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# Different trematode parasites in the same snail host: Species-specific or shared microbiota?

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## Abstract

The concept that microbes associated with macroorganisms evolve as a unit has swept evolutionary ecology. However, this idea is controversial due to factors such as imperfect vertical transmission of microbial lineages and high microbiome variability among conspecific individuals of the same population. Here, we tested several predictions regarding the microbiota of four trematodes (*Galactosomum otepotiense*, *Philophthalmus attenuatus*, *Acanthoparyphium* sp. and *Maritrema novaezealandense*) that parasitize the same snail host population. We predicted that each parasite species would harbour a distinct microbiota, with microbial composition similarity decreasing with increasing phylogenetic distance among parasite species. We also predicted that trematode species co-infecting the same individual host would influence each other's microbiota. We detected significant differences in alpha and beta diversity, as well as differential abundance, in the microbiota of the four trematode species. We found no evidence that phylogenetically closely related trematodes had more similar microbiota. We also uncovered indicator bacterial taxa that were significantly associated with each trematode species. Trematode species sharing the same snail host showed evidence of mostly one-sided bacterial exchanges, with the microbial community of one species approaching that of the other. We hypothesize that natural selection acting on specific microbial lineages may be important to maintain differences in horizontally acquired microbes, with vertical transmission also playing a role. In particular, one trematode species had a more consistent and diverse bacteriota than the others, potentially a result of stronger stabilizing pressures. We conclude that species-specific processes shape microbial community assembly in different trematodes exploiting the same host population.

KEYWORDS
bacterial exchanges, community similarity, microbiota, parasite, snail, trematode

## 1 | INTRODUCTION

The classical and simplified view of phenotypes as a combination of additive genetic effects and random environmental effects is slowly shifting to incorporate the impact of microbiomes on phenotypic diversity (Bruijning et al., 2022; Peixoto et al., 2021). Even though quantification of phenotypic variability attributable to microbiomes is challenging, there is strong evidence in support of different microbiomes being associated with different phenotypes within the same species or population (Jorge, Dheilly, Froissard, & Poulin, 2022; Kapheim et al., 2015; Takacs-Vesbach et al., 2016). In general, microbiomes can be highly variable among individuals of the same species, but microbiotas are unlikely to simply be a random assemblage of the available microbes in the organism's environment (Hahn et al., 2022; Jorge, Dheilly, Froissard, Wainwright, & Poulin, 2022; Jorge, Froissard, Dheilly, & Poulin, 2022; Salloum et al., 2023). In addition, mathematical modelling has shown that it is plausible for selection to act on both horizontally and on vertically acquired microbiota (Roughgarden, 2020).

With respect to the main sources of microbes, organisms may acquire microbes from their diet and environment (horizontal transmission), from their parents (vertical transmission) or a combination of the two (Candela et al., 2012; David et al., 2014; Ebert, 2013). However, for parasites with many life stages, horizontal microbial transmission can have multiple sources, such as the different hosts throughout their life cycle, as well as the environment (e.g. in free-swimming, infecting stages), and even co-infecting parasites within the same host individual (Jorge, Dheilly, Froissard, Wainwright, & Poulin, 2022). In addition, parasites such as trematodes transition through a complex life cycle and may acquire microbes vertically from previous life stages, and thus possibly also vertically across generations (Jorge et al., 2020).

Here, we compared the microbial community composition of the same life stage of four different digenean trematode species infecting the same snail host species in the same environment to test whether common microbiota patterns or microbial exchanges occur among different parasites within the same host. We hypothesize that if the four different trematode species have highly variable microbial communities with no significant differences, then this is likely the result of a random assemblage of the available microbial pool in their host or the environment, and thus natural selection is unlikely to have shaped their respective microbiota. Alternatively, if the different trematode species possess distinct microbial communities, this could be evidence of (i) evolutionary associations maintained by vertical inheritance, in which case we may expect decreasing similarity between the microbial communities of any two trematode species with increasing phylogenetic distance between these parasites; or (ii) trematodes with similar physiologies horizontally acquire and maintain similar microbes. Furthermore, we interrogate whether there is a more similar microbiota between parasites co-infecting the same individual host by characterizing the microbiota of two trematode species in single- and co-infected hosts. We hypothesize that microbial exchanges occur between co-infecting trematodes and that specific characteristics of the various parasite species (e.g. mouth-feeding vs. diffusion-feeding) result in asymmetrical exchanges, such that the microbiota of a parasite that feeds on co-infecting parasites becomes more similar to that of the consumed species.

The model host–parasite system used here involves *Zeacumantus subacarinatus* mud snails, which serve as the first intermediate hosts of the trematodes *Galactosomum otepotiense*, *Philophthalmus attenuatus*, *Acanthoparyphium* sp. and *Maritrema novaezealandense* (Martorelli et al., 2004, 2006, 2008). *Zeacumantus subcarinatus* snails are also intermediate hosts of other trematode species (Leung et al., 2009; Martorelli et al., 2006), but these were not included in our study due to low prevalence/absence in the snails we collected. Importantly, the first three trematode species above develop into rediae within the snail host, whereas *M. novaezealandense* develops into sporocysts. Rediae possess a mouth and can actively feed on host tissue as well as kill and ingest other co-infecting trematodes (Leung & Poulin, 2011; Sousa, 1992), whereas sporocysts have no mouth and can only passively absorb nutrients. Adults of these trematodes live in gulls and other shore birds, in which they reproduce sexually and release their eggs in host faeces or bodily fluids (Leung et al., 2009). After accidental ingestion by the mud snail, a single egg hatches into a larva that undergoes many rounds of asexual multiplication, giving rise to a large colony (of either rediae or sporocysts) occupying 30%–40% of the inside of the snail shell, and producing cercariae, i.e., the next infective stage in the life cycle (Fredensborg et al., 2005; Hechinger et al., 2008). Often, a trematode may infect a snail already harbouring a colony of another trematode species; in such cases, co-infection ensues, with the two colonies existing in physical contact and competing over months or years for space within the snail host (Lloyd & Poulin, 2012).

Here, we use this model system to test the predictions that: (i) different trematode species have distinct microbial communities even if they share the same host species and are sampled at the same time and place; (ii) the differences in the composition of their microbial communities will increase with greater phylogenetic distance among the trematode species; (iii) microbial exchanges and sharing occurs between pairs of trematode species sharing the same individual snail, which would be indicated by a more similar microbiota than in single infections and (iv) these exchanges will be asymmetrical, with species having rediae (mouth-feeding) acquiring more microbes from species with sporocysts (non-mouth-feeding) than the other way around.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample collection, processing and sequencing

All equipment used in field trips and sample processing was sterilized with bleach (1:10 dilution, soaking overnight). In March 2022, *Z. subcarinatus* snails were collected at low tide in Lower Portobello Bay, Dunedin, New Zealand (45° 4,904,800 S, 170° 4,001,200 E), and

placed alive in sterile containers with seawater from the collection site. In addition, cotton swabs were used to take environmental samples (two swabs of the sand substrate and two of water) and two negative controls (swabs exposed to air for 5 s), which were stored in PowerBead tubes (QIAGEN) and kept in dry ice until storage at −70°C upon arrival at the laboratory. Two additional environmental samples were taken prior to cercarial shedding, from the seawater of the containers where the collected snails were kept in the laboratory. To identify infected snails, cercarial shedding was induced by placing the snails in individual wells of sterile culture plates with seawater from the collection site and incubating at 25°C for 24 h. Plates were then screened under a dissecting microscope, released cercariae were identified based on morphology and snails infected with *Philophthalmus attenuatus*, *Maritrema novaezealandense*, *Acanthoparyphium* sp. and *Galactosomum otepotiense* were sorted into separate sterile containers and kept alive in aerated seawater from the collection site until further processing.

Dissections were carried out under a laminar flow hood with UV- and heat-sterilized equipment. To remove potential epibionts and external contamination, each snail was placed in a Petri dish with 70% ethanol and the shell was brushed with interdental brushes. This process was repeated a second time in another Petri dish, and dissections were carried out in a third Petri dish containing sterilized PBS. The shell was broken with flat pliers, and five cercariae-producing rediae/sporocysts were randomly removed with a pipette and placed in a culture plate well containing PBS. To remove external contamination, barrier tips were used to pipette rediae/sporocysts up and down in PBS, a process repeated a second time in another well before placing the larvae in a third well and using fresh barrier tips to collect and release the larvae into a PowerBead tube (QIAGEN). A small piece of snail organ tissue adjacent to the parasitic infection was collected and subjected to the same process as the parasites. Collected trematodes and snail tissue were stored at −70°C until DNA extraction. Negative controls (blanks) include the PBS solution (one for each bottle, total of three bottles), the third PBS wash of trematode and snail tissue (randomly taken during processing as a control for external contamination, one for the snail tissue and one for each trematode species, totalling eight controls) and two swabs exposed to the laboratory environment for 5 s.

DNA extraction and library preparation were carried out as in Jorge et al. (2020) including the use of ZymoBIOMICS microbial community standards, but amplicons were purified using AMPure at a ratio of 0.8 solution to PCR product. All samples were multiplexed and sequencing was carried out targeting the V4 hypervariable region of the bacterial 16S SSU rRNA gene with the primers 515F-806R (Apprill et al., 2015; Parada et al., 2016) using an Illumina MiSeq platform and v3 reagent cartridge (250 bp, paired-end) at the Otago Genomics & Bioinformatics Facility. Raw sequencing reads were deposited in SRA (BioProject PRJNA972185).

## 2.2 | Bioinformatics and statistical analyses

De-multiplexed sequences were quality checked using FastQC v0.11.9 (Andrews, 2010), following which adaptors, primers and overrepresented sequences were removed with the *cutadapt* plugin (Martin, 2011) implemented in QIIME2 v2021.4 (Bolyen et al., 2019), with 0 error rate and minimum length of 240 bp. Sequences were forward- and reverse-trimmed by 13 bp and denoised using the *dada2* plugin in QIIME2 (Callahan et al., 2016). To assign taxonomy, we trained the SILVA database version 138.1 targeting the region SSURef_NR09 (Quast et al., 2013) on our dataset using the Naïve Bayes classifier in QIIME2 and the following parameters: sequences minimum length of 900 bp for Archaea, 1200 bp for Bacteria and 1400 bp for Eukaryota, dereplicated with the default *uniq* mode, using the forward primer sequence GTGYCAGCMGCCGCGGTAA and reverse primer sequence GGACTACNVGGGTWTCTAAT. Feature tables were filtered to remove contamination (all features found in blanks), mitochondria, chloroplasts, eukaryotes and features without a phylum assignment. Our contamination filtering may lead to discarding sequences from the dataset that were found in blanks and were not contaminants (e.g. due to sample cross-contamination), but this conservative approach was taken to avoid spurious signals in downstream analyses. Rarefaction curves (with a maximum depth of 4000) were used to define depth filters by comparing alpha diversity metrics (Faith's PD, Shannon Diversity, and Observed Features) at different depths and defining a cut-off where the increase in diversity levelled off in relation to depth for parasite and snail samples (Figure S1). Data quality was evaluated in QIIME2 by comparing the observed composition of ZymoBIOMICS microbial community standards against their expected composition (before filtering). The resulting filtered feature table was generated with a minimum total feature frequency of 500 and features with a minimum frequency of 2, excluding nine samples from the dataset. Taxonomy was assigned based on the trained SILVA database using the *feature-classifier* plugin with *sklearn* mode in QIIME2; amplicon sequence variants (ASVs) were aligned with MAFFT (Katoh & Standley, 2013) using the *phylogeny* plugin in QIIME2 and rooted and unrooted phylogenetic trees were built with FastTree2 (Price et al., 2010). QIIME2 filtered output files were loaded into R v4.1.3 (R-Core-Team, 2022) using the qiime2R package v0.99 (function *qza_to_phyloseq*) and the file2meco package v0.4.0 (function *qiime2meco*) (Bisanz, 2018; Liu et al., 2021). Phyloseq v1.38.0 (McMurdie & Holmes, 2013) was used to group ASVs into higher taxonomic ranks (family, order, and phylum). All analyses were undertaken with the package microeco v0.11.0 (Liu et al., 2021) unless otherwise specified.

Variation in microbial species diversity within samples (alpha diversity) and (dis)similarities of microbial communities among different samples (beta diversity) were estimated among trematode species, among snails parasitized by different trematodes, among trematode–snail host pairs, between trematodes in co-infection with another trematode species vs. single infections, and among the environment, snails and trematodes. Alpha and beta diversity were analysed over rarefied data (rarefied to even depth of 500) using the functions *call_diff* and *cal_betadiv* at phylum, order, family, and ASV ranks. The alpha diversity metrics assessed were observed richness, Shannon diversity and Faith's PD, and statistical significance was based on analyses of variance (ANOVAs) among

groups. The beta diversity metrics assessed were Bray–Curtis, Jaccard, weighted and unweighted Unifrac, and statistical significance was based on permutational multivariate ANOVAs (perMANOVAs run with the default configurations of 999 permutations and FDR correction for multiple testing). In addition, we calculated individual pairwise distances in microbiota composition among trematodes of the same or different species with the *cal_group_distance*, for the same metrics used in the beta diversity analyses (Bray–Curtis, Jaccard, weighted, and unweighted Unifrac). Pairwise phylogenetic distances between trematode species (based on available partial COI sequences, accessions FJ765457, FJ765485, FJ765489, and FJ765472; Leung et al., 2009) and families (based on available partial 28S sequences, accessions AY220630, AY222227, AY222248, and KT956940; Olson et al., 2003) were calculated with the function *cophenetic.phylo* of the ape v5.0 package (Paradis & Schliep, 2019), aligned with MAFFT v7.505 with default configurations (Katoh et al., 2002) and used in an unrooted neighbour-joining tree estimated with the *nj* function in the ape package. Association between phylogenetic and microbiota distances (phylosymbiosis) was tested using Mantel tests with 9999 permutations (*mantel.randtest* function in the package ade4 v1.7–20; Dray & Dufour, 2007) and Spearman correlation (*pairs.pannel* function of the package psych v2.2.9; Revelle, 2018) as the variables were not normally distributed (see histograms, q–q plots and Shapiro–Wilk results, Figures S2 and S3, Table S1).

Taxon abundance was calculated with non-rarefied data at phylum, order, and family ranks, for parasite species and their snail hosts. Venn diagrams at ASV and family levels were used to summarize the number of unique and shared ASVs/taxa among trematodes, snail hosts, and the environment. Non-rarefied data were also used in tests of differential abundance to identify taxa driving microbial community differences among all trematode species, in pairwise comparisons between trematode species and between trematodes and their snail hosts, at phylum, order, and family ranks. There are various approaches to test for microbial differential abundance, all of which are known to return different results depending on factors such as features of each method (e.g. input data requirements, data transformation, distribution models) and of the data (e.g. sparsity, effect size between conditions, depth of sequencing) (Cappellato et al., 2022; Nearing et al., 2022; Paulson et al., 2013). Thus, to attain a more thorough evaluation of the data, the methods used for differential abundance analyses were ALDEx2_kw (Fernandes et al., 2014), for which benchmarking studies show an above-average performance when compared with a number of other methods (Nearing et al., 2022; Yang & Chen, 2022) and the corncob v0.3.0 package (Martin et al., 2022), which enables estimation of differential abundance and variability (i.e. overdispersion) simultaneously, and has also featured as a good option in benchmarking studies (Nearing et al., 2022; Yang & Chen, 2022). Per-taxon differential abundance including low-prevalence taxa was tested with the method metastat (White et al., 2009), which handles sparse samples using Fisher's exact test and corrects for multiple testing with the False Discovery Rate. Finally, for each parasite species and for the snails infected by them, indicator taxa in the microbiota were searched with the package indicspecies v1.7.12 (De Caceres & Legendre, 2009). These indicators are bacterial taxa associated with particular parasite species, such that the parasite species is inferred based on the bacterial taxa it associates with. The indicator species analysis was run using an abundance table (count of ASVs) and the function *combinespecies* for a combined search among all species (max.order = 2, default 999 permutations to test significance).

Due to the low prevalence/absence of co-infection involving different combinations of the four trematode species in the snails we sampled, the only pair of co-infecting trematodes assessed was *Maritrema* and *Philophthalmus* (found in six snails, in addition to the ones single-infected by either *Maritrema* or *Philophthalmus*). For all analyses, significance was based on a $p$-value ≤ .05 accounting for multiple tests based on the False Discovery Rate. Scripts, metadata and filtered data are available from FigShare (Salloum, 2023 [dataset]).

## 3 | RESULTS

The filtered dataset consisted of 138 samples and 7065ASVs, ranging in coverage from 511 to 290,870 (mean = 10,074; SD = 31,439). Of these, six were environmental samples (5,058 ASVs, mean coverage = 103,861; SD = 76,786), 69 were snail hosts (1,060 ASVs, mean coverage = 5,001; SD = 4,836) and 63 were parasites (963 ASVs, mean coverage = 3,554; SD = 3,040). Of the latter, 22 were single-infecting *Maritrema* (307 ASVs, mean coverage = 3,824; SD = 2,274), 12 were single-infecting *Philophthalmus* (232 ASVs, mean coverage = 685; SD = 194), 10 were single-infecting *Galactosomum* (170 ASVs, mean coverage = 4,058; SD = 1,881) and 9 were single-infecting *Acanthoparyphium* (124 ASVs, mean coverage = 6,126; SD = 3,334). Of the six sampled snails with co-infection of *Maritrema* and *Philophthalmus*, five snails passed filters (117 ASVs, mean coverage = 7,835; SD = 8,529), the six co-infecting *Maritrema* (189 ASVs, mean coverage = 4,940; SD = 5,500) and four of the six co-infecting *Philophthalmus* (100 ASVs, mean coverage = 1,549; SD = 776). No co-infections of other pairs of species were sampled (see methods). Unfiltered negative controls ranged in coverage from 363 to 35,570 (mean = 16,101, SD = 10,161). According to the Mock Community Standard quality control analyses, results at class level and above were highly accurate, but analyses below order level should be interpreted with caution due to the decline in taxon accuracy rate (Figure S4).

The microbial composition of trematodes and snails was significantly different from that of the environment, as supported by all alpha and beta diversity analyses at all taxonomic ranks considered (Figure 1, Tables S2 and S4). The Venn diagrams show many ASVs and bacterial families unique to each trematode species which were not present in the snail hosts or the environment (Figure 2); even though the pool of all trematode species shared 93 ASVs (0.9% of the total) and 47 families (8.2% of the total) with the pool of snails (Figure 2a,b), only 2 ASVs and 28 families were shared among all trematode species and the snails (Figure 2c,d).

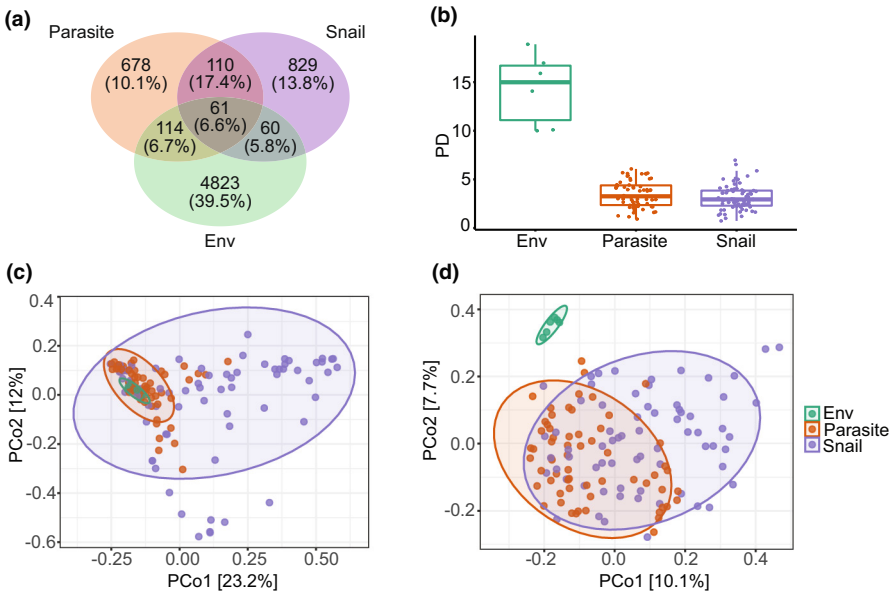


**FIGURE 1** Microbiota richness and composition of the environment, parasites and snails. (a) Venn diagrams based on number of ASVs unique or shared among groups (parasites and snails include co-infections). Percentages are the ratio sequences:total sequences; (b) Alpha diversity at family level, based on Faith's PD; (c) PCoA of weighted Unifrac beta diversity metric, at family level; (d) PCoA of unweighted Unifrac beta diversity metric, at family level. "Env", environment; "Snails", all snail hosts pooled; "Parasites", all trematode species pooled.

## 3.1 | Contrasting the microbiotas of the four trematodes and their hosts in single infections

The abundance of bacterial phyla, orders and families differed among parasite trematodes (Figure 3, Figures S3 and S4). Based on the bar plots of relative abundance, the most prevalent bacterial phyla were the same in the microbiota of the four trematode species (Proteobacteria, Firmicutes, Actinobacteriota and Bacteroidota). Tests of differential abundance returned significant results among the four parasite species at all taxonomic ranks assessed (Tables S5 and S6), as described for each parasite below, together with indicator taxa results (Tables S7 and S8). Details for differentially abundant bacteria and indicator taxa of parasites are provided in the Supplementary Material (Box S1). Indicator taxa had high specificity (probability that a parasite individual belongs to a specific trematode species when the microbial indicator is found), associated with high predictive value of the bacterial taxa as indicator of a particular parasite species. However, the sensitivity (probability of finding the indicators in trematode individuals) was relatively low, with a maximum of 60% for *Halarcobacter* and for *Pseudoalteromonas* in *Galactosomum* trematodes, and lower for other indicator-parasite combinations. No indicator was found for *Maritrema*. Indicator taxa were also found in snails infected by different trematode species (except by *Maritrema*), also with high specificity and low sensitivity (Table S7).

### 3.1.1 | *Galactosomum*

The phylum Campylobacterota occurred at significantly higher abundance in *Galactosomum* than in other trematodes, and *Halarcobacter*, a bacterial genus of this phylum, was detected as an indicator for *Galactosomum* (Figures S6 and S7, Tables S6–S8). Proteobacteria were in higher abundance in *Galactosomum* than in *Philophthalmus*, and the latter had the lowest abundance of Proteobacteria among the four trematodes (Table S6).

At order rank, Enterobacterales (phylum Proteobacteria) were detected in higher abundance by the three methods used, and also as an indicator of *Galactosomum* (families Vibrionaceae, Pseudoalteromonadaceae, Granulosicoccaceae, Alteromonadaceae and Rhodobacteraceae, Tables S5, S7, and S8). Mycoplasmatales (and the family Mycoplasmataceae), which were present in other trematodes, were absent from *Galactosomum* (Figures S5 and S6, Table S12).

At family rank, *Galactosomum* had significantly higher abundance of Vibrionaceae (order Enterobacterales), in agreement with this family's detection as an indicator for this trematode (Figure 3, Tables S5, S7, S8, and S13). Pseudoalteromonadaceae and Crocinitomicaceae were only found in this trematode and were indicator taxa of this species (Figure 3, Tables S8 and S13). This is the family of an indicator taxon detected at species level (*Lishizhenia caseinilytica*, phylum Bacteroidota; Table S7). Rhodobacteraceae were more abundant in *Galactosomum* than in *Philophthalmus* and detected as an indicator of *Galactosomum*, both in single occurrence and in association with other bacterial taxa (Table S7). Arcobacteraceae were more abundant in *Galactosomum* than in the other trematode species, and more abundant in *Galactosomum* than in its snail host (Tables S8 and S13).

Fokiniaceae bacteria were prevalent in snails infected by *Galactosomum*, and the snail hosts had higher abundance of this bacterial family than their infecting parasite (Figure 3, Tables S8 and S13). Some indicator taxa of *Galactosomum*-infected snails compared with other snails are also indicators of this trematode when compared with other trematode species (families Vibrionaceae, Pseudoalteromonadaceae, Alteromonadaceae and Rhodobacteraceae, the genus *Halarcobacter* and the species *Lishizhenia caseinilytica*, Table S7).

### 3.1.2 | *Philophthalmus*

At phylum rank, there were significantly less Proteobacteria in *Philophthalmus* than in other trematode species (Table S5). Bacteroidota were found as less variable in *Philophthalmus* than in other trematode species (Table S5).

At order rank, *Philophthalmus* had more Rhodobacterales than other trematodes (Table S12). Within this order, the family Rhodobacteraceae was found as an indicator of *Philophthalmus*, and was significantly more abundant and less variable in this trematode than in others (Tables S5, S7, S8, and S13). Rhodobacteraceae were also more abundant in *Philophthalmus* than in its infected snail hosts (Tables S5, S8, and S13). Another family with higher abundance in *Philophthalmus* than in other trematodes (*Galactosomum* and *Acanthoparyphium*) was Microtrichaceae (Actinobacteriota phylum), also an indicator of *Philophthalmus* (Tables S7, S8, and S13).

Snails infected by *Philophthalmus* were found with more Firmicutes than snails infected with other trematodes and had the second highest variability of this bacterial phylum (second to *Maritrema*, Table S5). *Philophthalmus*-infected snails also had a higher prevalence of Fokiniaceae bacteria (similar to *Galactosomum*-infected snails), which belong to the Rickettsiales order (Proteobacteria phylum), with more Fokiniaceae than their infecting trematodes (Figure 3, Table S13). Mycoplasmataceae were found in higher abundance in snails infected by *Philophthalmus* (and those infected by *Maritrema*) than in infections by other trematodes, and the genus *Mycoplasma* was an indicator taxon of *Philophthalmus*-infected snails when associated with Alphaproteobacteria, but not of the trematode (Table S7). The Mycoplasmataceae family was also abundant

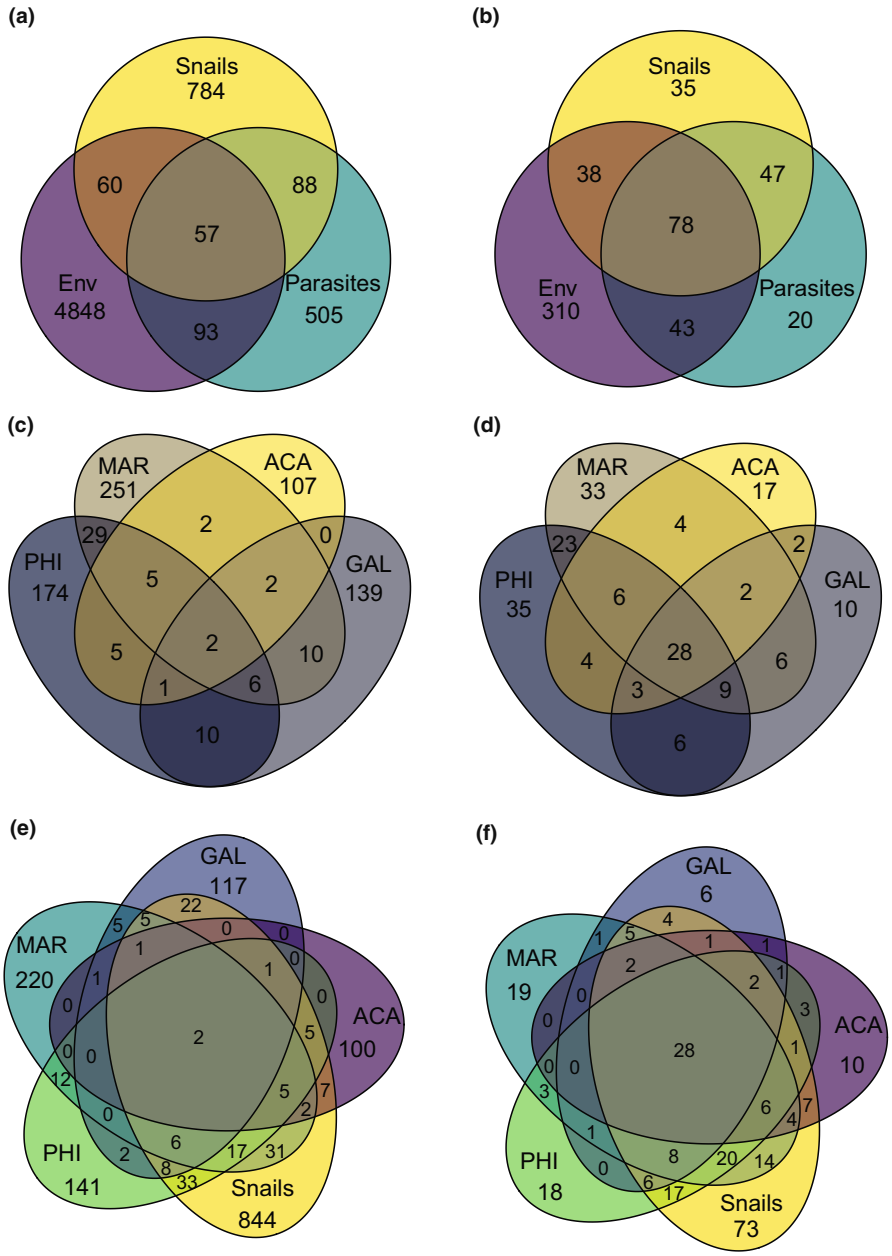


**FIGURE 2** Venn diagrams at ASV (left column) and family (right column) levels, excluding co-infections; (a) Unique and shared ASVs among trematodes, snails and the environment (Env); (b) Unique and shared bacterial families among trematodes, snails and the environment (Env); (c) Unique and shared ASVs among the four trematode species; (d) Unique and shared bacterial families among the four trematode species; (e) Unique and shared ASVs among the four trematode species and snails; (f) Unique and shared bacterial families among the four trematode species and snails. ACA, *Acanthoparyphium*; GAL, *Galactosomum*; MAR, *Maritrema*; PHI, *Philophthalmus*.

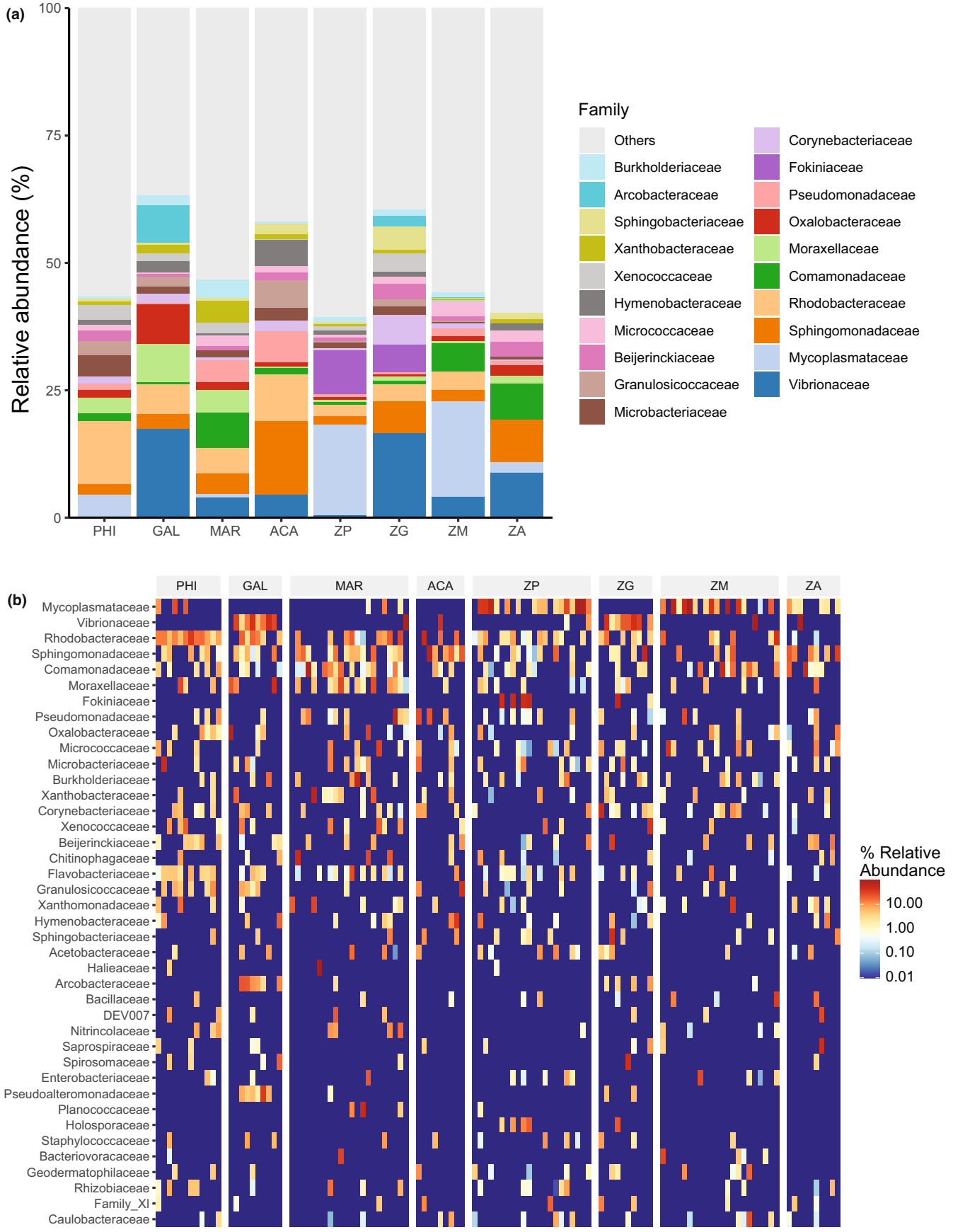

**FIGURE 3** Taxonomic composition of parasites and snails. (a) Bar plots of taxonomic composition including the 20 most abundant families, pooled across individuals based on mean relative abundance; (b) Heat map showing the relative abundance of the 40 most prevalent bacterial families in parasites and snails. ACA, *Acanthoparyphium*; GAL, *Galactosomum*; MAR, *Maritrema*; PHI, *Philophthalmus*; ZA, *Acanthoparyphium*-infected *Zeacumantus* snails; ZG, *Galactosomum*-infected *Zeacumantus* snails; ZM, *Maritrema*-infected *Zeacumantus* snails; ZP, *Philophthalmus*-infected *Zeacumantus* snails.

in *Maritrema*, *Philophthalmus* and *Acanthoparyphium* trematodes (Table S13).

### 3.1.3 | *Acanthoparyphium*

At phylum rank, Proteobacteria was found to be significantly more abundant in *Acanthoparyphium* than in other trematode species (Table S5). Within this phylum, the family Sphingomonadaceae was more abundant in *Acanthoparyphium* than in other trematodes and was also detected as an indicator of this trematode species and of its snail hosts (Figure 3, Tables S8 and S13). Another Proteobacteria indicator of *Acanthoparyphium* is of the family Beijerinckiaceae (*Methylobacterium-Methylorubrum*), which was more abundant than in *Galactosomum*, but less so than in *Philophthalmus* trematodes and *Acanthoparyphium*-infected snails (Tables S7, S8, and S13). The family Comamonadaceae was found in higher abundance in *Acanthoparyphium*-infected snails (followed by *Acanthoparyphium* trematodes) than in the other infections and trematode species (Table S13).

### 3.1.4 | *Maritrema*

At phylum rank, Proteobacteria had higher variability in *Maritrema* than in other trematode species and higher abundance than in *Philophthalmus* trematodes (Table S5). The phylum Firmicutes had the second highest abundance in *Maritrema*-infected snails (highest abundance in *Philophthalmus*-infected snails) and the highest variability in *Maritrema*-infected snails (Table S5). Firmicutes were also more abundant in *Maritrema* than in *Galactosomum* and *Acanthoparyphium* (Table S6). Within Firmicutes, the Mycoplasmataceae family had a higher abundance in *Maritrema*-infected snails than in the other trematode infections (Table S13), followed by the abundance of this family in *Maritrema*, *Philophthalmus*, and *Acanthoparyphium* trematodes.

The microbiota of each of the four trematode species also differed in terms of richness, returning significantly different alpha diversity estimated from all used metrics at order and family ranks (Figure 4a, Table S2). In particular, *Philophthalmus* harboured the microbiota with higher alpha diversity of the four trematodes (Figure 4a). Pairwise comparisons between a trematode species and the snails infected by that species supported differences in terms of richness (Shannon diversity) for *Maritrema* vs. its snail hosts and for *Philophthalmus* vs. its snail hosts, but not for the other two trematode species or for other alpha diversity metrics (Table S3). Snails infected with different trematode species had a similar level of alpha diversity in their microbiota (Figure 4b, Table S2).

In terms of beta diversity, there was no correlation between differences in microbial composition and phylogenetic distances between parasite species/families (no phylosymbiosis, Figure S7, Table S9). Significant beta diversity differences among the microbial communities of parasite species were found in all metrics at order and family ranks (except for Bray–Curtis distances between *Maritrema* and *Acanthoparyphium*, Table S4). Family-level unweighted Unifrac distances involving *Acanthoparyphium* were larger than distances between any other pair of trematodes, and *Galactosomum* was closer to *Philophthalmus* than to the other species (Figure 4c). However, at phylum level, the only significant beta diversity result was in the comparison between *Galactosomum* and *Philophthalmus* (Bray–Curtis distance, Table S4). The microbiota of snails infected with different parasite species also had compositional differences, as indicated by significant beta diversity results for at least two metrics at order and family levels (Table S4). Family-level unweighted Unifrac distances were larger between snails infected with *Galactosomum* and *Maritrema*, and distances were also large between *Acanthoparyphium*-infected and *Philophthalmus*-infected snails (Figure 4d). Overall, differences among trematode microbial communities were more pronounced than among snails infected by different trematodes (Figure 4c–f). Pairwise beta diversity analyses between snails and their infecting trematodes supported differences in the microbial composition of *Maritrema*, *Philophthalmus*, *Acanthoparyphium* and their snail hosts (Table S4). *Galactosomum* had a unique microbial community composition when compared with the other trematodes, but its microbial community was similar to that of its snail host (non-significant results for all beta diversity metrics at all taxonomic ranks tested, Table S4).

## 3.2 | Co-infections

Co-infecting *Maritrema* had a microbial community more similar (in terms of alpha and beta diversity) to *Philophthalmus*, largely differing from the microbiota of *Maritrema* in single infections (Figure 5). This pattern was supported by all beta diversity metrics at order, family, and ASV ranks (except for unweighted Unifrac at family rank, Table S10), and by all alpha diversity metrics at all taxonomic ranks assessed (except Shannon diversity at phylum level, Table S2). In terms of alpha diversity, co-infecting *Maritrema* had higher richness in their microbiota than *Maritrema* in single infections, approximating the richness levels of *Philophthalmus* (Figure 5b). Beta diversity of *Philophthalmus* in co-infections with *Maritrema* did not differ from *Philophthalmus* in single infections (Figure 5d,e, Table S8). However, the unweighted Unifrac distance between co-infecting *Philophthalmus* and co-infecting *Maritrema* was smaller than the distance between *Maritrema* and *Philophthalmus* in single infections (Figure 5e).

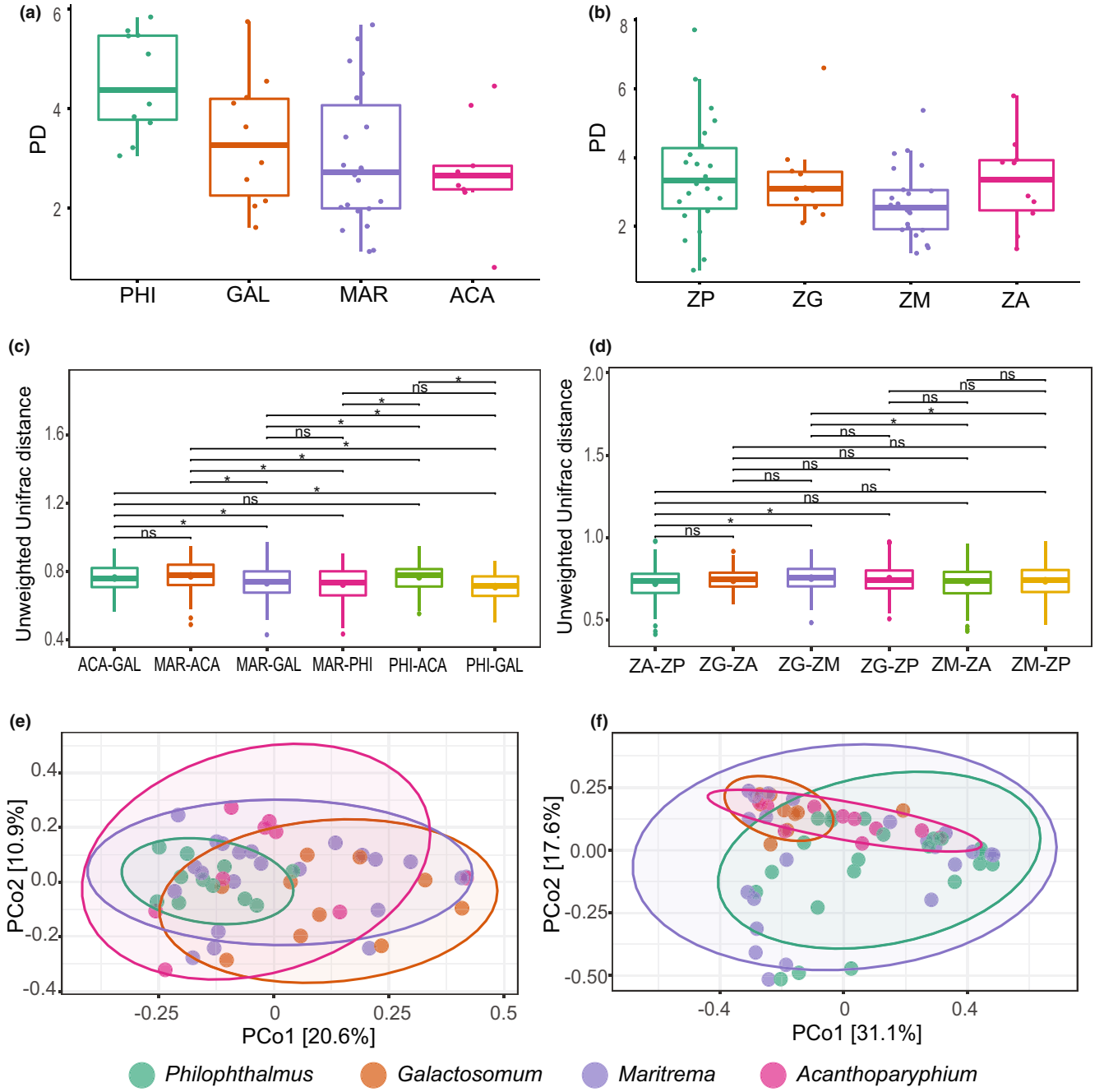


FIGURE 4 Alpha and beta diversity of parasites' microbiota and those of their snail hosts at family taxonomic level. (a) Faith's PD alpha diversity among different parasite species; (b) Faith's PD alpha diversity among snails infected by the different parasites (c) Unweighted Unifrac pairwise distances between parasite species; (d) Unweighted Unifrac pairwise distances between snails infected by the different parasites; (e) PCoA of weighted Unifrac distances among parasite species; (f) PCoA of weighted Unifrac distances among snails infected by different parasites. ACA, *Acanthoparyphium*; GAL, *Galactosomum*; MAR, *Maritrema*; PHI, *Philophthalmus*; ZA, *Acanthoparyphium*-infected *Zeacumantus* snails; ZG, *Galactosomum*-infected *Zeacumantus* snails; ZM, *Maritrema*-infected *Zeacumantus* snails; ZP, *Philophthalmus*-infected *Zeacumantus* snails. Significance codes: "*", significant (corrected $p$-value < .05); "ns", non-significant.

Venn diagrams showed 10 bacterial families shared between co-infecting *Maritrema* and *Philophthalmus*, three of which are not present in the snails (Figure 5a). There were shared families among single-infecting *Maritrema* and single-infecting *Philophthalmus*, but sample sizes for single-infecting trematodes were larger than for co-infecting ones. Differences in the microbiota of co-infecting parasites compared to single infections (Figure 5b, Figures S8 and S9) were further supported by tests of differential abundance (Tables S5 and S11). At phylum rank, Deinococcota were less abundant in single-infecting *Maritrema* than in co-infecting *Maritrema*, while Bacteroidota were more abundant in single-infecting *Maritrema*, and Actinobacteriota and Proteobacteria were more variable in

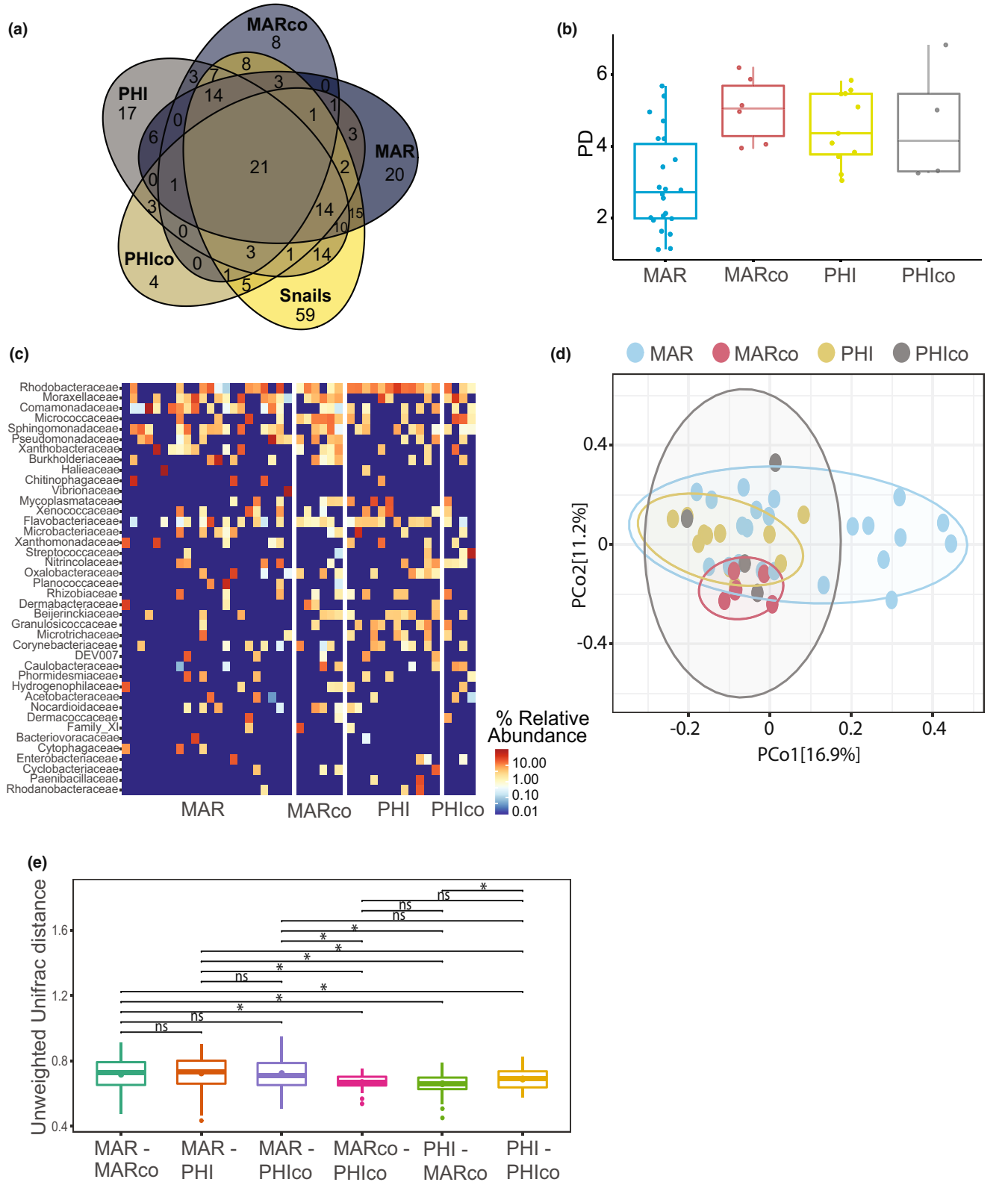


**FIGURE 5** Comparison of *Maritrema* and *Philophthalmus* microbiota in single and co-infections. (a) Venn diagram at family level, showing the number of unique and shared taxa among groups; (b) Faith's PD alpha diversity for parasites in single and co-infections, at family taxonomic level; (c) Heat map showing relative abundance of the 40 most prevalent bacterial families of parasites in single and co-infections; (d) Unweighted Unifrac distances among parasites in single and co-infections, at family taxonomic level; (e) PCoA of weighted Unifrac distances among single and co-infecting *Maritrema* and *Philophthalmus*. MAR, single infecting *Maritrema*; MARco, co-infecting *Maritrema*; PHI, single infecting *Philophthalmus*; PHIco, co-infecting *Philophthalmus*. Significance codes: "*", significant (corrected *p*-value < .05); "ns", non-significant.

single-infecting *Maritrema* (Table S5). The phylum Bdellovibrionota was more variable in single-infected snails than in co-infected snails (Table S5).

At order rank, Chitinophagales (phylum Bacteroidota) was more abundant and more variable in single-infecting *Maritrema*, and Flavobacteriales (phylum Bacteroidota), Pseudomonadales (phylum Proteobacteria), Burkholderiales (phylum Proteobacteria), and Micrococcales (phylum Actinobacteriota) were more variable in single-infecting *Maritrema* (Table S5). Within Micrococcales, the Micrococcaceae family was more abundant in single-infecting Maritrema (Table S5). The family Erwiniaceae (order Enterobacterales) was also found in higher abundance in single-infecting *Maritrema* (Table S13).

Single-infected snails had more Rhizobiales (Proteobacteria phylum) and Verrucomicrobiales (Verrucomicrobiota phylum) and were more variable in these two orders as well as in Bacteriovoracales (Bdellovibrionota phylum, Table S5). Within the latter, the family Bacteriovoracaceae had higher variability in single- than in co-infected snails (Table S5). No differential abundance test returned a significant result when comparing single-infecting and co-infecting *Philophthalmus* (Tables S5 and S11).

## 4 | DISCUSSION

As research into parasite microbiomes increasingly seeks to elucidate their impact on host–parasite interactions and disease severity (Dheilly et al., 2017, 2019), whether different parasite species from the same higher taxon have distinct microbial communities remains unclear. Here, we compared the microbial community of four different trematode species infecting the same host species and collected on the same day from the same locality. We identified significant differences in both bacterial composition and abundance among trematodes and among hosts infected by different trematode species. Analyses of indicator taxa uncovered different bacteria that were more likely present in the microbiota of each of those trematodes and of their snail hosts (except *Maritrema*). Contrary to one of our expectations, phylogenetically closely related trematode species did not have more similar microbiota. Snail host genetic and environmental variability were minimal in this study since all snails were collected at the same location and time. Furthermore, individual snails of a single population were expected to have a similar genetic makeup, given previous evidence of strong population structure and low genetic diversity in this direct-developing species (Keeney et al., 2009). Thus, the effects of host genetics on its microbial variability and that of its parasites (Easson et al., 2020; Steury et al., 2019) were not expected to be strongly determinant in this dataset.

Host/environment were potentially responsible for shared microbiota patterns among different parasite species, given some overlap in bacterial composition among trematode species, and among trematodes, the environment, and their snail host. However, the significant differences in alpha and most beta diversity metrics, and differential abundance of specific bacterial taxa indicate a more similar microbiota within parasite species than between species. Such microbiota differences were not correlated with phylogenetic distances among the trematodes, in line with the previous finding that microbiotas in marine invertebrates are less structured following phylogenetic proximity than in vertebrates (Boscaro et al., 2022).

Based on beta diversity distances, *Acanthoparyphium* had the most divergent microbiota among the four species assessed, and *Philophthalmus* and *Galactosomum* had a closer microbiota than other trematode pairs. Different diets have been previously correlated with microbiota differences in invertebrates (Muegge et al., 2011; Youngblut et al., 2019), but the diet of *Acanthoparyphium*, *Galactosomum*, and *Philophthalmus* should be similar (mouth-feeding rediae within the same snail species), whereas that of *Maritrema* (no mouth, passively absorbing sporocysts) should not be very different. Thus, dietary differences may also be insufficient to explain microbiota differences among these four species. *Galactosomum* is the trematode with the most similar microbiota composition to that of its snail host. In this specific case, it is possible that the exchange of microbes between the snail host and *Galactosomum* is more important than in the case of the other trematodes. However, as all four trematode species are exposed to the same environment, observed differences likely reflect a combination of factors such as species-specific physiology (Amato et al., 2019; Song et al., 2020), community assembly processes (e.g. vertical transmission) and potentially natural selection on specific horizontally acquired microbes (Rosenberg & Zilber-Rosenberg, 2021; van Vliet & Doebeli, 2019). Furthermore, it is possible that each trematode species induces slightly different immune responses in their snail hosts, leading to species-specific effects on the composition of their microbial community.

Each trematode species also had different levels of diversity (i.e. different bacterial taxa composing the microbial communities of all conspecific parasites) and of consistency (i.e. inter-individual variability in the bacterial taxa present/absent within a species) in their microbiota. Overall, consistency is low among individual trematodes of the same species, as supported by low specificity in indicator taxa analyses. This pattern has been observed in many animal microbiomes, including other helminths and humans (Hahn et al., 2022; Jorge, Dheilly, Froissard, & Poulin, 2022; Sanna et al., 2022). Among these trematodes, *Philophthalmus* microbiota was highly diverse but comprised a relatively more consistent taxonomic composition than the other parasites assessed. On the other side of the spectrum of microbiota stability is *Maritrema*, for which no indicator species were found, potentially due to a lack of consistency among bacterial taxa associating with each individual trematode.

Given the similar diversity levels found in the small number of co-infecting *Philophthalmus* and larger number of single-infecting *Philophthalmus*, this trematode is potentially less susceptible to microbial community differences induced by co-infecting parasites. This relatively stable microbiota composition in *Philophthalmus* is well aligned with previous findings of a core microbiota across different geographical localities (Jorge, Dheilly, Froissard, Wainwright, & Poulin, 2022). However, *Philophthalmus* microbiota stability is unlikely to be due to vertical transmission, as this mode of

bacterial transmission through the trematode life cycle is imperfect (Greiman & Tkach, 2016) and the microbiota composition among different *Philophthalmus* larval stages is largely different (Jorge, Dheilly, Froissard, Wainwright, & Poulin, 2022). Factors commonly evoked to explain microbiota stability that could help explain it in *Philophthalmus* include competition and cooperation among bacterial taxa, natural selection, and the balance between ecological interactions and the mobility of genes encoding resistance to stress (Coyte et al., 2022).

As for *Maritrema*, the low stability in their microbiotas was evidenced by the high impact of co-infections on its alpha and beta diversity, as well as differences of bacterial taxa abundance between single- and co-infections. A larger influence of the surrounding environment is likely (i.e. presence of *Philophthalmus* in the same snail host). However, such influence does not mean *Maritrema* microbiotas are random assemblages of an available microbial pool, as *Maritrema* had a significantly different microbial community composition from that of its snail host. Additionally, this lower consistency in microbiotas across *Maritrema* individuals may also be due to the fact that in our study population, nearly half of the snails infected by *Maritrema* are known to harbour two or more clonal colonies, i.e., they were initially infected by ingesting more than one egg of the parasite (Keeney et al., 2007). Therefore, genetic variation among the *Maritrema* individuals we sampled from each infected snail may account for the high variation of their microbial communities. No comparable information is available for the other trematode species in our study, however, based on data from other trematodes in their snail hosts (e.g. Theron et al., 2004), the proportion of multi-clonal infections is likely much lower.

Comparisons of *Maritrema* and *Philophthalmus* in single- and co-infections revealed that *Maritrema* in co-infections had a more similar microbiota to that of *Philophthalmus* than to its own species in single-infections, but this was not true for *Philophthalmus*. *Maritrema* and *Philophthalmus* co-infecting the same snail had smaller distances among their microbiotas (unweighted Unifrac) than *Maritrema* and *Philophthalmus* in single infections. This supports one of our predictions, i.e., that microbial exchanges can occur between co-infecting parasites and render their microbiota more homogeneous. However, this finding contradicts our other prediction: we expected the asymmetry to involve greater one-way exchanges of bacteria from *Maritrema* to *Philophthalmus*, the species with rediae, and not the other way around, as observed. Differences in the microbiota of co-infecting *Maritrema* compared to single-infecting *Maritrema* were further supported by the differential abundance of specific bacterial phyla. The same is not applicable to *Philophthalmus*, for which single and co-infecting specimens had a largely similar microbiota. Intrinsic biological differences between *Maritrema* and *Philophthalmus* do not explain these results. Given that *Philophthalmus* rediae are mouth-feeding and can ingest *Maritrema* (Kamiya & Poulin, 2013), changes in the opposite direction (co-infecting *Philophthalmus* microbiota assimilating that of *Maritrema*) were expected. However, this lack of change in co-infecting *Philophthalmus* microbiota is well aligned with the finding that *Philophthalmus* microbial community composition is seemingly more diverse and more stable, potentially constraining detectable changes and decreasing variability among individual trematodes, whether in single or co-infections.

The findings of this study support the notion that the microbiota associating with parasites is different from that of their parasitized hosts and of their environment and that species-specific factors can influence their assembly, diversity and stability (Hahn et al., 2022; Jorge et al., 2020). They also suggest that the microbial communities of each trematode species may respond differently to its surrounding environment and also differ in the level of microbial exchange with their hosts. This has implications for parasitological research in general: specific components of host and parasite microbiomes are expected to interact and modulate infection success (Dheilly et al., 2017, 2019; Poulin et al., 2023; Salloum et al., 2023), but even parasites of a single class (e.g. digenean trematodes, as used here) infecting the same host population may be differentially susceptible to changes in their host/environmental microbial communities. Assessments of microbial communities associating with more parasite species at different stages in their life cycles and in different hosts could help better understand the contribution of various microbial sources to parasite microbiotas, as well as the resilience of their microbial communities to differences in their surroundings.

While limiting as much as possible differences associated with season, geography, the host environment and genetics, our results reveal a different, species-specific bacterial composition and abundance in each trematode species, with no evidence that phylogenetic relatedness among trematodes affects the similarity of their microbial communities. Thus, even though microbiota variability can be high, deterministic processes (e.g. natural selection) could be at play in defining successful microbial colonizers in these different species. Furthermore, co-infecting parasites sharing the same individual host can exchange bacteria, resulting in the microbiota of the recipient becoming more similar to that of the donor. However, the asymmetric direction of these exchanges is not simply predictable based on the distinct feeding mechanisms used by the co-infecting species. Nevertheless, variability in the taxonomic composition of microbiotas does not necessarily translate to variability in the microbiome's functional capacity, and selection could be more relevant at the functional level (Doolittle & Booth, 2016). Differences in the microbiota of infected snail hosts could be due to changes induced by the parasites themselves, responses of the snail host to parasitic infection or simple by-products of infection (Dheilly et al., 2015, 2017; Hahn et al., 2022). However, the bacteriota of each trematode species is different from that of the snail host and of its external environment. Functional inferences at this stage are merely speculative, but there are interesting ecological roles for members of the many differentially abundant families in these four trematode species, some of which have been associated with vertical transmission, pathogenesis, and symbiosis with helminth species (see Box S1). Of particular interest may be bacterial taxa that are known to synthesize secondary metabolites such as enzymes, peptides, pigments, and anti-microbials (e.g. Alteromonadaceae, Granulosicoccaceae, Pseudoalteromonadaceae, Box S1), which could directly affect host–parasite metabolic interactions. Potentially pathogenic taxa

that were previously found associated with helminths and molluscs (Rhodobacteraceae, Mycoplasmataceae, Rickettsiales, Box S1) could be using trematodes as vectors or be in obligate/facultative symbiosis with trematodes or snails. Going forward, higher taxonomic resolution and metagenomics approaches will be beneficial to unravel the role of specific microbiome components in the ecological interactions and evolution of parasites and their parasitized hosts.

## AUTHOR CONTRIBUTIONS

PMS, FJ and RP designed the study; PMS and RP collected samples; PMS undertook laboratory work, statistical analyses, and wrote the manuscript, with input from FJ and RP.

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## OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at (https://doi.org/10.6084/m9.figshare.22881482).

## DATA AVAILABILITY STATEMENT

Raw sequence reads are available in the SRA (BioProject PRJNA972185, BioSamples SAMN35067136 to SAMN35067307); bioinformatics scripts, filtered data, FastQC reports and metadata are available on Figshare (https://doi.org/10.6084/m9.figshare.22881482).

## BENEFIT-SHARING STATEMENTS

Benefits from this research accrue from the sharing of our data and results on public databases as described above.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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