community of all amphipods and that of the environmental samples. To do that, beta diversity was estimated for uninfected and infected amphipods using the Bray Curtis, Jaccard, Weighted and Unweighted Unifrac metrics, with significance based on perMANOVAs, at phylum, family and AVS levels. Alpha diversity was estimated using Observed richness, Shannon diversity index, and Faith's PD, with significant differences based on ANOVAs also at phylum, family and ASV levels. Congruence between taxonomic and phylogenetic diversity estimates (at ASV level) was checked with a correlation test between Shannon Diversity and Faith's PD, done with the lm function in R (R-Core-Team). In addition, Faith's PD standardised effect sizes at ASV level were estimated with the function phyloseq_phylo_ses and null model 'taxa.labels', using the R package metagMisc v. 0.5.0 (https://github.com/vmikk/metagMisc).

Differential abundance of taxa in amphipods of different infection status was tested at phylum, family and ASV levels with three methods: Aldex2_kw implemented in Microeco, with 999 bootstraps; LinDA (Zhou et al., 2022) implemented in the R package MicrobiomeStat v1.1 (https://cran.r-project.org/web/packages/MicrobiomeStat/index.html), with a filter to remove taxa with no statistical power (minimum relative abundance of 0.005 at ASV level, and of 0.001 at phylum and family levels); and Corncob v. 0.3.2 (Martin et al., 2020), with the "Wald" hypothesis testing procedure. Unique and shared taxa among amphipods and the environment were identified with the ps_venn function in MicEco v. 0.9.19 (https://github.com/Russel88/MicEco/). In addition, the second dataset (containing parasites, see Koellsch et al., 2024 for more details) was used to identify ASVs that were not present in the environment nor in any amphipod except in acanthocephalan-infected and acanthocephalan parasites, and separately in cestode-infected and cestode parasites. Finally, the main dataset (only containing amphipods) was used for bar plots of relative abundance based on group means, grouped as acanthocephalan-infected, cestode-infected, uninfected amphipods, and environmental samples.

The relative importance of various factors in explaining the composition of the amphipods bacterial community was assessed using generalized linear models (GLMs), in which alpha diversity (Observed diversity, Shannon and Faith's PD) was the response variable, and explanatory variables were amphipod infection status (uninfected, acanthocephalan-infected, or cestode-infected), amphipod size (area in cm^2), presence of eggs (binary variable), and acanthocephalan infection load (number of acanthocephalans infecting a single host). The model was fitted using the glm function in base R (R-Core-Team, 2022), significance was estimated with an ANOVA, and model fit based on the null deviance, residuals deviance, Akaike Information Criterion (AIC), and the distribution of residuals (normality assessed with Shapiro-Wilk tests implemented in base R). Separate GLMs were done for each alpha diversity metric used at phylum, family, and ASV levels.

3. Results

The filtered dataset consisted of 71 amphipods (39 uninfected, 21 acanthocephalan-infected and 11 cestode-infected), in addition to the two environmental samples, ranging from 550 to 916,980

ASVs per sample (Supplementary Table S1). DNA concentration prior to normalisation did not influence the number of ASVs recovered per sample (Supplementary Fig. S2). Rarefied curves show that the diversity of most samples plateau after 500 ASV counts (except from the environment and a few amphipods), and that there is no systematic bias between sample type and depth of sequencing (Supplementary Figs. S3-S4). The rarefied datasets used for alpha and beta diversity analyses lost 4,738 ASVs that were no longer present in any sample after random subsampling.

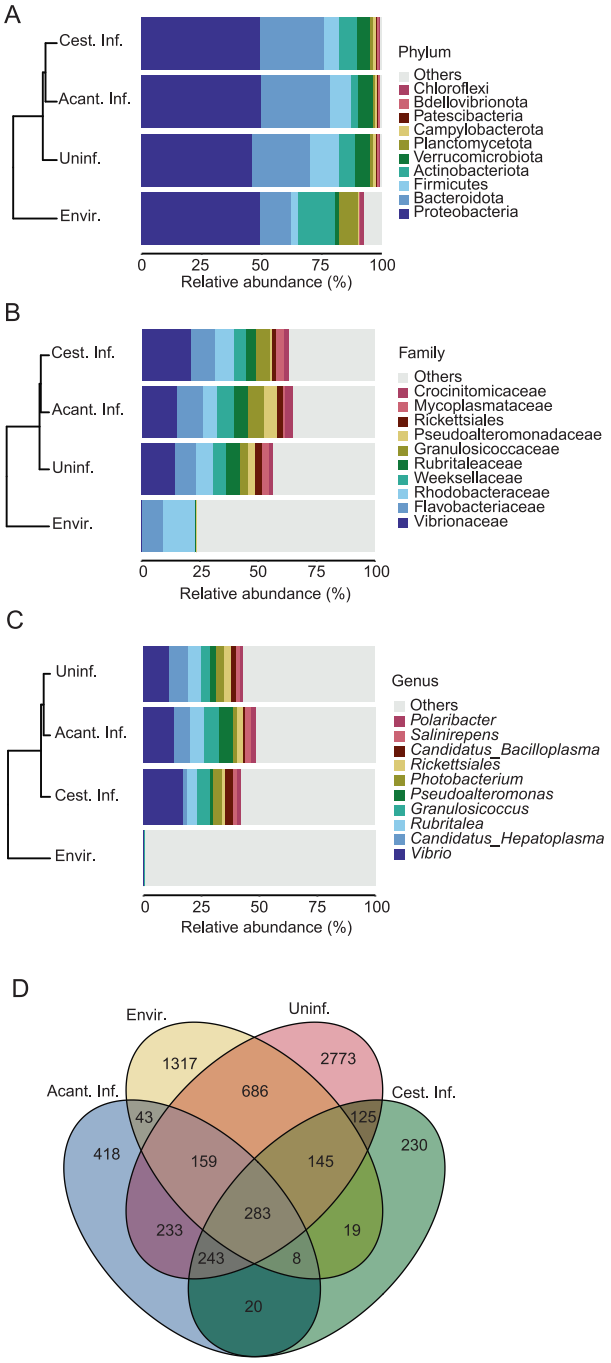

Fig. 1. Comparisons among amphipods and the environment. (A-C) Bar plots of relative abundance at phylum level (A), family level (B), and genus level (C), grouped by similarity. Only the 10 most abundant taxa are shown (the grey area represents the proportion of all other taxa that are not the 10 most abundant). (D) Venn diagram depicting the number of unique and shared amplicon sequence variants (ASVs) among groups. Acant. Inf., acanthocephalan-infected amphipods; Cest. Inf.. cestode-infected amphipods; Uninf., uninfected amphipods; Envir., environmental samples.

As the taxonomic resolution increased, so did the dissimilarity of the amphipods with the environment (Fig. 1A-C); although many ASVs were shared with the environment, there were many unique ASVs found only in amphipods (Fig. 1D). ASV taxonomic and phylogenetic diversity (Shannon and Faith's PD) were correlated ($r_{(71)} = 0.77$; P-value < 2.2e-16), and the Faith's PD standardised effect sizes were negative (except for four amphipods with a non-significant P-value, Supplementary Table S2). This indicates a clustering phylogenetic relationship among bacteria in each sample, in which different diversity metrics are expected to be congruent (Tucker and Cadotte, 2013; Mazel et al., 2016). Amphipods had significantly less diverse bacterial communities than the environment in all alpha diversity metrics at all taxonomic ranks tested (except for Shannon diversity at phylum level, Supplementary Table S3). Beta-diversity results also showed that the bacterial communities of amphipods were different from those of the environmental samples, as almost all beta-diversity analyses between amphipods and the environment were significant (except Weighted Unifrac and Bray Curtis at the phylum level, Supplementary Table S4). Effect sizes for comparisons of acanthocephalan-infected, cestode-infected and uninfected amphipod alpha and beta diversity ranged from 0.22 to 0.37 (Supplementary Table S5).

Comparisons of relative bacterial abundance showed increasing dissimilarity between acanthocephalan-infected and cestode-infected amphipods at lower taxonomic ranks, with cestode-infected being more similar to acanthocephalan-infected down to the family level, and then shifting to cluster with uninfected amphipods at the genus level (Fig. 1). Tests of differential abundance detected various significantly different taxa abundance and variability (dispersion) between infected and uninfected amphipods (Fig. 2, Supplementary Tables S6-S7). Specifically, the phylum Chlorofexi and the family 67–14 (of phylum Actinomycetota, class Thermoleophilia, order Solirubrobacterales) were more abundant in uninfected than in acanthocephalan-infected amphipods, and this result was repeated in two of the three methods used (corncob and LinDA, Fig. 2, Supplementary Tables S6-S7). In general, significant results in both LinDA and corncob methods showed that taxa in acanthocephalan-infected had lower abundance than taxa in uninfected and in cestode-infected amphipods. Exceptions were the family Granulosicoccaceae, which was less abundant in uninfected than in acanthocephalan-infected and cestode-infected amphipods, and the family Pseudoalteromonadaceae, which was more abundant in acanthocephalan-infected than in cestode-infected, but did not differ in uninfected amphipods (Fig. 2). The Aldex_kw method did not return any significant differential abundance results.

There were a few taxa identified only in the bacterial community of infected amphipods, regardless of the parasite infecting them (one family, namely Hymenobacteraceae, two genera, namely *Aquimarina* and an unknown genus of the family Micrococcaceae, and 20 ASVs in common between acanthocephalan-infected and cestode-infected amphipods but missing from uninfected amphipods and missing from the environmental samples (Fig. 1D, Supplementary Supplementary S8). In the second dataset (the one including parasites), we found 20 ASVs only shared among acanthocephalan-infected amphipods and acanthocephalans, of which nine were more abundant in the parasites than in their amphipod hosts (Supplementary Table S9). In addition, 10 ASVs were only found in cestode-infected amphipods and cestodes, of which four were more abundant in the cestode parasites (Supplementary Table S9).

Beta-diversity metrics considering presence-absence (Unweighted Unifrac and Jaccard) showed significant differences in the community composition of acanthocephalan-infected com-

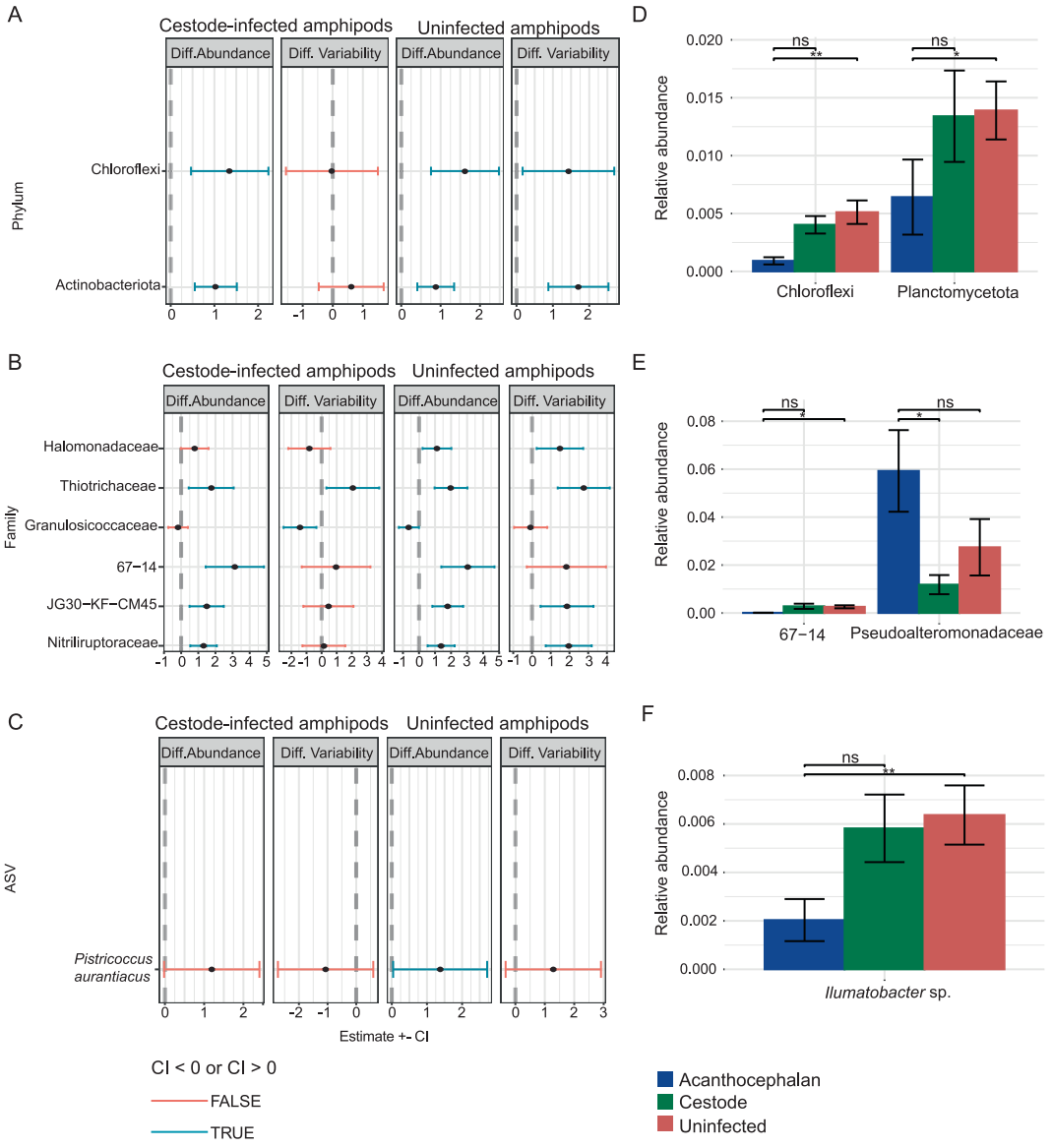

Fig. 2. Differential abundance test results at phylum (A and D), family (B and E) and amplicon sequence variant (ASV) levels (C and F). Only taxa with a significant result are shown. (A-C) Corncob results showing differential abundance and differential variability for cestode-infected compared with acanthocephalan-infected amphipods and for uninfected compared with acanthocephalan-infected amphipods. Each dot represents the model estimate and each whisker its confidence interval. Significant results are shown in blue (do not include 0 in their confidence intervals). The X axis refers to the estimates and confidence intervals. Diff. Abundance, differential abundance; Diff. Variability, differential variability (overdispersion). (D-F) LinDA results showing the relative abundance of the three infection groups (acanthocephalan-infected, cestode-infected, and uninfected amphipods). Statistical significance: *P<0.05; **P<0.01; ns, non-significant. CI, Confidence Interval. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pared with uninfected and with cestode-infected amphipods (except Jaccard at phylum rank), but no differences were found between uninfected and cestode-infected amphipods (Fig. 3A, Supplementary Supplementary S4). Thus, the main bacterial community composition differences concern acanthocephalan-infected amphipods compared with the two other groups. Presence-absence metrics also returned significant results when comparing uninfected amphipods with those infected with one acanthocephalan (except for Jaccard at phylum rank, Supplementary Table S4), and when comparing amphipods infected with one acanthocephalan with those infected with more than one (only Unweighted Unifrac at phylum rank was significant, Supplementary Table S4). No significant differences were observed between amphipods infected with more than one acanthocephalan and those which were uninfected (Supplementary Table S4). In addition, no significant beta-diversity differences were found for metrics considering taxa abundance among infected and uninfected amphipods (Bray Curtis and Weighted Unifrac, Supplementary Table S4), and the significant results are therefore based on metrics that are more sensitive to rare taxa (Jaccard and Unweighted Unifrac).

Acanthocephalan-infected amphipods had overall lower alpha diversity than cestode-infected amphipods (Fig. 3B, Supplementary Table S10). Significant differences between acanthocephalan-infected and cestode-infected amphipods were detected at the ASV level, and only for observed richness and Shannon diversity, which are metrics of taxonomic diversity (which do not account for phylogenetic diversity). Interestingly, acanthocephalan-infected amphipods were significantly different from uninfected amphipods at ASV and family levels for Faith's PD, which is a metric of phylogenetic diversity, as well as for observed richness at the family level (note that observed richness does not include abundance estimates as does Shannon diversity). In addition, slightly

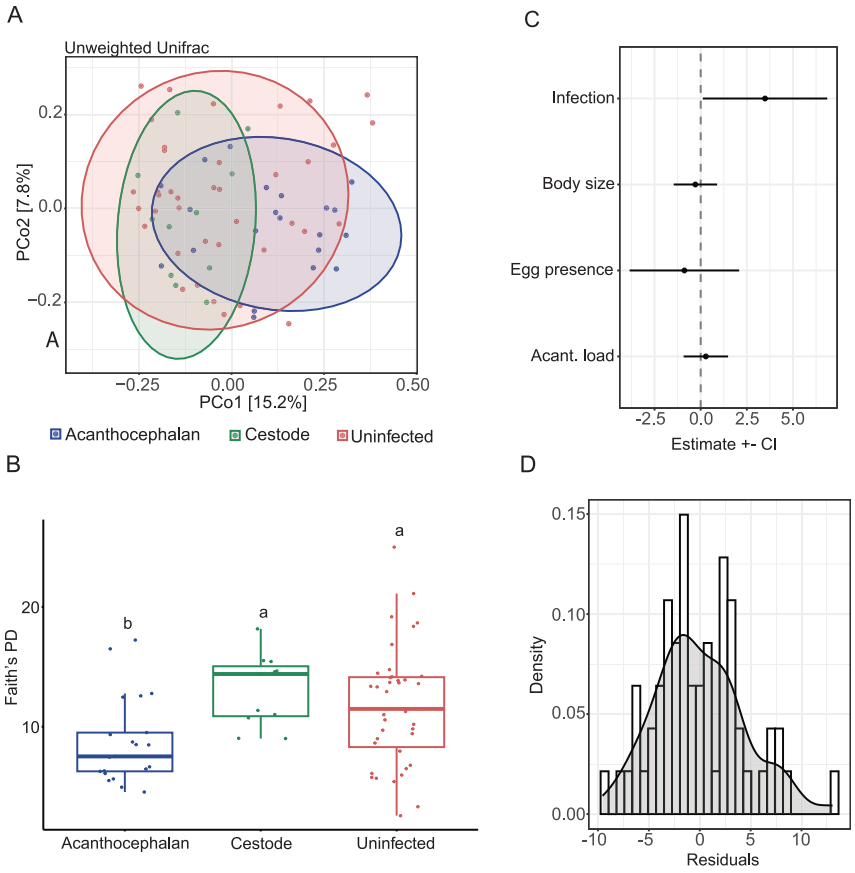

Fig. 3. Diversity comparisons among acanthocephalan-infected, cestode-infected, and uninfected amphipods and generalised linear model (GLM) results. (A) Principal Coordinate Analysis (PCoA) of beta diversity measured with unweighted Unifrac at amplicon sequence variant (ASV) level. (B) alpha diversity based on Faith's Phylogenetic Diversity (Faith's PD). The same letter (a or b) is assigned for groups having the same level of alpha diversity, and different letters for groups with different levels of alpha diversity. Significance is based on a corrected *P*-value < 0.05. (C) GLM result using Faith's PD alpha diversity at ASV level as a response variable and infection (acanthocephalan-infected, cestode-infected, and uninfected), amphipod body size, presence of eggs, and acanthocephalan load (Acant. load) as predictors. In the plot, estimates are represented as dots and their confidence intervals (CI) as whiskers. Predictors crossing the dotted line are non-significant. (D) Distribution of residuals from the GLM model shown in C.

lower Faith's PD levels were found in amphipods infected with a single acanthocephalan compared with both uninfected amphipods and those with more than one acanthocephalan (Supplementary Table S11). Finally, alpha diversity in cestode-infected amphipods only differed from uninfected amphipods at the phylum level, and only for Faith's PD (Supplementary Supplementary S10). Therefore, phylogenetic diversity was more relevant for differences between uninfected and acanthocephalan-infected or cestode-infected amphipods, while taxonomic diversity (based on richness and abundance) was more relevant for comparisons between infections (acanthocephalan-infected with cestode-infected).

The GLMs showed parasitic infection as the main factor explaining phylogenetic alpha diversity in the amphipods' hemolymph bacterial communities, while amphipod size, the presence of eggs, and the number of acanthocephalans infecting a single host (parasite load) were not significantly correlated with any alpha diversity metric (Fig. 3C-D, Supplementary Table S12). In addition, parasitic infection was also the main factor explaining richness at the family level. Lower deviance and AIC in the GLMs were observed at higher taxonomic ranks, and residuals were very close to the normal distribution, although a few Shapiro test results showed a slight deviation from normality (Shannon diversity at phylum, family, and ASV levels, and observed richness at phylum and ASV levels, Fig. 3D, Supplementary Table S12).

4. Discussion

Our results support an association of parasitic infection with the parasitised hosts' microbiomes, be it as a result of the hosts' responses to infection, a modification in host microbiomes induced by parasites, a pre-existing condition of the hosts leading to susceptibility to certain parasites, or a combination of all of the above. We observed significant differences in the bacterial communities of parasitised amphipods, both in terms of bacterial diversity, with an emphasis on rare taxa, and in the relative abundance of specific taxa. We also found significant differences in acanthocephalan-infected versus cestode-infected amphipods, supporting previous findings that changes in host microbiomes depend on the parasite identity (Ling et al., 2020; Hahn et al., 2022; Salloum et al., 2023b). The differences we observed are unlikely to have been determined by the parasitised hosts' environments, since all amphipods were collected simultaneously at the same location, and all alpha and beta diversity analyses showed a significant difference between the amphipods' bacterial communities and that of the environment. Furthermore, the diversity (richness and phylogenetic diversity) of the bacterial communities associated with hosts was more strongly correlated with parasitic infection than with other potentially microbiota-modulating factors (host size, presence of eggs and parasite load). These results support a potential role for the parasitised hosts' microbiomes in interactions with parasites.

Further investigations to unravel the power of the microbiome to modulate the evolutionary 'balance' between host resistance and parasite success is thus warranted.

Compared with amphipod size and presence of eggs, parasitic infection was the main factor explaining differences in phylogenetic diversity and taxonomic richness in their amphipod hosts, and was more relevant than parasite load. This is also supported by the significant difference in the bacterial communities of acanthocephalan-infected amphipods compared with cestode-infected and uninfected amphipods. In contrast, cestode infections seemed to affect their amphipod hosts' bacterial communities much less than acanthocephalan infections. Interestingly, the bacterial communities of acanthocephalan parasites have been found to differ from that of their amphipod hosts, but cestode parasites have similar bacterial communities to their amphipod hosts (Koellsch et al., 2024). *Plagiorhynchus allisonae* acanthocephalans have pied stilt and pied oystercatcher as definitive hosts (Bennett et al., 2023a). The dilepdid cestode is likely *Anomotaenia* sp., which has recently been described from *T. serrulata* amphipods and has red-billed and black-backed gulls as definitive hosts (Bennett et al., 2023b). Both parasites infect the intermediate amphipod hosts when their eggs are accidentally ingested; after hatching, larval parasites burrow out of the amphipod's gut to settle in its hemocoel. Larval stages of both parasites lack a digestive tract, absorbing food via diffusion. Therefore, except for the definitive host species, the life cycle of these two distantly related parasites is similar. Differences in their hosts' bacterial communities may not be due to life history differences among the two parasites, but rather to potentially different impacts acanthocephalans and cestodes have on the amphipods' microbes. In addition, there could be differences in the mechanisms underlying the exchange of microbial taxa between hosts and parasite species.

Acanthocephalan-infected and cestode-infected amphipods had many unique ASVs not shared with amphipods infected by the other parasite species, nor with uninfected amphipods or with the environment. In addition, there were ASVs only shared among parasites and their respective hosts, with approximately half being more abundant in the parasite than in the amphipod, and the other half being more abundant in the amphipod hosts than in their parasites. If we assume higher abundance as an indication of ASV origin, then it could be inferred that there is a two-way interaction in the bacterial communities of hosts and parasites: parasites are vectors of some bacteria to their hosts, while also acquiring other bacteria from them. Given the similarity in the broad bacterial community patterns of cestode-infected and uninfected amphipods, bacteria transference between hosts and parasites may not necessarily be due to large scale changes in their microbial communities and could be inherent to the bacterial taxon itself (e.g., if colonising the amphipod represents an advantage to the initially parasite-associated bacterium).

Our findings of lower alpha diversity and lower abundance of many bacterial taxa in acanthocephalan-infected amphipods indicate a large imbalance (hereafter dysbiosis) in the bacterial community associated with acanthocephalan infections. Acanthocephalans have been previously correlated with dysbiosis in fish microbiotas (Colin et al., 2022). Considering that microbiome differences in parasitised hosts are likely dependent on the host and parasite species (Ling et al., 2020; Hahn et al., 2022; Salloum et al., 2023b), it is interesting to find the same overall pattern (dysbiosis) caused by different acanthocephalan species in a definitive host (fish, Colin et al., 2022) and an intermediate host (*T. serrulata* amphipods). Other diseases in crustaceans can lead to opposite microbiota patterns (e.g., increase in crab hemolymph microbial abundance, Zhang et al., 2018). Further investigations should confirm whether host microbiota dysbiosis is characteristic of acanthocephalan infections and could be generalised to various parasite developmental stages and species of parasites and hosts. If so, we propose that a decrease in the host's microbiota alpha diversity (and lower bacterial abundance of various taxa in comparison with uninfected individuals, see Fig. 2) could serve as indicators of acanthocephalan presence, although factors other than acanthocephalan infection can lead to dysbiosis (see below).

In general, a decrease in bacterial community alpha diversity has been linked with an increase in stress (Rocca et al., 2018; Houtz et al., 2022). In humans, gut microbiome alpha diversity has been associated with development of the immune system, with lower alpha diversity resulting in lower levels of regulatory T-cells and decreased ability to survive enteric infections (Cohen and Wingert, 2023; Lubin et al., 2023). There is also evidence that infections of the central nervous system in humans are correlated with lower gut bacterial community alpha diversity, however the causal link is not clear (Grochowska et al., 2022). In animals with open circulatory systems, and specifically in crustaceans, hemolymph microbiota were shown to be sourced from the digestive system (Yang et al., 2015; Zhang et al., 2020). It was suggested that hemolymph microbiomes have roles in crabs' immune defence (Wang and Wang, 2015; Zhang et al., 2020). Thus, it is possible that the dysbiosis associated with acanthocephalan infections in these *T. serrulata* amphipods results from stress due to parasite-induced pathology. Alternatively, it is possible that acanthocephalans require a dysbiotic microbiota in their hosts for successful infection. Further investigations could assess whether acanthocephalans are able to infect non-dysbiotic hosts, or undertake trials to terminate acanthocephalan infections with probiotic therapy and restoration of a healthy bacterial community.

We found some bacterial taxa were only shared among infected amphipods (regardless of parasite identity) but not detected in uninfected amphipods or in the environment. These taxa are interesting, as some could be interacting with the parasitised hosts' defence mechanisms and have evolutionary consequences for host-parasite interactions. One example is the *Aquimarina* genus (here detected only in infected amphipods), which has taxa that produce secondary metabolites with algicidal (Chen et al., 2012) and antimicrobial activity (Dieterich et al., 2022) and are known to associate with other invertebrates (Couceiro et al., 2024). In lobsters, *Aquimarina* are pathogenic, causing damage to the pereopod (limbs) and making them more susceptible to other pathogens (Ooi et al., 2020). Here, we detected *Aquimarina* in infections with both parasites but not in uninfected amphipods. Thus, we suggest that *Aquimarina* could either be an opportunistic pathogen, taking advantage of the weakened immune system of parasitised amphipods, or the other way around (parasites would be taking advantage of *Aquimarina*-infected amphipods). Alternatively, an interesting hypothesis is that *Aquimarina* bacteria could be favoured by the presence of parasites (e.g., because of the parasite-induced microbiota imbalance, easing competition among microbes), and the parasite could benefit from the effect of *Aquimarina* on their crustacean host (e.g., weaker immune system and non-resistant hosts).

Other bacteria shared among infected amphipods (regardless of the parasite infecting them) included taxa known to associate with various animals, and that may play a role in a range of physiological and immune processes such as the stress response (e.g., *Neptuniibacter*; Diéguez et al., 2017; Wright et al., 2017), nutrient acquisition and digestion (e.g., *Rubritalea*, *Thalassotalea*, the genus *BD1-7_clade* of the family Spongiibacteraceae; Sheu et al., 2016; Holert et al., 2018; van de Water et al., 2018; Sizikov et al., 2020; Kim et al., 2020; Valdespino-Castillo et al., 2021), regulation of microbial communities (e.g., the predatory bacteria *Sericytochromatia, Peredibacter*, and the genus *OM27_clade* of family Bdellovibrionaceae; Orsi et al., 2016; Bratanis et al., 2020; Biagi et al., 2020 see also White, E., 2023. The ecology and evolution of non-

photosynthetic Cyanobacteria (Doctoral Dissertation). University of Queensland, Australia), and nitrogen fixing processes, (e.g., *Terasakiella*, the genus *SH-PL14* of the family Rubinisphaeraceae, and members of the phylum Planctomycetota, here matching the genus *BD7-11*; Winderl et al., 2008;Zani et al., 2021; Filek et al., 2022; Liu et al., 2023; Suarez et al., 2023). In particular, the presence of these potentially nitrogen-fixing bacteria in both acanthocephalan-infected and cestode-infected amphipods aligns with the finding that parasitic infection interferes with nitrogen cycling, increasing nitrogen release to the ecosystem (Mischler et al., 2016). Given the taxonomic breadth between parasites in our study (acanthocephalans and platyhelminth cestodes) and in Mischler et al. (2016) (platyhelminth trematodes), we anticipate that the previously described changes in nitrogen cycling are not (only) induced by the parasite itself, but also result from microbiome changes that impact nitrogen-fixing bacteria associated with the parasitised host. Interestingly, many of the significant differences in the bacterial community composition of acanthocephalan-infected, cestode-infected and uninfected amphipods were due to rare bacterial taxa. Although impossible to assess with the methods used here, rare microbial taxa have been found to significantly contribute to important ecosystem functions (such as nitrogen cycling) and to host defences against infection by different pathogens (Jousset et al., 2017).

In summary, our results support parasitic infection as the main factor explaining differences in their amphipod hosts' hemolymph microbiome. Parasite load was less relevant to the amphipod hosts' bacterial community richness than the presence of a specific parasite, as were amphipod size and egg presence. Various bacterial taxa related to important physiological and metabolic processes were detected in parasite-infected as opposed to uninfected amphipods. Amphipods infected with different parasite phyla that have a similar life history strategy showed differences in their associated bacterial communities, with a stronger impact of acanthocephalan than cestode infection on the hosts' bacterial communities. Many microbiome changes associated with parasitic infections are likely species-specific, but different acanthocephalan parasites may drive a common modification in the microbiome of various host species (decrease in alpha diversity). Further investigation is needed to understand whether the patterns and taxa detected in this study are also relevant in other populations and geographical locations, as well as to investigate the role of the various bacterial taxa in infected hosts, to ultimately determine how microbiome components can interact with hosts and parasites, potentially impacting their evolution.

CRediT authorship contribution statement

Célia Koellsch: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Robert Poulin:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Priscila M. Salloum:** Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis.

Data availability statement

Raw sequence reads are available in the SRA (BioProject PRJNA1070336); raw and masked images are available on Figshare (https://doi.org/10.6084/m9.figshare.25106309.v1); bioinformatics scripts, filtered data, and metadata are available on Figshare (https://doi.org/10.6084/m9.figshare.25557633).

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2024.08.005.

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