



# A combination of host ecology and habitat but not evolutionary history explains differences in the microbiomes associated with rotifers

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**Abstract** The holobiont concept places emphasis on the strict relationship between a host and its associated microbiome, with several studies supporting a strong effect of the quality of the microbiome on the host fitness. The generalities of the holobiont have been questioned for several invertebrates, including zooplankton. Here we assess the role of host ecology,

habitat, and evolutionary history to explain the differences in the microbiomes associated with rotifers, across a broad taxonomic spectrum and from different habitats. The analyses of 93 rotifer-associated microbiomes from 23 rotifer host species revealed that a combination of effects from the host ecology and its habitat seem to be stronger than host phylogenetic distances in explaining differences in microbial composition of the microbiomes. This pattern is in line with the idea of habitat filtering being a stronger explanation than co-evolution in shaping the relationship between a microbiome and its rotifer host.

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## Introduction

The strict association between multicellular eukaryotes like plants and animals with microbes that are beneficial for the host's survival and fitness opened a new avenue of research within the holobiont theory (Bosch & Miller, 2017). The strict association, a pattern termed phylosymbiosis, makes both the host and the associated microbiome co-evolve to the point that similarities in microbiome composition between hosts could reflect the phylogenetic relatedness of the hosts: both the host and the microbiome experience the same evolutionary history under the same evolutionary pressures (Lim & Bordenstein, 2020). Yet, a similarity between microbiome composition and host phylogenetic relationships could also be due to the correlated effect of host filtering: phylogenetically related hosts may have similar ecologies, indirectly filtering for similar microbiomes even if they did not evolve strictly associated (Mazel et al., 2018).

General conclusions are hampered by the fact that the inference on the potential processes may be biased by our skewed knowledge on the patterns, highly dominated by microbiomes from model species, mostly vertebrates (Youngblut et al., 2019; Kuziel & Rakoff-Nahoum, 2022), and by a focus on animals with a strong association with their microbiome, like corals (e.g. Pollock et al., 2018). The phylosymbiosis pattern of host-microbiome association seems to be much looser for several terrestrial and aquatic invertebrates (e.g. Hammer et al., 2017, 2019; Eckert et al., 2020). Specifically, zooplankton seem to have a transient and flexible microbiome, which is also highly affected by external conditions (Eckert et al., 2021). Amongst the zooplankton, one of the most diverse groups of animals on which to reliably test for phylosymbiosis is represented by the phylum Rotifera. Preliminary studies on zooplankton included also rotifers but were not able to disentangle the degree of existing phylosymbiosis and whether the observed patterns could be due to coevolution or to similarities in environmental conditions between host species (Eckert et al., 2021; Novotny et al., 2021). A better understanding of the association between bacteria and common abundant aquatic invertebrates like rotifers

would help elucidate the structure of food webs in general and also the role of zooplankton as carriers of potential human pathogens (Grossart et al., 2010; Di Cesare et al., 2022) within the rationale of the one health approach (Rabinowitz & Conti, 2013).

The aim of the study is to explore the relevance of the potential drivers of the community composition of bacteria associated with rotifers, with a focus on phylosymbiosis, expanding the taxonomic breath of previous studies and including different habitats, from freshwater to marine ones. The goal is to disentangle the role of host ecology, habitat, and evolutionary history to explain differences in microbiomes associated with rotifers.

## Methods

### The dataset

We gathered as much information as possible on rotifer-associated microbiomes, downloading public data (from e.g. Eckert et al., 2021; Novotny et al., 2021) and adding new data to obtain a large taxonomic diversity of rotifer hosts from different habitats (Table 1). Available data were searched manually and downloaded using the Python package `pysradb` (Choudhary, 2019). In addition, related metadata was downloaded using a custom script for R v4.0.2 (R Core Team, 2020). The scripts are available on GitHub at [https://github.com/CNR-IRSA-MEG/metaR/tree/master/lib/rotifer\\_microbiome](https://github.com/CNR-IRSA-MEG/metaR/tree/master/lib/rotifer_microbiome). New data was obtained from species directly collected in the field. Batches of 20–50 animals for each species were isolated and kept in distilled water for 30 min to allow their stomachs to empty, then washed three times with distilled water and placed in the MicroBead tubes of the UltraClean Microbial DNA Isolation Kit (MoBio Lab, Qiagen) for DNA extraction, as previously done to study rotifer-associated microbiomes (Eckert et al., 2021). Negative controls were included for most of the lab steps, including blanks from reagents and water used for DNA extraction.

Quantification of the total DNA was done with the QuBit Fluorometer (Invitrogen, Thermo Fisher Scientific). Amplicon libraries were prepared and sequencing was conducted by IGA Technology (Udine, Italy) using Illumina Nextera chemistry and a MiSeq platform. Universal bacterial primers

**Table 1** Information on the dataset, with rotifer species, number of microbiomes for each species, habitat, and geographical area of origin for each microbiome

Rotifer species	Number of microbiomes	Habitat	Origin	Lab culture versus environment	Source
<i>Adineta vaga</i> (Davis, 1873)	4	Freshwater	United Kingdom	Culture	Eckert et al. (2021)
<i>Brachionus calyciflorus</i> (Pallas, 1766)	5	Freshwater	The Netherlands	Culture	New
<i>Brachionus quadridentatus</i> (Herman, 1783)	1	Freshwater	Italy	Environment	Eckert et al. (2021)
<i>Dissotrocha macrostyla</i> (Ehrenberg, 1838)	1	Freshwater	Italy	Environment	New
<i>Epiphanes senta</i> (Muller, 1773)	1	Freshwater	Italy	Environment	Eckert et al. (2021)
<i>Euchlanis dilatata</i> Ehrenberg, 1830	2	Freshwater	Italy	Environment	Eckert et al. (2021)
<i>Keratella quadrata</i> (Muller, 1786)	2	Freshwater	Italy	Environment	Eckert et al. (2021)
<i>Keratella serrulata</i> (Ehrenberg, 1838)	2	Freshwater	Italy	Environment	Eckert et al. (2021)
<i>Keratella</i> sp.	11	Brackish	Baltic Sea	Environment	Novotny et al. (2021)
<i>Keratella valga</i> (Ehrenberg, 1834)	1	Freshwater	Italy	Environment	New
<i>Lecane elsa</i> (Hauer, 1831)	1	Freshwater	Italy	Environment	Eckert et al. (2021)
<i>Lecane inermis</i> (Bryce, 1892)	22	Freshwater	Poland	Culture	Eckert et al. (2021)
<i>Notommata allantois</i> (Wulfert, 1935)	1	Freshwater	Italy	Environment	New
<i>Notommata cerberus</i> (Gosse, 1886)	1	Freshwater	Italy	Environment	New
<i>Philodina citrina</i> (Ehrenberg, 1830)	1	Freshwater	Italy	Environment	New
<i>Polyarthra dolichoptera</i> (Idelson, 1925)	1	Freshwater	Italy	Environment	Eckert et al. (2021)
<i>Polyarthra</i> sp.	3	Freshwater	China	Environment	New
<i>Rotaria macrura</i> (Ehrenberg, 1832)	5	Freshwater	Italy	Culture and environment	Eckert et al. (2021)
<i>Rotaria rotatoria</i> (Pallas, 1766)	1	Freshwater	Italy	Environment	Eckert et al. (2021)
<i>Synchaeta baltica</i> (Ehrenberg, 1834)	19	Brackish	Baltic Sea	Environment	Novotny et al. (2021)
<i>Synchaeta monopus</i> (Plate, 1889)	6	Brackish	Baltic Sea	Environment	Novotny et al. (2021)
<i>Trichocerca</i> sp.	1	Freshwater	Italy	Environment	New
<i>Trichotria tetractis</i> (Ehrenberg, 1830)	1	Freshwater	Italy	Environment	New

S-D-Bact-0341-b-S-17, 5'-CCTACGGGNGGCWGCAG-3', and S-D-Bact-0785-a-A-21, 5'-GACTAC HVGGGTATCTAATCC-3' (Herlemann et al., 2011) with an amplicon size of 464 bp were used.

All raw reads generated in this and in previous studies were cleaned and clustered in the same pipeline. Adaptors and primers were clipped from the sequences using cutadapt v1.9.1 (Martin, 2011).

The Usearch/Uparse pipeline was used for sequence assembly, quality filtration, chimera check and removal, and preparation of the database of bacteria composition (Edgar et al., 2011; Edgar, 2013), following the protocols of Eckert et al. (2021) for the construction of zero radius operational taxonomic units (zOTUs, Edgar, 2016). For taxonomic assignment, the `utax` command was used with the Silva database v123 (Quast et al., 2012) requiring a minimum similarity of 80% for the taxonomic assignment. zOTUs assigned to non-bacterial taxa such as chloroplasts and mitochondria were removed from the dataset and since the dataset was composed of samples with very different read depth rarefaction to a threshold of 3000 reads was conducted (maximising number of retained samples but still providing enough reads for the analyses on bacterial diversity),

DNA sequence data for the phylogenetic analyses of the zooplankton hosts was obtained for a fragment of the mitochondrial COI and a fragment of the nuclear 18S rRNA, downloaded from GenBank (Table S1). Sequences were aligned independently for each marker using MAFFT v7 (Katoh et al., 2019). A concatenated alignment of 18S and COI was then used to reconstruct a time-calibrated phylogeny with BEAST v2.6.0 (Bouckaert et al., 2019) using the CIPRES portal (Miller et al., 2011). The parameters for the reconstruction comprised a GTR + G + I substitution model, a relaxed lognormal clock, a birth–death prior, a random starting tree, 100,000,000 generations, and sampling every 10,000 generations. The Markov chain Monte Carlo (MCMC) sample was checked for convergence in Tracer v1.7.1, and the trees were combined into a maximum credibility tree whilst keeping the target node heights with a 20% burn-in in TreeAnnotator v2.4.8.

### Microbiome diversity

Differences in microbiome between samples were measured as Bray–Curtis abundance-based dissimilarities in the R package `vegan` v2.5–7 (Oksanen et al., 2022) and visualised as non-metric multidimensional scaling (nMDS) and hierarchical clusters with average agglomeration method, with plots obtained with the R package `ggplot2` v3.3.3 (Wickham, 2016).

To assess the potential drivers of the differences in microbiome composition, we started by exploring whether rotifers living in freshwater and brackish

waters hosted a different microbiome. The analysis would be biased by the fact that brackish samples are highly dependent coming from one study from one site performed by one lab, whereas freshwater samples are obtained from different labs on different samples. Thus, we only visually explored the differences by plotting the habitat of origin on the cladogram of similarities in microbiome composition in comparison to the phylogeny of the host rotifers. The rationale is that differences between brackish Baltic samples and freshwater may overrule variation amongst sites and taxa within each habitat category, even if a formal statistical test would be biased by the sampling scheme.

To explore the effect of the differences between lab cultures and samples from the field, limiting the analyses to freshwater samples, we performed a Permutational Analysis of Variance (PERMANOVA) using the matrix of Bray–Curtis pairwise dissimilarities as the response variable and whether the sample originated from the field or from lab cultures as an explanatory variable. Given the high level of pseudoreplication in our data, with repeated microbiome data obtained from the same species, we randomly selected only one microbiome for each species.

To test the effect of phylogenetic relationships between hosts, we performed a Mantel test between the matrix of Bray–Curtis distances in microbiome composition for each zooplankton species (with a randomly selected sample for each species) and the matrix of patristic distances of the same zooplankton species from the phylogenetic tree of the hosts, obtained with the R package `ape` v5.0 (Paradis & Schliep, 2018). Given the potential bias on the microbiomes due to culturing animals in the lab, we repeated the analyses only on the microbiomes from rotifer hosts that were directly collected in the environment.

### Results

The final dataset passing all the quality filters and thresholds included information on bacterial composition from 93 microbiomes covering 23 rotifer species, both bdelloids and monogononts, from fresh and brackish waters, and from lab cultures or from animals collected directly in the environment (Table 1). From a total number of  $18 \times 10^6$  reads,  $7.5 \times 10^6$  were

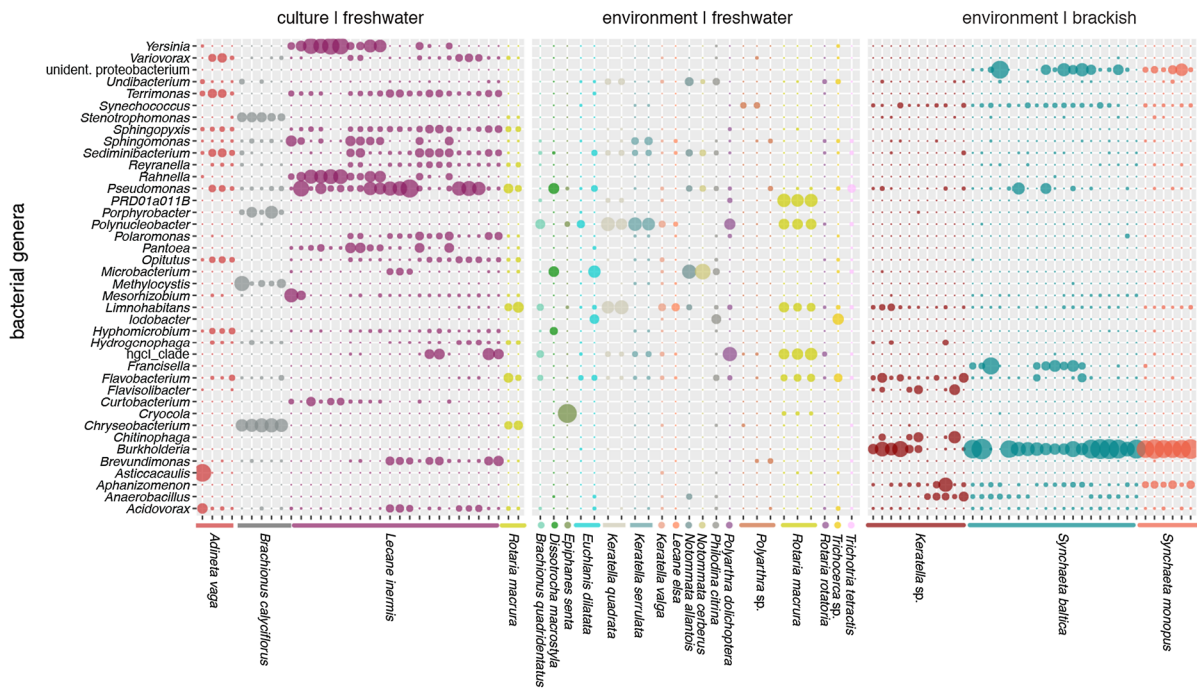
kept for the zOTU table after filtering. Then, 1344 zOTUs were removed after being identified as chloroplasts or mitochondria.

None of the bacterial genera that were frequent in the dataset (> 1000 reads) were ubiquitously present in all rotifer samples. The genera that were found in most rotifer microbiomes (>70% of samples) including those of rotifers from brackish water, freshwater culture, and environment were *Pseudomonas* (80%), *Acidovorax* (78%), and *Flavobacterium* (71%) (Fig. 1). Cultured *Lecane inermis* often had high number of reads affiliated with *Yersinia* and *Rahnella*, and both *Adineta vaga* and *L. inermis* contained many reads affiliated with *Terrimonas*, *Sphingopyxis*, *Sediminibacter*, and *Acidovorax*. The microbiota of cultured *Brachionus calyciflorus* was dominated by *Stenotrophomonas*, *Porphrobacter*, *Methylocystis*, and *Chryseobacterium*. The only genus that was common in all cultured rotifers was *Pseudomonas*. Environmental freshwater rotifers often had reads affiliated with

*Polynucleobacter*, *Limnohabitans*, and *Flavobacterium*. Brackish water rotifer microbiota were dominated by bacteria affiliated with *Burkholderia*, *Flavobacterium*, *Anerobacillus*, but also with autotrophs such as *Aphanizomenon* or *Synechococcus* (Fig. 1).

Differences in microbiome composition were visually different between rotifers from freshwater and brackish habitats, and from lab cultures and field samples (Figs. 2, 3). No effect on microbiome composition could be ascribed to the origin of the rotifer host as a lab culture or as an environmental sample across the 23 species in the dataset, with one randomly selected sample for each species (PERMANOVA:  $R^2=6.71$ ).

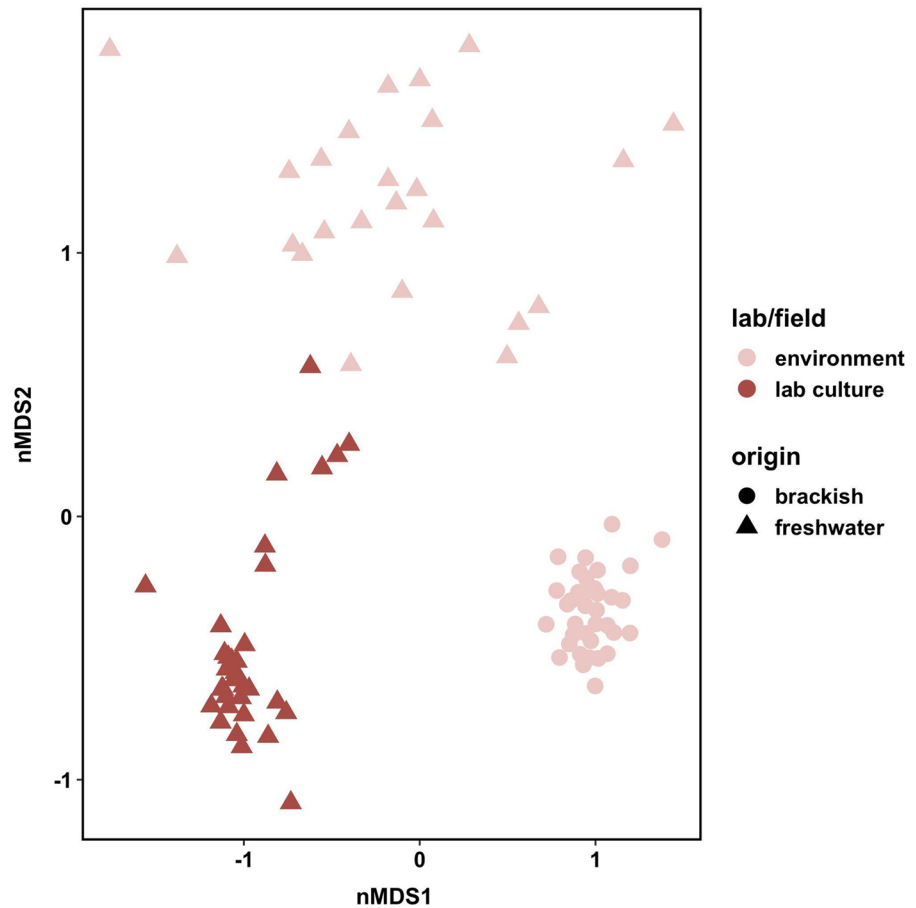
No effect was found on the differences in microbiomes between host rotifer species of phylogenetic distances between the 23 hosts species (Mantel test:  $r=0.093$ ,  $P=0.119$ ). Reducing the dataset to only species from environmental samples, the correlation slightly increased (Mantel test:  $r=0.120$ ,  $P=0.098$ ).



**Fig. 1** Read abundance of the 40 most common bacterial genera in the dataset (> 1000 reads) in the different samples. Samples are grouped by rotifers from cultures, freshwater rotifers

from the environment, and brackish rotifers from the environment. Colours of the bubbles denote the different rotifer species. Size of the bubbles is proportional to the number of reads

**Fig. 2** Non-metric multidimensional scaling (nMDS) plot of the differences in microbiome composition between samples, with shape according to their freshwater or brackish origin and colour (same as in Fig. 3) according to their lab or environmental origin



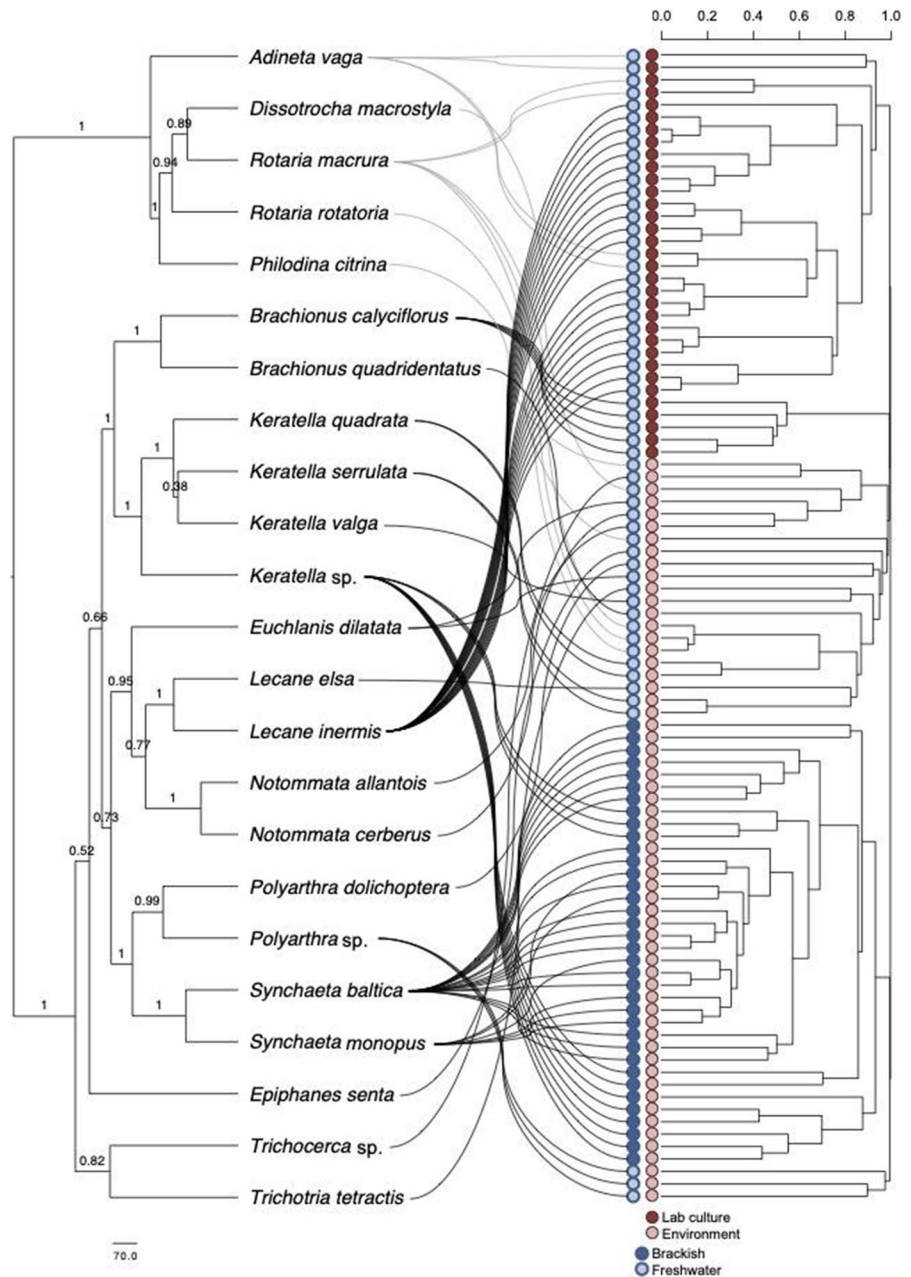
## Discussion

Our survey of rotifer-associated microbiomes revealed that ecological differences between host rotifer species may explain the differences in the rotifer-associated microbiomes more than co-evolution between the microbiome and the rotifer host. The most relevant driver of the differences in rotifer-associated microbiomes was the sample of origin of the rotifers, with one of the major distinctions being between freshwater and brackish samples (Figs. 1, 2, 3). Regarding brackish samples, *Burkholderia* is indeed known to be associated with plants and animals, but also to be found free living in marine habitats (Stoyanova et al., 2007): it was found abundant and diverse in brackish samples but almost absent in freshwater samples and lab cultures. *Polynucleobacter*, *Microbacterium*, and other genera were found in several freshwater environmental rotifers, but never in brackish rotifers. Microbiomes from lab cultures also

had a clear signature, with several genera (*Yersinia*, *Variovorax*, *Terrimonas*, etc.) that were not found in environmental samples. The most common bacterial taxa found in the microbiomes, e.g. *Pseudomonas*, *Acidovorax*, and *Flavobacterium* are common environmental taxa (Newton et al., 2011). Composition of aquatic bacterial communities are known to be affected by laboratory conditions (Eilers et al., 2000) and the rotifer-associated microbiomes from cultured rotifers confirmed such pattern. We thus support the previous inference of a strong effect of the type of water of origin on the zooplankton microbiome (Eckert et al., 2021).

Each species seems to have a strong effect on the differences between microbiomes, according to the potential for coevolution between hosts and the bacterial communities associated with them (Lim & Bordenstein, 2020). Yet, such effect could be misleading in our survey, given that different microbiome samples from the same species in our dataset do not

**Fig. 3** Tanglegram of host-microbiome relationships. The host phylogeny on the left is a Bayesian ultrametric tree with branch length and scale bar proportional to evolutionary rates in a combined COI + 18S alignment and with numbers on branches representing posterior probabilities. The cladogram on the right represents clustering of dissimilarities in microbiome composition between samples. Each sample, connected with a curved line to the host species (in grey for Bdelloidea and black for Monogononta) is marked with different colours depending on whether it is from freshwater or brackish water and from lab culture or from the environment



really originate from different rotifer populations, but mostly from different lab cultures of the same species (e.g. *Adineta vaga*, *Brachionus calyciflorus*, *Lecane inermis*) or different times of sampling of the same site (e.g. *Synchaeta baltica*, *Keratella pecializ*). Interestingly, *Rotaria macrura*, for which we had microbiomes from environmentally collected animals and from lab cultures, revealed rather different microbiomes between the two groups of origin, but similar

within each group (Fig. 3), reinforcing the effect of sample of origin more than that of the host species per se. The strong effect of the water of origin, in addition to that of the species, is in line with the expectation that microbes could be more affected by environmental stressors than their animal hosts (Hammer et al., 2019), especially in a fluctuating environment like that of surface continental and brackish waters (Scheffer & Carpenter, 2003).

Another aspect of the survey we performed on rotifer-associated microbiomes is the support for an absence of correlation between differences in microbial composition and phylogenetic distances between hosts. Interestingly, for very short phylogenetic distances between hosts, notably for microbiomes from different samples within the same species, similarities between microbiomes can vary across a wide range from very similar to highly dissimilar (Fig. 3). All previous analyses including rotifers (e.g. Eckert et al., 2021; Novotny et al., 2021) did not have a large enough taxonomic breadth to allow any inference on such a relationship: our survey revealed that even increasing sample size, no phylosymbiosis seems to exist for rotifers. On the contrary, strong correlations were found for example for corals (Pollock et al., 2018; O'Brien et al., 2020), *Hydra* (Franzenburg et al., 2013; Bosch & Miller, 2017), sponges (Hentschel et al., 2002; Turon et al., 2019), or fish (Doane et al., 2020; Sylvain et al., 2020). The host-microbiome relationship affected by evolutionary distances of the hosts, termed phylosymbiosis (Lim & Bordenstein, 2020), is known to be potentially explained as a simple ecological filtering process, with host traits and ecology co-varying with host phylogeny, without any long-term coevolutionary mechanisms (Mazel et al., 2018; Kohl, 2020). Our survey on rotifers was not able to support evolutionary effects, but only ecological effects as patterns, and potentially also as a relevant process.

Overall, the survey of rotifer-associated microbiomes revealed a combination of effects of host ecology and habitat, but not of host evolutionary history to explain the differences in the microbiomes associated with rotifers. Yet, the currently available data do not have the necessary level of independent replication within species to allow any strongly supported inference on the relative role of the host ecology and evolutionary history. Thus, the quest for a more quantitative answer is still open, with the potential for a dataset with larger taxonomic coverage and more populations for each species to allow obtaining more definitive answers.

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**Data availability** All new raw 16S sequence data for the microbiomes are available in NCBI SRA under Accession No. PRJNA866905 for the new sequences and PRJEB39191, PRJNA611635, PRJNA655592, PRJNA655595 for the previously published sequences. All R scripts and datasets used for the analyses are available at [https://github.com/CNR-IRSA-MEG/metaR/tree/master/lib/rotifer\\_microbiome](https://github.com/CNR-IRSA-MEG/metaR/tree/master/lib/rotifer_microbiome).

#### Declarations

**Conflict of interest** No competing interests exist.

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