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### ► To cite this version:

Eglantine Mathieu-Bégné, Simon Blanchet, Olivier REY, Eve Toulza, Charlotte Veyssière, et al.. A longitudinal survey in the wild reveals major shifts in fish host microbiota after parasite infection. *Molecular Ecology*, 2023, 32 (5), 10.1111/mec.16901 . hal-04013020

**HAL Id: hal-04013020**

**<https://hal.science/hal-04013020>**

Submitted on 3 Mar 2023

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# A longitudinal survey in the wild reveals major shifts in fish host Microbiota after parasite infection

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## **Abstract**

Recent studies have highlighted associations between diseases and host microbiota. It is yet extremely challenging –especially under natural conditions- to clarify whether the host microbiota promotes future infections, or whether changes in host microbiota result from infections. Nonetheless, deciphering between these two processes is essential for highlighting the role of microbes in disease progression. We longitudinally surveyed, in the wild, the microbiota of individual fish hosts (*Leuciscus burdigalensis*) both before and after infection by a crustacean ectoparasite (*Tracheliastes polycolpus*). We found a striking association between parasite infection and the host microbiota composition restricted to the fins the parasite anchored. We clearly demonstrated that infections by the parasite induced a shift in (and did not result from) the host fin microbiota. Fin microbiota further got similar to that of the adult stage, and the free-living infective stage of the parasite during infection with a predominance of the *Burkholderiaceae* bacteria family. This suggests that *Burkholderiaceae* bacteria is involved in a co-infection process and possibly facilitate *T. polycolpus* infection. We reveal novel mechanistic insights for understanding the role of the microbiota in host-parasite interactions, which has implications for predicting the progression of diseases in natural host populations.

## **Keywords:**

Host-parasite interaction, Co-infection, Bacteria community, Individual survey, Metabarcoding.

## Introduction

Understanding the factors shaping host susceptibility to pathogens allow anticipating disease risks. Infection has often been described as the result of tri-partite interaction between parasites virulence, host resistance and environmental factors (Wolinska & King, 2009). However, recent studies demonstrated that infected hosts are associated with specific microbiota (Avelo & Norberg, 2018; Bär et al., 2015; Portet et al., 2018; Reynolds et al., 2015; Speer, 2022).

Differences in microbiota among individuals hence provide a novel mechanism to explain infection risk within host populations. Parasitism could be seen as a multi-faceted interaction between the environment, the host, the parasite and their associated microbiota (Bordenstein & Theis, 2015; Dheilly, 2014).

A key question recently raised by Speer (2022) (Speer, 2022) is whether “the microbiome differentially attract parasites or [...] reflect the infection status of its host” (i.e., the causal role of host microbiota during infection). Differences in host microbiota *before* infection may modulate host susceptibility to parasite infection, making the host microbiota central to predict individual infection risks (Chavez et al., 2021; Speer, 2022). For instance, host microbiota can provide optimal conditions for parasite development (Britton & Young, 2014; Buffie & Pamer, 2013; Hayes et al., 2010) or constitute a cue for the parasite to locate its hosts (Chavez et al., 2021; Speer, 2022). Different microbiota between infected and uninfected hosts can also occur *after* parasite infection. For instance, parasites while disrupting host immune system can modulate host microbiota composition (Beatty et al., 2017; Lee et al., 2014). Parasites can also indirectly facilitate colonization by opportunist pathogenic bacteria or fungi from the surrounding environment by weakening host resistance (Mooney et al., 2015). Finally parasites can directly carry new microbes that co-infect the host, while benefiting parasite fitness (Dheilly et al., 2015; Reynolds et al., 2015). Specific association between microbiota communities and parasites have been described in the wild in several plant and animal species (Andre, 2002; Boissière et al., 2012; Stensvold & van der Giezen, 2018). Nonetheless, a pressing challenge is to distinguish the mechanisms leading to these associations in wild populations.

A powerful approach to decipher causality in parasite-microbiota-host interactions is to survey the microbiota of individual hosts before and after infection (Afrin et al., 2019; Blanchet, Thomas, et al., 2009; Gaulke et al., 2019; Jenkins et al., 2018). Most host microbiota surveys have focused on changes occurring during or after parasite exposure, which limits our understanding of the mechanisms sustaining relationships between parasites, hosts and the microbiota (but see, Jenkins et al., 2018; White et al., 2018). Also, to investigate the mechanisms leading to changes in host microbiota after parasite infection, both host-associated microbiota and microbiota from any potential colonization sources must be investigated. Understanding the role of host-associated microbiota during parasitic infection is therefore a challenging task, especially in the wild, where controlling confounding factors is difficult. However, investigating the role of host-associated microbiota during parasitic infection constitutes a promising avenue for realistic and novel insights into the complex links between parasites, hosts, the environment, and their microbiota.

Here, we tested whether variation in the host microbiota composition is associated with parasitic infection, and we questioned whether this variation pre-exist to infection or is induced by the infection. We focused on an ectoparasite (the freshwater Crustacean *Tracheiastes polycolpus*) that has emerged in Western Europe in the 1920's and that infects several native fish species (Lootvoet et al., 2013; Rey et al., 2015). The parasite anchors and feeds on fins where secondary infections by bacteria and fungi are often observed (Blanchet, Rey, et al., 2009). We tracked the microbiota of the fin mucus of its main fish host species, the rostrum dace (*Leuciscus burdigalensis*), both before and after infection at the individual host (fin) level. We first tested whether infected hosts displayed different microbiota compared to non-infected ones. We then tested whether fin microbiota may facilitate the infection by *T. polycolpus* by comparing fin microbiota before parasite infection between hosts that became infected or remained noninfected. We assumed that, if fin microbiota is a cause of infection, specific bacterial communities before infection should be associated with those of infected hosts. We further investigated the temporal dynamics of microbiota during infection to test whether the infection by *T. polycolpus* resulted in changes in fin bacteria community. If so, we expected that, during infection, the microbiota of infected hosts will shift away from those of non-infected hosts and we tested whether these changes resulted from colonisation of opportunistic environmental bacteria and/or from colonisation from the parasite.

## **Material and Method**

### **Ethics approval**

The experiment was performed in agreement with the national legislation (Arrêté Préfectoral 22/05/2018) and did not require ethical approval.

### **Biological history of the interaction**

*Tracheliastes polycolpus* is a copepod that feed on fish fins causing degradations and inflammations (Loot et al., 2004). Only females are parasitic, whereas males are microscopic free-living forms. Once anchored on a fin, the parasite develops two egg sacs (Mazé-Guilmo, 2016). After a few weeks, eggs hatch and release free living larvae that rapidly develop into a *copepodite* infective stage (Mazé-Guilmo, 2016). Larvae are not in direct contact with the environment until hatching. Therefore, the microbiota of *T. polycolpus* larvae -until hatching- is mainly transmitted from the mother, which makes this model particularly powerful to study parasite-associated microbiota.

### **Experimental design**

We tracked the microbiota of each individual fin of captivity-raised rostrum dace (*L. burdigalensis*) in a semi-wild caging situation before and after infection. Twenty cages (30x50x80 cm, mesh size=1 cm), each containing four dace (80 dace in total) were set up within a 200-m long section in the Arize River in southwestern France (X=366422, Y=4770813, WGS 84/UTM zone 31N) during summer 2018. Cages were spaced one from the other of a one meter minimal distance in microhabitats previously identified as hotspots of infections (deep whirlpool areas, Mathieu-Bégné et al., 2021) so as to maximize the chance of infection. Cages were upstream-downstream orientated so as to favor a natural flow in each cage. Water temperature ranges from ~13°C to ~17 °C throughout the experiment and was constant over the entire river section (no substantial differences among cages). Dace were F2 individuals from wild ancestors bred at the INRAe hatchery facility in Rennes (U3E, France). All dace shared a similar genetic background (i.e., they hatched from the same pool of genitors), were of the same age (2-years old fish, sub-adult to adult stage) and were similar in weight and body size (15±3 g and 110±8 mm) to limit confounding factors. They were brought back from Rennes in early spring, raised in groups of 10 fish in height large outdoor tanks at the SETE laboratory (Moulis, France). The tanks were filled with dechlorinated tap water and included enrichment elements such as gravel at the bottom of each tank and artificial refugees for the wellbeing of the fish. Dace were fed *ad libitum* before the onset of the experiment. None of them were previously exposed to *T. polycolpus* (but their ancestors were in the wild). Fish were introduced in their cages *in situ* the 9<sup>th</sup> of July 2018. Once every two days, cages were brushed (to avoid mesh clogging and to let natural particles including food and infective larvae circulate) and dace were visually inspected for infections (expected to occur after four weeks of caging (Mathieu-Bégné et al., 2021)) using an Aqua Scope II™ (i.e., underwater viewing device). After ~3 weeks of acclimatization, dace were collected from their cages and fin mucus was sampled to characterize fin microbiota *before infection*. All individuals were anesthetized (using benzocaine), we verified that no infection has occurred yet, and the mucus was sampled on each of the seven fins (anal, caudal, dorsal, pelvic, and pectoral) and on the body using sterile cotton swabs (Copan Diagnostics®). Fish were weighted, measured, and individually tagged using colored elastomers (Northwest Marine Technology®) injected at fin insertions. Fish were then gently replaced in their cage. From that period, cages were checked daily for *T. polycolpus* infection, and a second swab sampling was performed on surviving daces one to three weeks after the first swabbing. All fish were extracted from their cages, measured, weighted, checked for *T. polycolpus* infection, and mucus was sampled on each fin and on the body to characterize fin microbiota *after* infection. Suspicious aspects of the fish fins such as those caused by other fin ectoparasites (e.g., *Gyrodactylus* sp.) were not observed, and hence only *T. polycolpus* infections were recorded. However, eight dace were not considered in the final analyses either because they were found dead or either because they displayed abnormal behavior (difficulty to maintain a vertical position in the cage or a horizontal position in the water column). This resulted in 57 dace at the end of the experiment including 13 infected dace. When a parasite was found (34 parasites in total), it was gently removed and stored in a 50-ml vial containing water from the river for transporting them alive to our lab facilities. Water samples were also collected at each cage to characterize the environmental bacteria. All swab samples were stored at -40°C until DNA extraction.

To sum up, host microbiota was sampled on all host fins and body before and after infections.

Four main fin categories can be defined according to their status after the infections: (i) microbiota from infected fins (n = 18 fins from 13 fish), (ii) microbiota from healthy (uninfected) fins sampled on an infected fish (i.e., *T. polycolpus* generally infects 1 or 2 fins out of the seven fins, letting the others uninfected -healthy-, n = 60 fins from 13 fish), (iii) microbiota from healthy (uninfected) fins sampled on healthy fish in a cage where at least one infected fish was observed (n = 144 fins from 24 fish), and (iv) microbiota from healthy fins sampled on healthy fish in cages where no infected fish was observed (n = 66 fins from 11 fish).

### ***Parasite microbiota***

The microbiota of all parasites was characterized from the trunk of adult parasites and from infective larvae reared in the laboratory. When parasites carried mature egg sacs (7 parasites, corresponding to 14 egg sacs), they were brought alive to the laboratory. The two egg sacs were separated from the parasite trunk using sterile scalpels. Trunks of adult parasites were stored at -40°C until DNA extraction, and each egg sac was placed in a single 2mL well from a sterile 24-well plate (as in Mazé-Guilmo, 2016). Wells were filled with 2 mL natural mineral water (renewed every day) until hatching. Well plates were stored in a thermostat incubator at a temperature matching the one measured in the field during sampling (16°C ± 1 °C). The thermostat incubator was enlightened according to a 12:12 day-night cycle. Once egg sacs hatched, larvae were collected, stored in 2mL Eppendorf® and kept at -40°C until extraction (~225 larvae, pooled by egg sac).

### ***DNA extraction and 16S rRNA gene amplicon sequencing***

We followed the DNA extraction and amplification protocol described in (Bestion et al., 2017). Bacteria DNA of each sample were extracted using a standard protocol for gram-positive bacteria based on the Qiagen DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany). After extraction, the concentration of the resulting gDNA was 10-20 ng/μL. DNA was amplified using PCRs realized in a final volume of 20 μL including 2 μL of DNA, 10 μL of AmpliTaq Gold™ 360 Master Mix (ThermoFisher Scientific), 0.5 μM of each primer and 3,2 μg of bovine serum albumin. Initial denaturation was realized at 95°C for 10 minutes followed by 30 cycles of denaturation (95°C for 30 s), annealing (57°C for 30 s) and elongation (72°C for 90 s) and finally followed by a 7 min final elongation step at 72°C. We amplified the V5-V6 region of the 16S rRNA gene (295 bp) using a pair of universal bacteria primers (BACTB-F: GGATTAGATACCCTGGTAGT; and BACTB-R: CACGACACGAGCTGACG, Fliegerova et al., 2014). Samples were identified with unique combination of 8-base tags added in 5' end of each primer. PCR products were purified (QIAquick PCR purification Kit, Qiagen GmbH, Hilden, Germany) and pooled. A single library of the amplicon multiplex was prepared accordingly to the MetaFast protocol. Sequencing was performed at the GeT-PlaGe facility (Toulouse, France) using the sequencer Illumina MiSeq and 2x250 bp paired-end sequencing technology. Positive controls (Zymobiomics Bacteria Community DNA Stan) and negative controls (blanks, and air samples realized during the sampling campaign and during extraction) were included in the sequenced multiplex to investigate extraction and amplification success as well as marker universality, and potential contamination, respectively. Overall, 759 samples were sequenced.

### ***Bioinformatic analysis and filtering***

Illumina sequencing data were demultiplexed using the OBITools suite of scripts (Boyer et al., 2016). Data were then processed using FROGS (Escudie et al., 2017). FROGS first includes a pre-processing step that de-replicates and filters amplicons according to their length. We set a range of possible amplicon size of 200-350 bp, which fits the size of amplicons for the V5-V6 region of the bacterial 16S rRNA gene. Clustering in Operation Taxonomic Units (OTUs) was then realized using the swarm algorithm implemented in FROGS with aggregation distance set to three (Mahé et al., 2014). Chimera and singletons were removed. The taxonomic affiliation of each OTU was done using BLAST on the Silva database up to the species level and equally good affiliation were denoted as multi-affiliation (v132, Quast et al., 2012). Finally, using the R package *Phyloseq* (McMurdie & Holmes, 2013), OTU composition of each sample was rarefied to 2 000 sequences per OTU to allow samples comparisons and we further filtered out OTUs occurring in less than 3 samples and under a frequency of 1‰ (excluding all the negative and blank controls) and 4 603 OTUs were retained.

## **Statistical analysis**

### *Association between parasitic infection and host microbiota*

We first tested whether fin microbiota of host differ when the host is infected by comparing the microbiota among the four categories of fin samples *after* infection (see above, categories *i* to *iv*). We measured beta dissimilarity based on the Bray-Curtis distance to test for differences in community composition and assessed significance of through a Permutational Multivariate Analysis of Variance (adonis2 function, from the R package *vegan*, Oksanen et al., 2011) followed by pairwise comparisons (pairwise.adonis2 function from the R package *pairwiseAdonis*, Arbiz Martinez, 2020). Source of beta diversity (*balanced variation in species abundances* or *abundance gradient*) was assessed using the R package *betapart* (Baselga, 2013). We also measured alpha-diversity based on the Shannon index and tested differences of fin microbiota richness through a Kruskal-Wallis test followed by pairwise comparisons.

### *Fin microbiota before parasite infection*

To test whether differences in microbiota were associated with fin mucus existed prior to parasite infection, we focused on the fin microbiota sampled before infection. We relied on the same approach as previously, based on measures of alpha-diversity and beta dissimilarity to assess community differences in term of richness and composition.

### *Fin microbiota after parasite infection*

To test whether parasite infection triggers changes in host microbiota, we investigated the temporal dynamics of fin bacteria communities during infection. We expected that temporal shifts (from *before* to *after* infection) in fin microbiota should differ between infected and healthy fins, and that shifts should be stronger in magnitude in infected- than in healthy fins. We conducted a Community Trajectory Analysis (CTA, De Cáceres et al., 2019) based on a Discriminant Analysis of Principal Component ran on OTU relative abundances using the R package *adeget* (Jombart, 2008). The four categories of fins microbiota, both before and after infection, were used as grouping variables. Trajectories through time for each fin were drawn using the R package *vegclust* (De Cáceres & De Cáceres, 2010) and using the DAPC axis as a projection plan. For each trajectory we tested differences in trajectory length using a KruskalWallis test combined with a post-hoc test correcting for false discovery rate. Similarly, we tested for differences in trajectory direction (relying on a comparison of pairwise Euclidean distances between trajectories) using a MANOVA (multivariate analysis of variance) followed by pairwise permutation tests (1000 permutations, corrections for false discovery rate) using the R packages *vegan* and *RVaideMemoire* (Hervé, 2019; Oksanen et al., 2011).

### *Potential sources for host microbiota changes after infection*

To identify potential source of host microbiota changes after infection, we developed an approach inspired by (Roguet et al., 2018). First, we aimed to identify OTUs discriminating healthy and infected fins after infection. We built a Random Forest model (hereafter RF model) classifying healthy and infected fins according to their respective OTU relative abundances. To simplify the model and the interpretation, we only considered the microbiota sampled on healthy fins from fish in a cage where no infection occurred compared to infected fins that we contrasted to the microbiota sampled from infected fins (i.e., the two most contrasted modalities). We set the number of trees to 10 000 and estimated the best *mtry* value to 134. Because we had an unbalanced experimental design, we constrained the number of samples used to achieve classification to two third of the minimum sample size (i.e., 12) and the remaining third was used to estimate the model error referred as to Out Of Bag (OOB) error. We then identified OTUs that contributed the most to the discrimination between infected and healthy fins using the *rfPermute* R package to compute p-values through permutation tests (1 000 permutations) (Archer, 2019). The most contributing OTUs were those with Gini-index p-values lower than 5% (Breiman, 2001). We further evaluated the ability of these candidate OTUs to discriminate fins by building a refined RF model (number of trees=4000 and *mtry*=26) and compared the OBB error rate of the refined RF models to that of the RF model including all OTUs. Secondly, we searched for potential sources of OTUs characterizing the microbiota of healthy and infected fins by screening the microbiota composition of the environment (i.e., water samples after infections), the adult parasites and the parasite larvae. We hypothesised that if one of these sources contributes significantly to either the healthy or infected fin

microbiota, the relative abundances of candidate OTUs found in the potential source(s) should significantly match those found in either healthy or infected fins. We used the refined RF model described above and classified the samples from the three potential sources as either healthy or infected fins. The most likely source(s) should be classified as either healthy or infected fins consistently through samples. Significance of the final classification was tested with a Chi-squared test.

## **Results**

### ***Association between parasitic infection and host microbiota***

We compared the host microbiota *after* infection among the four fin categories: infected fins, healthy fins from an infected fish, healthy fins from a healthy fish in a cage where at least one infected fish was observed, and healthy fins from a healthy fish in cages where no infected fish was observed. We identified significant differences in microbiota composition between infected fins and healthy fins (either from infected fish, healthy fish or healthy cage) ( $F=4.395$ ;  $df=3,277$  and  $p=0.001$ ). Pairwise comparisons revealed differences between each fin categories except for the comparison between healthy fin from a healthy fish in an infected cage and healthy fin from an infected fish (Table 1). This indicates that infected fins differed in their microbiota composition compared to all other types of healthy fins, but also that some differences existed among some healthy fins from the different categories. Nonetheless, the dissimilarity estimates were twice higher for comparisons involving infected fins and healthy fins (mean Bray-Curtis=0.353) than for comparisons involving two types of healthy fins (mean BrayCurtis=0.186, Table S1), showing that differences between infected fins and healthy fins were higher than among categories of healthy fins. This was confirmed through a visual inspection of the relative abundance of the most predominant bacteria families, as the strongest differences were observed for infected fins, with a noticeable predominance of bacteria from the *Burkholderiaceae* family in infected fins, while healthy fins (any categories) were characterized by a predominance of *Neisseriaceae* (Fig. 1). The partitioning of dissimilarity revealed that all the dissimilarity measured among fin categories was attributed to balanced variation in species abundances, which highlighted changes in the relative abundance of bacteria OTUs among fin categories (nestedness), rather than changes in the composition of bacteria OTUs (turnover) (Table S1). We did not find a consistent association between OTU richness and parasite infection. We indeed identify significant alpha-diversity differences (chi-squared = 16.809,  $df = 3$ ,  $p\text{-value}<0.001$ ) but pairwise comparisons revealed significant differences only between healthy fins of fish kept in healthy cage and other categories of healthy fins (on fish kept in a parasitized cage, or on a fish that have been parasitized on another fin, see Table S2). At the contrary no significant alpha diversity differences were observed between healthy fins and parasitized fins (see supplementary Fig S1 and Table S2).

### ***Fin microbiota before parasite infection***

We did not identify significant differences in microbiota composition of host fins *before* infections occurred ( $F=1.371$ ;  $df=3,214$  and  $p\text{-value}=0.074$ ). The microbiota of fins that turned out to be infected later on were similar at the onset of the experiment to those of fins that stayed un-infected (irrespective of the category of fins). The most abundant bacteria families were the same in the different categories of fins before infection (Fig. 2). Similarly, no significant differences in term of alpha diversity were observed between the different categories of fins (chi-squared = 0.62159,  $df = 3$ ,  $p\text{-value} = 0.892$ ).

### ***Host microbiota composition after parasite infection***

We found significant differences between the trajectories of bacteria communities associated with infected fins compared to those associated with healthy fins. Specifically, we found that changes –from *before* to *after* infection– in bacteria communities of infected fins were significantly stronger in magnitude (i.e., difference in term of trajectory lengths) than changes measured in all other healthy fin categories (Kruskal-Wallis chi-squared = 69.168,  $df = 3$ ,  $p\text{value}<0.001$ ; Fig. 3, Table S3). Similarly, the trajectory direction observed for the bacteria communities of infected fins was significantly different from that of all other categories of healthy fins (Manova  $F=131.8$ ;  $df=3,206$ ;  $p\text{-value}=0.001$ ; Fig. 3, Table S3). Overall, these results suggest that infections strikingly modified the microbiota of fins on which the parasite anchors, and that these changes were more important than those naturally occurring over time in fins that remains healthy (even when these fins were sampled on an infected fish) (Fig. 3).

### **Potential source for host microbiota changes after infection**

The first RF model classifier (based on all OTUs) achieved satisfying assignment success (OOB was 13.41% and class errors were 12.50% and 16.67% for healthy and infected fins respectively). Up to 188 OTUs significantly contributed to the decrease in the Gini index, and hence to the purity of the RF model (Table S4). The significance of these candidate OTUs was further validated by the refined RF model relying only on these 188 candidate OTUs, since the refined model achieved an even lower OOB error (OOB was 7.32 % and class errors were 6.25 % and 11.11% for healthy and infected fins respectively). We were hence able to classify infected vs. healthy fins after infection based on a few highly discriminating OTUs (188) with a high accuracy.

When considering these candidate OTUs, we found that the *Burkholderiaceae* family was abundant in the microbiota of infected fin, adult parasites and parasite larvae (16%, 15% and 5% respectively), whereas this family was rare in the environment (0.8%) and in the microbiota of healthy fins (0.5%) (Fig. 4). We further investigated the similarity of potential sources of bacteria (i.e., environment, adult parasite, infective parasite larvae) either with healthy fins or infected fins using the refined RF classifier model. Attributions resulting from the RF classifier were significantly different from randomness (Chi-squared=36.383, df=2, p-value<0.001). Specifically, we found that the microbiota associated with parasite adult and larvae were classified more often than by chance in the same class as infected fins (Chi-squared = 30.118, df = 1, p-value < 0.001 and Chi-squared = 13, df = 1, p-value = 0.001 respectively, Fig. 5). For instance, two of the four OTUs that mostly contributed to the purity of the RF classifier (i.e., OTU-15 and OTU-10, Fig. S2-a) showed higher relative abundances in infected fins, in adult parasites and more strikingly in parasite larvae, and both belonged to the *Burkholderiaceae* family (Table S4 and Fig. S2-b). For the two other OTUs (1 and 3), their abundances were on the contrary lower in the infected fins, adult parasites and parasite larvae than in the environment and on the healthy fins. These two OTUs belonged to the *Neisseriaceae* and the *Actinomycetaceae* families respectively (Table S4).

### **Discussion**

We tested whether infection by an ectoparasite of freshwater fish was associated with a particular host microbiota, and assessed the causal role of host microbiota in parasitic infection. We identified striking differences in the microbiota composition of host fins associated with parasite infection. Host microbiota differences did not exist prior to infection but were rather triggered during infection and were restricted to the fin where the parasite anchored (i.e., they were not generalized across the host). The microbiota of infected fins also shared strong similarities with parasite microbiota (in particular the larvae), which likely suggests a coinfection dynamic.

The composition of host-associated microbiota changed when hosts were infected by *T. polycolpus*. We compared the host microbiota of infected fins from that of healthy fins from various categories, which allowed testing whether the effects of parasite infection on the host microbiota are local (only the infected fin) or more general (the infected host and/or neighbor fish). Microbiota differences existed between healthy fins after infections, but the strongest community dissimilarity was observed between infected fins and all type of healthy fins. This suggests that the effects of parasite infection on host microbiota is local. We further showed that these dissimilarities resulted from balance variation in species abundance (nestedness-like patterns) (Baselga, 2013) which is congruent with the lack of differences in term of OTU richness. Infected fins were characterized by a higher abundance of *Burkholderiaceae* bacteria, whereas healthy fins were mostly characterized by *Neisseriaceae* bacteria. These conclusions were confirmed by the Community Trajectory Analysis and the Random Forest models. The former analysis established a specific community trajectory over time of infected fins compared to that of healthy fins that were relatively homogeneous among healthy fins from the different categories. The RF models accurately discriminated healthy fins (from healthy fish in healthy cages) and infected fins based on the relative abundance of a set of candidate OTUs that included OTUs from the *Burkholderiaceae* and *Neisseriaceae* families. All these lines of evidence strongly suggest that the fin microbiota of infected dace differ from that of healthy dace (and fins), which aligns with previous studies having investigated the association between parasitism and host microbiota (Avelo & Norberg, 2018; Bär et al., 2015; Portet et al., 2018; Reynolds et al., 2015).

We further showed that the differences in fin microbiota observed between infected fins and healthy fins resulted from infection rather than pre-existed to it. When comparing the same host fins

before and after the infection period, we indeed identified important changes in host microbiota that occurred only for fins that became infected. On the contrary, prior to infection, no significant differences were noticeable between fins that became infected and those that remained healthy, and the most abundant families were stunningly the same (Fig. 3). Previous studies suggested that some host microbiota can promote parasite infection, by providing suitable conditions for parasite development (Hayes et al., 2010), by lowering host resistance to parasite (Britton & Young, 2014; Buffie & Pamer, 2013) or by serving as cues used by the parasite to track their host (Chavez et al., 2021; Speer, 2022). In our study, both host and environmental factors were experimentally controlled. Thus, if some microbiota may favor future parasitic infection, this microbiota might be built upon multiple factors, notably prior differences between host in term of genetic background and/or life history traits, and/or differences in the environment in which hosts are living.

Hosts microbiota changed locally at the infected fin level rather than at the infected host level. Changes in the host microbiota over the time of infection have already been reported in other host-parasite interactions (Afrin et al., 2019; Gaulke et al., 2019). Yet, these changes in microbiota can result from different scenarios (Mooney et al., 2015; Reynolds et al., 2015; Swe et al., 2014). *Tracheliastes polycolpus* infection could have for instance weakened the host immune response (Lochmiller & Deerenberg, 2000; Sandland & Minchella, 2003), which may favor fin colonization by opportunistic bacteria from the environment (Mooney et al., 2015). In that case, the general health status of the host is impacted, and opportunistic colonization by bacteria is expected on the entire host (i.e., a “generalized” response). Alternatively, *T. polycolpus* could have disrupted the fin bacteria communities according to a *weapon strategy* (i.e., a “localized” response, eg., (Swe et al., 2014)). In that case, microbiota disruption is directly caused by the parasite (not by the infection process) that can bring new bacteria taxa. Dace microbiota changes occurred on the fin where *T. polycolpus* is anchoring, which suggests that *T. polycolpus* is following a *weapon strategy*.

Finally, we investigated the source of host microbiota after infection and deduced that host microbiota was probably transmitted (at least partly) directly by the parasite. The microbiota of infected fins was highly similar to that of both adult parasite and the free-living infective parasite larvae. We can suppose that some specific bacteria strains, including from the *Burkholderiaceae* family are intimately linked to the host-parasite couple and are maintained via some transfers from the infective parasite larvae and the adult parasite to the host. We believe that these bacteria are transmitted and maintained in the parasite larvae since those examined in this study were hatched and reared in a common and controlled environment before their microbiota were analysed (with no contacts with the host nor natural environment). Those shared bacteria between infected fin and *T. polycolpus* may result from a co-infection dynamics between the parasite and its associated microbiota (Dheilly et al., 2015; Reynolds et al., 2015). A taxonomic investigation further revealed that the *Burkholderiaceae* family, which was characteristic of infected fin, was also quite prevalent in parasite microbiota (adult and larvae, 15% and 5% respectively). Contrastingly, this family was extremely rare in the microbiota of healthy fins and of the environment (<1%). In agreement with a *weapon strategy* (Reynolds et al., 2015), we suggest that bacteria from the *Burkholderiaceae* family are carried by the parasite and transmitted directly to the host during infection, which results in a major modification of the microbiota of infected fins. Those *Burkholderiaceae* bacteria could be commensal of *T. polycolpus* or beneficial for the parasite. For instance, in (Flórez et al., 2017) the authors shown that the symbiotic *Burkholderia gladioli* bacteria, provided two antifungal compounds to its beetle host while being also vertically transmitted to the larvae stages. However, the functional role of a bacteria species remains very heterogeneous within bacteria families, and it is difficult to draw clear conclusions on the role of *Burkholderiaceae* bacteria for *T. polycolpus*. Future work avenues hence relate to i) specifying the candidate bacteria species that are facilitating *T. polycolpus* infection and ii) their functional role in the infection process.

Abundant literature regarding host microbiota related to parasitic infections provide evidence of correlative links between host microbiota and parasite infections (Jenkins et al., 2018; Lutz et al., 2022; Speer, 2022; Stensvold & van der Giezen, 2018). Our results provide one of the few evidences that it is possible to dissect more accurately the way the microbiota participates to the host-parasite interaction in natural settings. In particular, the host microbiota seems to be more than the extended phenotype of the host whose changes are triggered by parasite infection. We are notably emphasising that parasite associated bacteria are very likely to be shared with the host during infection. Hence, microbiota (in particular that of the parasites) appears to be an important intermediary in host-parasite

interactions, if not an active actor of these interactions. Our study adds upon other empirical evidences showing that some components of host-parasite interactions are actually directly mediated by the microbiota. For instance Chavez et al. (2021) showed that host cue used by the some skin-penetrating nematodes to localize their host are actually emitted by host microbiota rather than by the host *per se*. Hence, we argue that host but also parasite microbiota are critical components of host-parasite interactions and notably of their dynamics. For instance, one could question the role of host microbiota during repeated infections. If a parasite such as *T. polycolpus* actively participate to host skin microbiota changes, infected host with these altered microbiotas could be more prone to future infections. Our results hence pave the way for future research avenues integrating host and parasite microbiota as a critical actors or host-parasite interaction.

To conclude, we conducted one of the first individual-based surveys describing changes in host microbiota over the course of a parasite infection under natural conditions. We unambiguously demonstrated that the parasite *T. polycolpus* induces shifts of host microbiota composition at the fin level. We suggest a possible co-infection dynamic between parasite and its associated bacteria and that a complex interaction between host, parasite, environment, and respective microbiota is shaping the infection process. We argue for further investigations of these complex interactions in natural setting to improve our knowledge of the relative role of parasite-associated microbiota in infection success.

### **Acknowledgements**

We sincerely thank the people who dedicated their time to this project. We are very grateful to Kéoni Saint-Pé, Murielle Richard, Alexandre Garreau and Eloise Duval who contributed to the experiment and data collection. We also thank Thierry Paulin who gently allowed us to conduct our caging experiment on his property and Antoine Gallard who provided the dace along with instructions to take good care to them. We thank the reviewers for insightful comments.

This work was founded by the Labex Tulip Project 2019, Parasite-microbiota interaction, Variability in fish fins-associated microbiota: a serious side effect or a real chance for parasites? EMB was supported by a PhD grant from the French Ministry for Education and Sciences. This study was supported by the « Laboratoire d'Excellence (LabEx) » TULIP (ANR-10-LABX-41).

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**Data accessibility and benefit sharing statement**

Dataset necessary to interpret and replicate the analyses conducted in this study along with raw sequencing data and a description of fish used in the experiment have been released in a figshare repository (<https://doi.org/10.6084/m9.figshare.20073005>, Mathieu-Bégné et al. 2023)

No benefit is to be reported for the present study.

**Authors' contributions**

EMB, SB, OR, ET and GL developed the original idea, supervised and coordinated the study.

EMB, SB, ML, OS and GL conducted the caging experiment and realized the sampling. CV, SM and GL prepared the libraries for sequencing. EMB and ET defined the bioinformatic protocol. EMB ran the statistical analysis. EMB, SB, OR, ET and GL wrote the manuscript. All authors approved the final version of the manuscript, and all agree to be held accountable for the content therein.

**Competing interest statement**

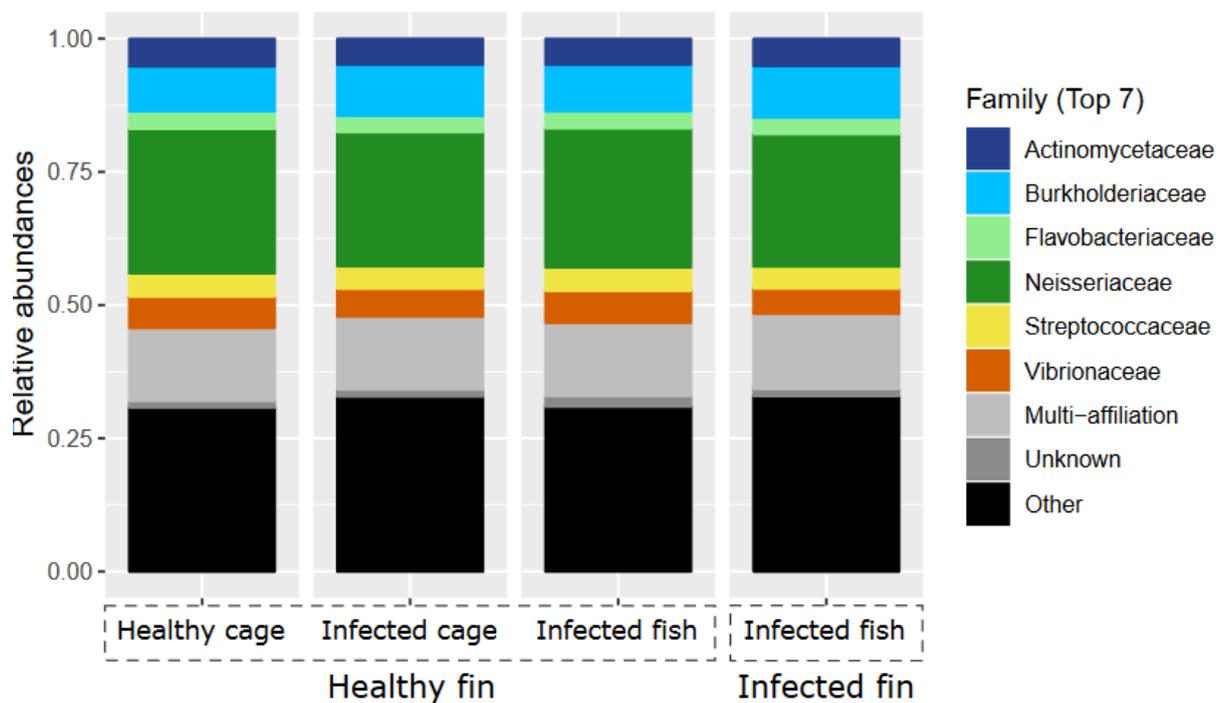
We have no competing interest.

**Figure captions**

**Table 1: Permutational Multivariate Analysis of Variance pairwise comparisons.** For each comparison (a–f) degree of freedom (Df), test statistic (F) and the p-value are given.

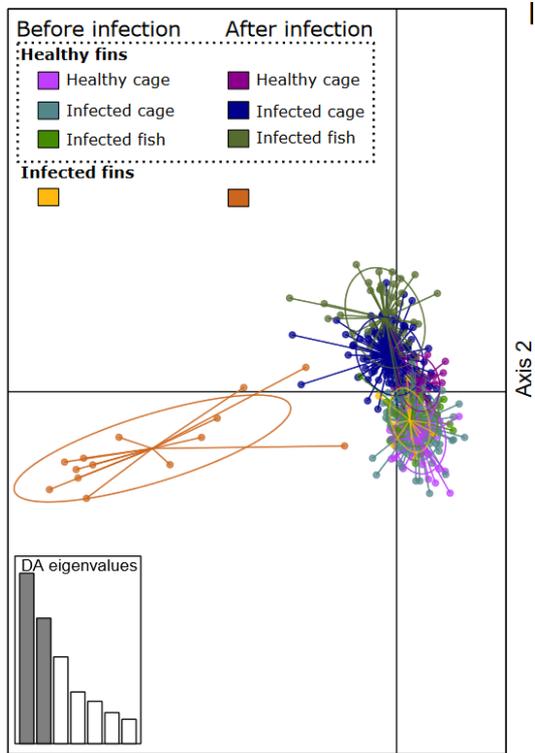
	<b>Df</b>	<b>F</b>	<b>Pr(&gt;F)</b>
<b>a) Infected fin vs healthy fin from an infected fish</b>	1,760	3.966	0.001
<b>b) Infected fin vs healthy fin from a healthy fish in an infected cage</b>	1,155	6.501	0.001
<b>c) Infected fin vs healthy fin from a healthy fish in a healthy cage</b>	1,810	6.943	0.001
<b>d) Healthy fin from an infected fish vs healthy fin from a healthy fish in an infected cage</b>	1,198	1.776	0.051
<b>e) Healthy fin from an infected fish vs healthy fin from a healthy fish in a healthy cage</b>	1,123	5.245	0.001
<b>f) Healthy fin from a healthy fish in an infected cage vs healthy fin from a healthy fish in a healthy cage</b>	1,202	3.943	0.002

**Figure 1:** Microbiota composition of host after infection. For each category of fin (infected fin, healthy fin from an infected fish, healthy fin from an healthy fish in an infected cage and healthy fin from an healthy fish in a healthy cage) microbiota composition is drawn at the family level for the seven most prevalent families. Relative abundances over the total bacteria community are displayed.

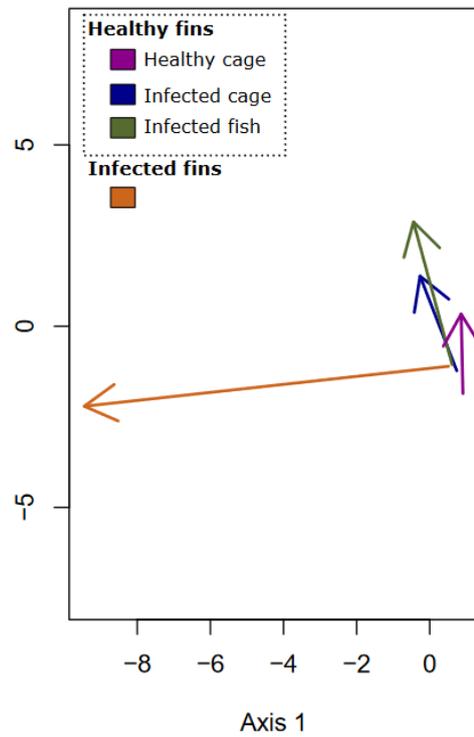


**Figure 2:** Microbiota composition of host before infection. For each category of fin (infected fin, healthy fin from an infected fish, healthy fin from an healthy fish in an infected cage and healthy fin from an healthy fish in a healthy cage) microbiota composition is drawn at the family level for the seven most prevalent families. Relative abundances over the total bacteria community are displayed.

a)

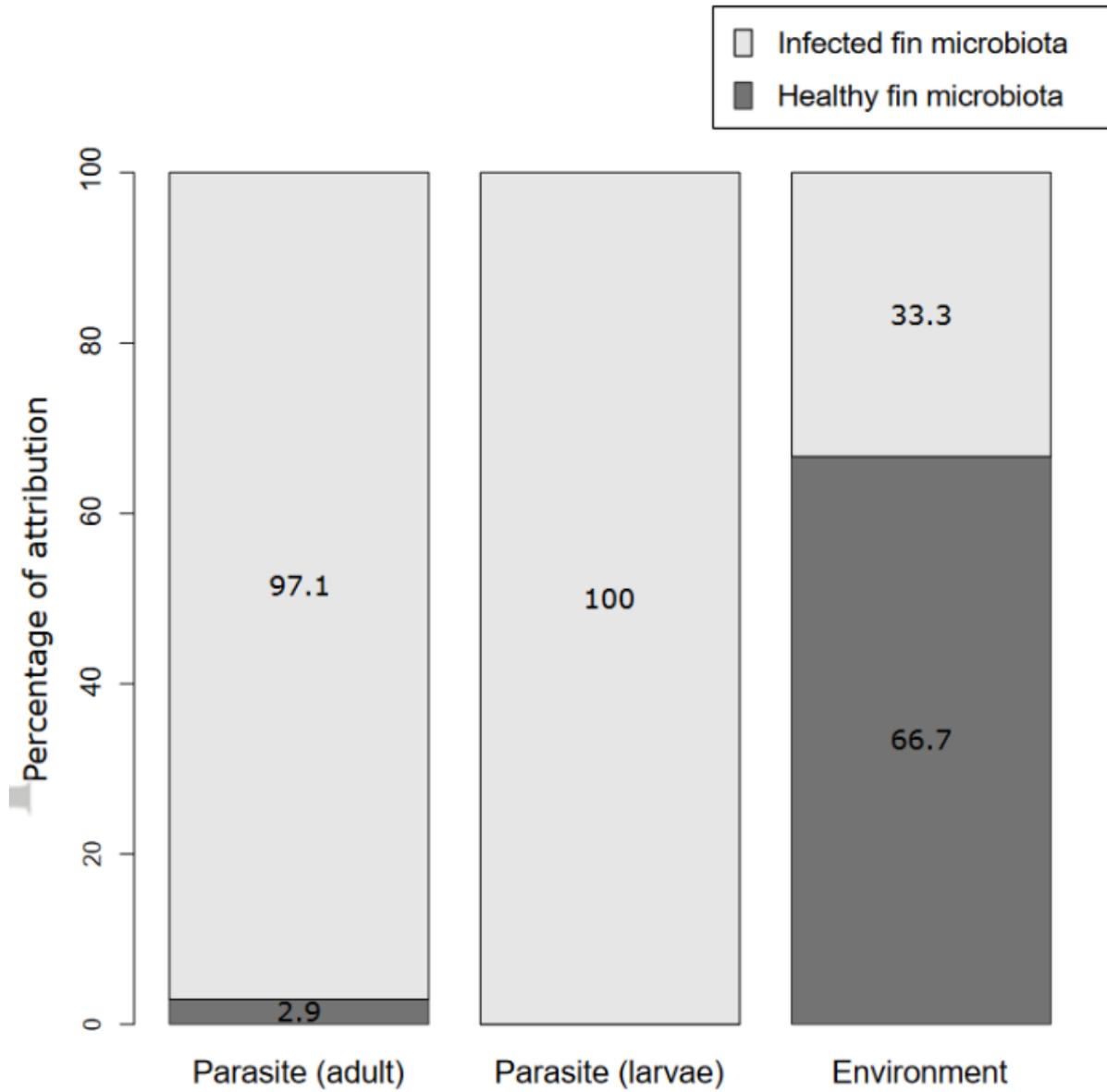


b)





**Figure 4:** Microbiota composition, associated with infected fins, healthy fins, adult parasite, parasite larvae and surrounding environment. Microbiota composition is drawn at the family level and for candidates OTUs significantly discriminating healthy fin associated microbiota from infected fin associated microbiota. Relative abundances over the total community are displayed.



**Figure 5:** Barplot showing the percentage of samples belonging to each potential source (environment, adult parasite and parasite larvae) attributed either to the same class as microbiota associated with healthy fin or as microbiota associated with infected fin. Attributions are based on the random forest model built on candidate OTUs and classifying microbiota associated with healthy or infected fins.

