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Bacterial community dynamics following antibiotic exposure in a trematode parasite

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ABSTRACT

Parasites harbour rich microbial communities that may play a role in host-parasite interactions, from influencing the parasite's infectivity to modulating its virulence. Experimental manipulation of a parasite's microbes would be essential, however, in order to establish their causal role. Here, we tested whether indirect exposure of a trematode parasite within its snail intermediate host to a variety of antibiotics could alter its bacterial community. Based on sequencing the prokaryotic 16S ssrRNA gene, we characterised and compared the bacterial community of the trematode *Philophthalmus attenuatus* before, shortly after, and weeks after exposure to different antibiotics (penicillin, colistin, gentamicin) with distinct activity spectra. Our findings revealed that indirectly treating the parasites by exposing their snail host to antibiotics resulted in changes to their bacterial communities, measured as their diversity, taxonomic composition, and/or the relative abundance of certain taxa. However, alterations to the parasite's bacterial community were not always as predicted from the activity spectrum of the antibiotic used. Furthermore, the bacterial communities of the parasites followed significantly divergent trajectories in the days post-exposure to antibiotics, but later converged toward a new state, i.e. a new bacterial community structure different from that pre-exposure. Our results confirm that a trematode's microbial community can be experimentally altered by antibiotic exposure while within its snail host, with the dynamic nature of the bacterial assemblage driving it to a new state over time after the perturbation. This research opens new possibilities for future experimental investigations of the functional roles of microbes in host-parasite interactions.

1. Introduction

The rich microbial communities, or microbiota, harboured by animals are increasingly recognised as playing major roles in their development, health and behaviour (Diaz Heijtz et al., 2011; Feldhaar, 2011; Ezenwa et al., 2012; McFall-Ngai et al., 2013). In the context of host-parasite interactions, symbiotic microbes of the host can affect the expression of its immunity against parasites, and the outcome of infection (Hooper et al., 2012; Koch and Schmid-Hempel, 2012). Hosts are not alone in harbouring microbes, however: parasites ranging from arthropods to helminths also possess their own microbiota, distinct from those of their hosts or the external environment (Wilkinson et al., 2016; Ben-Yosef et al., 2017; Sinnathamby et al., 2018; Hahn et al., 2020; Jorge et al., 2020, 2021). This has important implications; for instance, parasite-associated microbes represent potential targets for the development of novel chemotherapeutics against parasitic diseases (Jenkins et al., 2019).

Microbial communities within parasites may also affect the parasite's infectivity, virulence, or other aspects of its phenotype and interaction with the host (Dheilly et al., 2015, 2019). However, to demonstrate the causal roles of microbes in shaping the biology of parasites and their interactions with their host, one would require an experimental approach in which the microbial community of a parasite is manipulated (e.g., experimental removal of certain microbial taxa) and the parasite's performance is subsequently monitored in comparison with a control group. For example, using such an approach, Martinson et al. (2020) showed that treating a parasitic nematode with antibiotics that eliminated certain bacteria from its microbial community caused a severe reduction in the parasite's infection success. Experimental manipulation of parasite

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microbial communities can be logistically difficult, however. For instance, exposure of parasites to antibiotics may be difficult to achieve in-vitro outside of the host, such that the only way to administer the treatment may also affect the microbial communities harboured by the host itself. Disruption of the microbial communities in either the host or parasite from their "stable" state can have implications for the host-parasite association, and distinguishing between effects originating from the two microbial communities can be important. In this context, working with trematodes presents some advantages. Most trematodes multiply asexually within a snail intermediate host, to produce and release cercariae, the infective stages that continue the life cycle in the subsequent host (Galaktionov and Dobrovolskij, 2003). Therefore, even if exposure of infected snails to antibiotics alters the snails' microbial communities, as long as the antibiotics also induce changes in the parasites' microbial communities, their consequences for the rest of the life cycle can still be quantified by tracking the performance of the parasites after they leave the snail.

Exposure of hosts and parasites to antibiotics can nevertheless have unforeseen consequences. Microbial communities are notoriously dynamic, from their original assembly to their recovery following disturbances (Costello et al., 2012). Resilient microbial communities can recover from a disturbance event at either taxonomic and or functional levels, i.e. by returning to the same taxonomic composition or by recruiting new taxa that perform functions previously assumed by the original members of the community (Dogra et al., 2020). Furthermore, disruptions caused by antibiotics may in fact kill the target microbes, but may also lead to several other changes to the community by creating opportunities for the remaining members to increase in abundance or for colonisation by new microbes (Costello et al., 2012). However, lacking knowledge of microbe-microbe interactions, it is difficult to predict whether the microbial community will be permanently altered, or will return to its previous state. The stability and resilience of microbial communities may depend on which specific microbes are eliminated, as removal of some microbes may have differential disruptive impacts on community structure (Gould et al., 2018). Thus, it is crucial to monitor microbial community composition over time following treatment with antibiotics.

Here, we investigate how the bacterial communities of a parasitic trematode react to different antibiotic treatments, and if and how they recover in similar ways. We tested four different antibiotic treatments and assessed the changes they induced in the bacterial community composition of *Philophthalmus attenuatus* exposed indirectly to each treatment. We focused on bacterial communities within the cercariae-producing stages of the parasite, i.e. rediae, exposed to antibiotics while inside their snail host (*Zeacumantus subcarinatus*). We determined the degree of community resilience of these communities among the different treatments measured as the degree to which the post-disturbance community returned to its former state in terms of community composition. Finally, we compared the degree of bacterial community disruptions incurred by both parasites and their respective hosts. Overall, our study provides a proof of concept that exposure of parasites within their host can induce alterations to the parasites' bacterial communities.

2. Material and methods

2.1. Study design and sample preparation

Zeacumantus subcarinatus snails (shell length 14–21 mm) were collected by hand from Portobello Bay near Dunedin, South Island, New Zealand (45° 49′ 48″ S, 170° 40′ 12″ E), during the 2020 austral summer. Two types of environmental samples (water, n = 2; substrate, n = 2) and a corresponding control (n = 2) were collected with sterile cotton swabs, saved separately in a PowerBead Pro Tube (QIAGEN Ltd, New Zealand), frozen in dry ice, and stored in a −80 °C freezer once back in the laboratory. Sea water was also collected in sterile containers for maintenance of snails during the experiment. In the laboratory, within 1 day after collection, snails were placed in individual sterile wells of tissue culture plates with natural sea water, and incubated for 2 days at 25 °C under light to identify *P. attenuatus*-infected individuals through cercarial shedding. Only snails infected solely with *P. attenuatus* (i.e. not those also infected by other trematode species) were used in this study. Infected snails were then placed in a common sterile 5 L plastic container with seawater and oxygen for 24 h.

The experimental design consisted of characterising the bacterial communities of *P. attenuatus* rediae and of tissue from their host snail at three different time points: pre-antibiotic treatment (T0), post-antibiotic treatment (T3), and at 77 days after the beginning of the experiment (T77; see Fig. 1A). Fifteen snails were randomly selected for characterisation of bacterial communities at T0, while a total of 180 infected snails (36 snails per treatment; see below) were randomly selected for the antibiotic manipulation experiment. Prior to antibiotic exposure, each snail was labelled with a coloured numbered bee tag (Queen Numbering Kit, Ecotrek, New Zealand). At the end of antibiotic exposure (T3), 10 snails were randomly sampled from each treatment for bacterial community characterisation. All remaining snails were placed in a common sterile 5 L plastic container with natural seawater and oxygen until the end of the study. They were fed ad libitum with thoroughly rinsed sea lettuce (*Ulva* spp.) obtained from the same site where snails were collected. At the end of the experiment (T77), 10 snails were randomly sampled from each treatment for bacterial community characterisation. Samples of the sea water from the communal tanks in which snails were kept at T0 and T77 (Fig. 1A) were taken with sterile cotton swabs. Metadata for all samples including sample type, treatment and time point are given in Supplementary Tables S1–S7.

2.2. Antibiotic treatment

Three different antibiotic solutions were used according to their activity spectra: Penicillin G potassium salt (5 g L^{-1}, Sigma P8721; Sigma-Aldrich, New Zealand) targeting Gram-positive bacteria, Colistin sulphate salt (25 mg L^{-1}, Sigma C4461) targeting Gram-negative bacteria, and Gentamicin (50 mg L^{-1}, Sigma G3632) targeting both Gram-positive and Gram-negative bacteria and mycoplasma. The combination of Colistin and Penicillin (2.5 g L^{-1} and 12.5 mg L^{-1}, respectively) was used as a fourth treatment. Antibiotic doses were chosen based on recommendations from the manufacturer of the antibiotic or based on other studies on other organisms. For colistin, a preliminary experiment was conducted prior to the main study because no reference for a dose was available in the literature. The highest concentration that would not kill the tested snails was chosen. The antibiotic solutions were prepared using natural seawater, and were sterilised by filtration with a 0.2 μm sterile filter. A fifth solution served as the control and consisted of filtered natural seawater. Snails from the same treatment were maintained in a common sterile 5 L plastic container, with the antibiotic solutions and control seawater replaced every 24 h for 3 days; afterward, snails were kept in natural seawater.

2.3. Sample collection, processing and sequencing

Three different sets of samples corresponding to time points T0, T3 and T77 were independently prepared for isolation, extraction and sequencing. Prior to dissections, snails were brushed with a sterile interdental brush in 70% EtOH, and rinsed thoroughly in

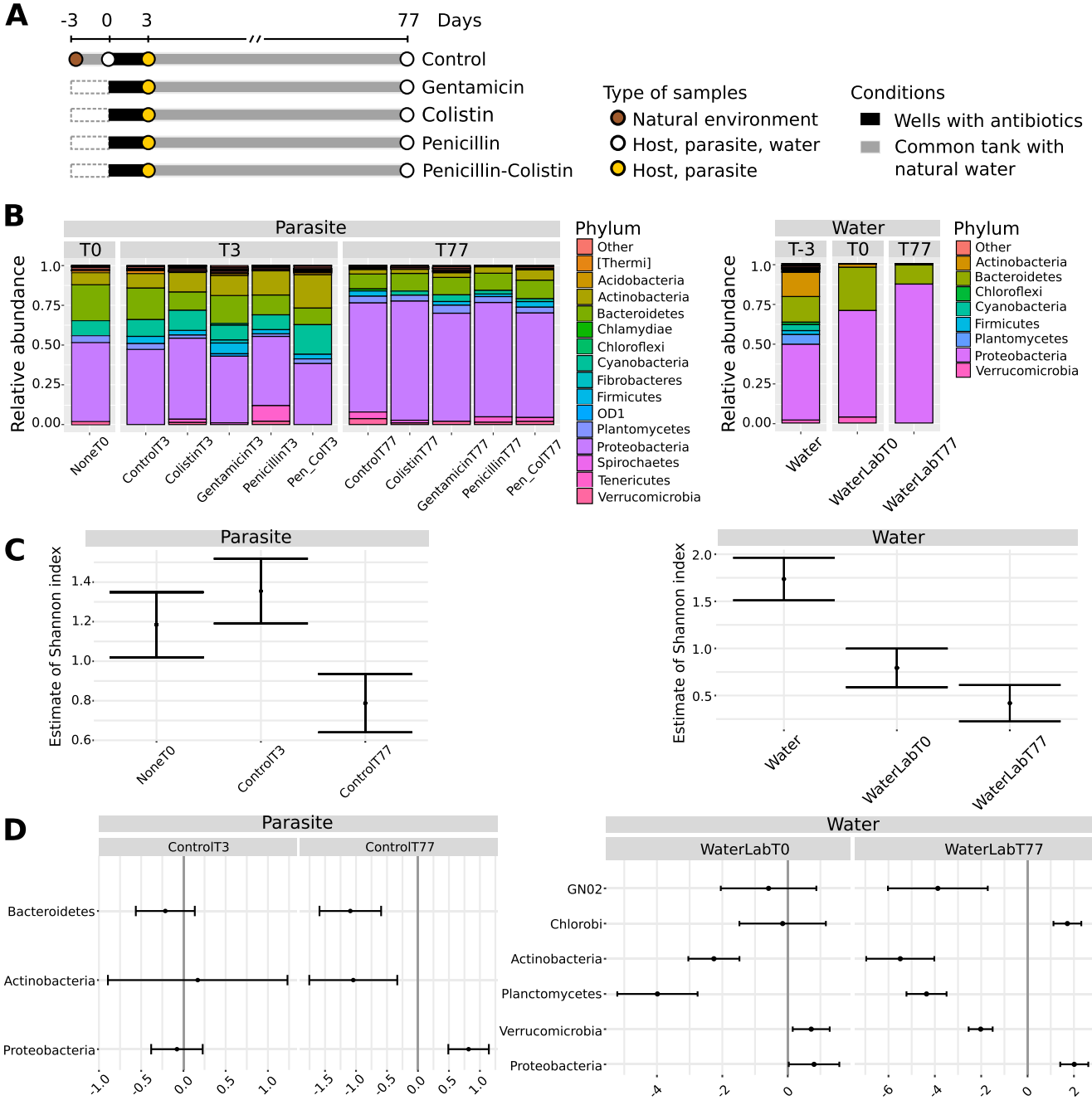

Fig. 1. Laboratory conditions can affect bacterial community composition. (A) Design of the antibiotic experiment. Circles denote sampling points for parasites, hosts or water for 16S amplicon data. Time points: –3 – sampling in natural habitat, 0 – pre-antibiotic treatment, 3 – post-antibiotic treatment, 77 – end of experiment. Type of samples: brown circle – natural environment (water and substrate); white circle – host, parasite and water; yellow circle – host and parasite. Conditions: black – snails kept in individual wells, grey – snails kept in a communal tank. (B) Relative abundance of phyla across parasite and water samples at the three sampling points. 'Other' represents all taxa whose relative abundances are less than 1% of the total abundance. pen_col, penicillin and colistin. (C) Alpha diversity dynamics as estimated with Shannon diversity index at phylum level along the duration of the experiment for both parasite and water samples. Error bars represent the 95% confidence interval for the estimated means. (D) Differential abundance of phyla along the course of the experiment compared to first parasite and water samples. For water, the first sampling point represents water sampled in the natural habitat. The magnitude of model coefficients is shown on a log-odds scale; error bars represent the 95% prediction interval.

sterile PBS under a sterile laminar flow cabinet. Parasite samples consisted of individual rediae isolated from snail tissue (T0, n = 28 rediae; each T3 treatment n = 9–10, T3 control n = 9; each T77 treatment n = 10, T77 control n = 10); only large, cercariae-producing rediae were used in this study, and not the smaller morphs (see Leung and Poulin, 2011). Small sections of snail gonad tissue adjacent to where parasite samples were collected were also isolated (T0, n = 15; each T3 treatment n = 2–3, T3 control n = 2; each T77 treatment n = 3, T77 control n = 3). All samples were cleaned from surface microbiota by repeatedly pipetting up and down in PBS in sterile wells. Samples of the surface microbiota for each sample type were collected from the resulting 'washing' (75 µl, two samples per parasite and host treatment group). At the end of each isolation procedure a control sample of the PBS solution was taken to account for any possible contamination. DNA from parasite, snail host tissue and environmental swabs was extracted using the DNeasy PowerSoil Pro Kit (QIAGEN), with modifications recommended for cells difficult to lyse by the Earth

Microbiome Project DNA Extraction Protocol (Marotz et al., 2017). Together with the isolated biological samples, ZymoBIOMICS microbial community standards samples (MCS and MCS DNA), and reagent-only samples were also included at both extraction and amplification steps. Metabarcoding libraries targeting the V4 hypervariable region of the prokaryotic bacterial 16S ssrRNA gene were prepared as described in Jorge et al. (2020). Each barcoded libraries pool was sequenced on an Illumina MiSeq platform using the V2 reagent cartridge (250 bp, paired-end) through the Otago Genomics & Bioinformatics Facility (New Zealand).

2.4. Bioinformatics

All bioinformatics procedures were performed using various built-in plugins from the QIIME2 software package (qiime2-2020.2-py36-linux, Bolyen et al., 2019). Paired-end reads from each library pool were processed and quality filtered separately. Adapters and primers were removed from raw sequences using the plugin *cutadapt* (with 0 error-rate and minimum length of 240 bp; Martin, 2011), and quality filtered using the *dada2* plugin (Callahan et al., 2016). In order to be able to later combine different sequencing runs, the quality profile plots were inspected for each run separately and the required trimming parameters selected, however the most stringent values of trimming parameters were applied across the three sequencing runs. The resulting amplicon sequence variant (ASV) tables were filtered to exclude non-bacterial, mitochondrial, chloroplast, ASVs without a phylum assignment, contaminants (i.e. ASVs found in blanks and exclusive to the laboratory environment), ASVs found in the corresponding cleaning PBS for each life stage, and samples with low sequencing depth (i.e. frequency lower than 1000 and/or with less than eight ASVs) using the *feature-table* plugin. The final dataset was assembled by merging the three feature tables and representative ASVs. Different taxonomic levels were assigned to the ASVs using the plugin *feature-classifier* (Bokulich et al., 2018) against the Greengenes 16S rRNA reference database (13_8 release) pre-trained on the 515F/806R region (Pedregosa et al., 2011). We further estimated closed-reference operational taxonomic units (OTUs) at a 97% similarity threshold against the 99% identity clustered Greengenes database using the VSEARCH QIIME2 plugin to predict Gram staining phenotype in BugBase (Ward, T., Larson, J., Meulemans, J., Hillmann, B., Lynch, J., Sidiropoulos, D., Spear, J., Caporaso, G., Blekhman, R., Knight, R., Fink, R., Knights, D. 2017. BugBase predicts organism level microbiome phenotypes. bioRxiv https://doi.org/10.1101/133462.).

2.5. Statistical analyses

All analyses were conducted in the R environment (R Core Team, 2021); unless otherwise specified, P values <0.05 are considered significant. We used ecological network regression models to test the effect of the two experimental factors (treatment or time) on estimates of Shannon diversity and Bray-Curtis distances using the R packages DivNet and Breakaway (Willis et al., 2017; Willis and Martin, 2020) at Family, Class and Phylum levels. Since many taxa do not occur in all samples, a perturbation parameter value of 0.5 was included in all analyses using the function *netdiv()* (Cao et al., 2019; Willis and Martin, 2020). To model differential abundance of taxa between groups, two different methods were used: Count Regression for Correlated Observations (corncob, Martin et al., 2020), and the linear discriminant analysis effect size (LEfSe, Segata et al. 2011). Corncob uses a beta-binomial model to assess both differential abundance and differential variability of microbial taxon abundances across groups. We tested all taxa using the function *differentialTest()*, while accounting for multiple comparisons. LEfSe analysis is a class comparison method that estimates the sizes of the significant variations. LEfSe was run using the default normalised abundance data method, with multiple testing correction options, as implemented in the package microbiomeMarker v. 0.0.1.9000 (Cao, Y. 2021. https://github.com/yiluheihei/microbiomeMarker).

Group divergence was estimated as the average dissimilarity of each sample from the corresponding group mean (by treatment or time) with the function *divergence()* from the R package microbiome v.1.8.0 (Lahti, L., Shetty, S. 2017. http://microbiome.github.com/microbiome).

BugBase was used to determine differences in abundance (using centred log-ratio transformation) of Gram-negative versus Gram-positive bacteria among the different groups using a Kruskal-Wallis test with Benjamini-Hochberg adjustment.

Using the methods described above, we started by investigating the effect of the experimental conditions on bacterial community compositions in rediae and snails by comparing estimates of taxonomic community and Shannon diversity at T0 with control groups at T3 and T77. Variation in environmental conditions (i.e. water) was also compared between water from the sampled site versus water in the laboratory (T0 and T77).

We then assessed whether the antibiotics modified the bacterial communities according to their activity spectra, and the extent of those changes, by comparing T3 treated samples with their respective T3 controls, and making comparisons among antibiotic-treated T3 samples. Specifically, the effect of the antibiotic gentamicin was evaluated mainly by comparing prevalence of Mycoplasmataceae among treated groups, while variation in the relative abundance of Gram staining phenotype was used to evaluate the effect of all antibiotics. Estimates of differentially abundant taxa between the control and treatments were also obtained using the corncob and LEfSe methods.

Finally, we estimated the degree of dissimilarity and variability between the different time points and treatment groups to evaluate the resilience of those modified bacterial communities based on group divergence and Bray-Curtis distances, considering the variance in diversity estimates.

2.6. Data accessibility

Data used in this study are available through the NCBI BioSample Submission Portal as Bioprojects ID: **PRJNA707308 and PRJNA786706,** and their associated Sequence Read Archive data (SRA, https://www.ncbi.nlm.nih.gov/sra/).

3. Results

3.1. Data

From the initial 178 samples collected and processed (not including blank and control samples), 141 were retained after data quality control. The analysed 16S rRNA gene fragment from the three time points, T0, T3 and T77, yielded a combined average of 6134 reads (range: 1015 to 73,766, with the highest values from environmental samples) per sample after quality control. The resulting 5114 ASVs were classified into 824 genera, belonging to 449 taxonomic families. The parasite bacterial communities across the sampling points (n = 102) were dominated by Proteobacteria (55.6% ± 14.8%; Fig. 1B).

3.2. Did the experimental procedure impact bacterial community composition?

The laboratory conditions seem to have impacted the bacterial community composition of the parasite. Rediae from snails in con-

trol groups at T3 and T77 differed in mean microbial diversity from those at T0 as estimated with the Shannon index. After the 3 days in individual wells (T3), rediae in the control group showed an increased bacterial diversity relative to that estimated from the initial T0 (at phylum, class, family and genus levels: $P < 0.01$, Fig. 1C, Supplementary Tables S1–S7). However, at the end of the experiment (T77) the average diversity per redia was lower than at T0 (at phylum and class level: $P < 0.01$, Supplementary Tables S1–S7). The longer time spent in the laboratory led to greater dissimilarity of the bacterial communities, as estimated with Bray-Curtis, with rediae from T3 being more similar to those of T0 than those at T77 (Supplementary Tables S1–S7). According to corncob estimates, no phylum showed differential abundance between T0 and T3, but T77 communities had a decrease in Bacteroidetes and Actinobacteria and an increase in Proteobacteria compared with T0 communities (Fig. 1D). Bacteroidetes in both T3 and T77 communities were found to be differentially variable from those at T0. Similar analyses conducted with LEfSe found many more bacterial taxa showing statistically significant differences in abundance among the three time points, however at a higher taxonomic level the results were often consistent with corncob estimates. LEfSe estimates also supported that rediae at T0 had an enrichment of Bacteroidetes compared with subsequent time periods, and that those at T77 had an enrichment of Proteobacteria. Additionally, LEfSe also indicated that rediae at T77 had increased abundance of Tenericutes, while those at T3 had an increase in Cyanobacteria and Actinobacteria.

Analyses of bacterial community composition in laboratory water compared with that estimated from the natural environment also showed differences in diversity and abundances (Fig. 1). The highest diversity was found in the natural environment, while over the course of the experiment the water in which infected snails were kept had lower bacterial diversity (Fig. 1C). There were also several taxa which decreased in abundance while others increased compared with the bacterial community composition found in the natural environment (Fig. 1D). The significant increase in taxa belonging to the phylum Proteobacteria and the decrease in Actinobacteria in water samples from the communal laboratory tank at T77 parallel the corresponding increase and decrease also observed in the parasite samples from the same time points, albeit with a higher degree of differentiation.

3.3. Were the antibiotic treatments successful at eliminating their targeted bacteria taxa?

To test whether indirect exposure to antibiotics had an effect on the bacterial community composition of parasites within their snail hosts, we analysed community differences among groups at the end of the 3 day treatment (T3). Gentamicin was the broadest spectrum antibiotic used targeting both Gram-positive and -negative bacteria as well as Mycoplasma. All samples treated with this antibiotic did not contain any ASV assigned to the family Mycoplasmataceae, while in all other groups there were several samples containing representatives of this family (Fig. 2A). However, when assessing impacts of antibiotics on bacterial community composition based on Gram-staining classification, the results were less clear. According to BugBase's estimates based on phenotypic predictions, there were no significant changes in Gram-staining bacterial phenotype between treatment groups and the control, but there were among treatment groups (Fig. 2B). While generally penicillin mainly targets Gram-positive bacteria, penicillin-treated rediae showed significantly higher average abundance of Gram-positive bacteria than seen in gentamicin- and colistin-treated parasites. This increase in Gram-positive bacteria in penicillin-treated samples was estimated to be driven by a decrease in Proteobacteria (Gram-negative phylum), and other taxa with relatively low abundances (Fig. 2B).

The antibiotic-induced perturbation led to different changes in bacterial community diversity depending on the antibiotic used (Fig. 2C), although the direction of change varied with the taxonomic level evaluated. At both phylum and class levels, colistin-treated rediae had a lower bacterial diversity compared with the controls (phylum: estimate = −0.164, $P < 0.0001$, class: estimate = −0.148, $P < 0.0001$). At those two taxonomic levels, there was a discrepancy regarding which treatment produced an increase in diversity in comparison to that in rediae from the control group (penicillin-colistin for phylum: estimate = 0.046, $P = 0.004$, gentamicin for class: estimate = 0.043, $P = 0.025$). However, at the family level, rediae in all treatments showed a significant increase in bacterial diversity compared with those in the control group; similar findings were also obtained at the genus level (Supplementary Tables S1–S7). It is worth mentioning that when not considering treatment as a covariate (i.e. considering all samples from the same treatment as in fact originating from a different population) in the computations at the family level, the estimate was of a significant decrease in bacterial diversity in rediae from all treatments compared with the control group (data not shown).

When comparing abundance and variability of bacterial taxa between rediae in the control group and those in different antibiotic treatment groups, only Gram-negative taxa seem to vary. While according to BugBase penicillin seems to cause an overall decrease in Proteobacteria when compared with all other groups, estimates with corncob did not provide statistical support for that decrease in Proteobacteria when compared with the control (Fig. 2D). They did, however, for the penicillin-colistin treated parasites. On the other hand, a different Gram-negative phylum, Bacteroidetes, had statistically significant lower abundance in rediae treated with penicillin, colistin or penicillin-colistin compared with controls (Fig. 2D). No other taxon was found to significantly differ in terms of abundance and/or variability between gentamicin-treated rediae and control ones. These results are overall supported by LEfSe estimates, highlighting the overrepresentation of Bacteroidetes in the control rediae compared with those treated with colistin, penicillin or penicillin-colistin (results not shown). Based on this method, only for colistin- and penicillin-treated rediae was there statistical support for an overall higher abundance of taxa compared with controls (colistin: Oceanospirillaceae, penicillin: six taxonomic clades belonging to the phylum Tenericutes).

When considering dissimilarities between the rediae in treated groups and controls, penicillin-colistin was the treatment containing the most dissimilar bacterial community composition, while gentamicin was the treatment showing the least dissimilarities (Fig. 2E).

3.4. How resilient are the parasite's bacterial communities?

After the antibiotics-induced disruption (T3), we investigated the potential for recovery of the bacterial communities present in the parasite. We observed that exposing parasites to antibiotics led to an increased divergence among the different samples at T3. However, while the different samples at T3 were significantly more divergent than at T0 and T77, this was only supported statistically in analyses at the family level, but not at class and phylum levels. These dissimilarities later decreased during the recovery stage, and after more than 70 days post-treatment, we observed that samples from the different treatments were less divergent, with no statistically different divergence from the level estimated among T0 samples at family, class and phylum levels (Fig. 3A, Supplementary Fig. S1). In contrast, within the different treatment

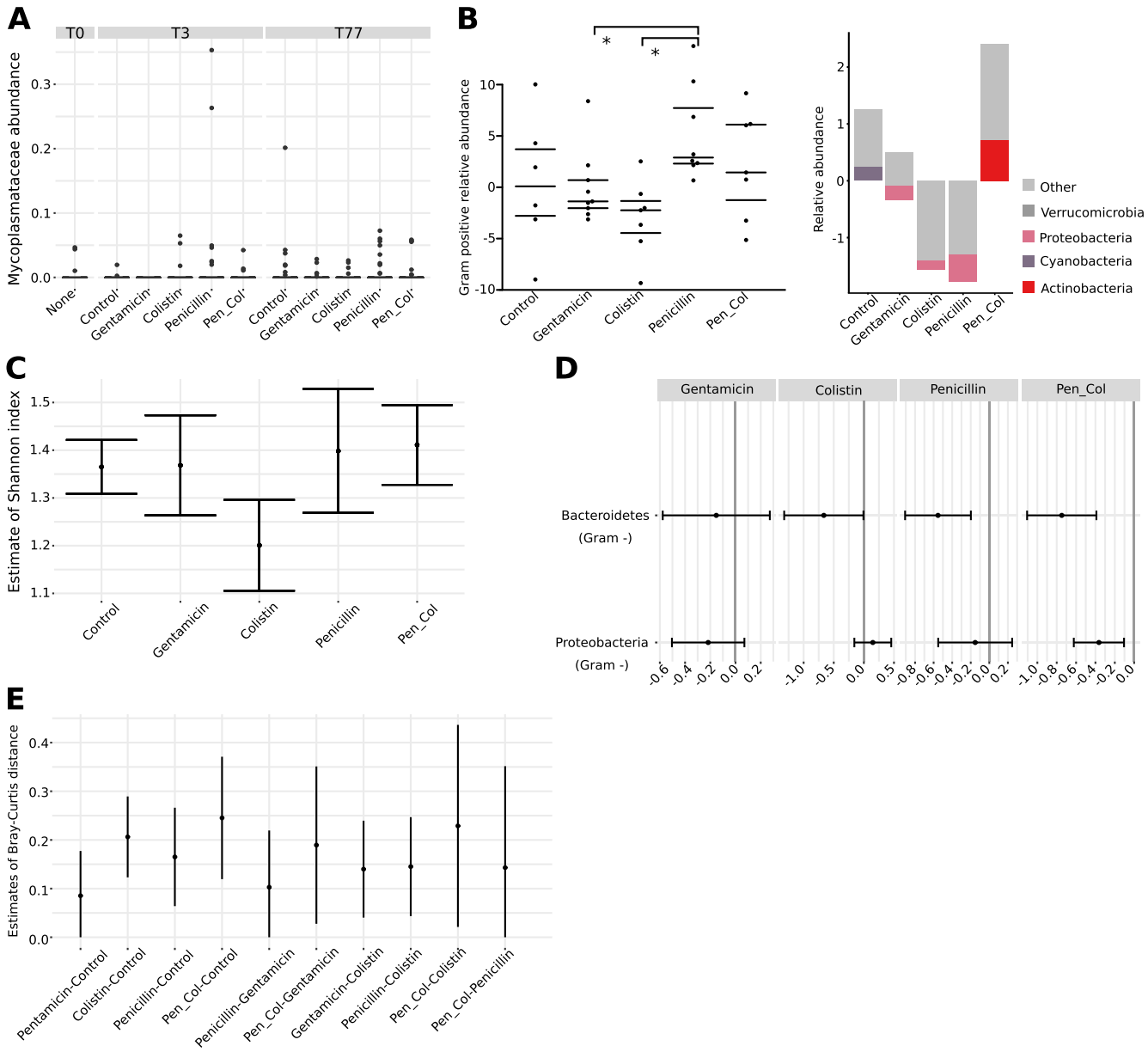

Fig. 2. Antibiotic exposure alters parasite bacterial community composition. (A) Relative abundance (proportion of amplicon sequence variants, or ASVs) of Mycoplasmataceae in samples of the different treated groups and sampling periods. Each dot represents one sample in which Mycoplasmataceae were found. (B) Relative abundances of Gram-positive bacteria and corresponding phyla contribution for control and treated groups after the antibiotic exposure (T3). *$P < 0.05$, significant difference in the relative abundance of Gram-positive bacteria between groups. 'Other' represents all taxa whose relative abundances are less than one-tenth of the most abundant taxon. (C) Alpha diversity as estimated with Shannon diversity index at phylum level for the control and treated groups after the antibiotic exposure (T3). Error bars represent the 95% confidence interval for the estimated means. (D) Differential abundance of phyla in antibiotic treated groups compared with the control after the antibiotic exposure (T3). The magnitude of model coefficients is shown on a log-odds scale; error bars represent the 95% prediction interval. (E) Bray-Curtis dissimilarity estimates for treatment group level comparisons at phylum level. Error bars represent the 95% confidence interval for the estimated means. pen_col, penicillin and colistin.

groups, gentamicin-exposed parasites had the highest microbial community divergence among samples, being statistically higher than that estimated for the baseline at T0 (Fig. 3B, Supplementary Tables S1–S7). While this higher divergence among gentamicin-treated samples was also higher than in the control group, it was only statistically supported at the family level, but not at class and phylum levels (family: $P < 0.0001$, class: $P = 0.231$, phylum: $P = 0.360$).

Knowing that the diversity of bacterial communities in rediae decreased with time post-exposure across all treatment groups (Supplementary Fig. S2), we then tested whether the bacterial communities returned to a taxonomic composition similar to the one estimated at T0. For this, we compared the three time points, i.e. T0 (communities before perturbation), T3 (communities immediately after the impact of antibiotic exposure), and at T77 (communities after a recovery period). Based on Bray-Curtis dissimilarity distances at genus, class and phylum levels, the communities at those three points increased in dissimilarity with increasing time in the laboratory, with T3 being least dissimilar to T0 and T77 most dissimilar to T0 (Fig. 3C). However, the opposite was true when communities were compared at the family level (Supplementary Tables S1–S7). When comparing T3 and T77 bacterial communities at both family and phylum levels, we estimated a greater dissimilarity than when comparing either of those to T0

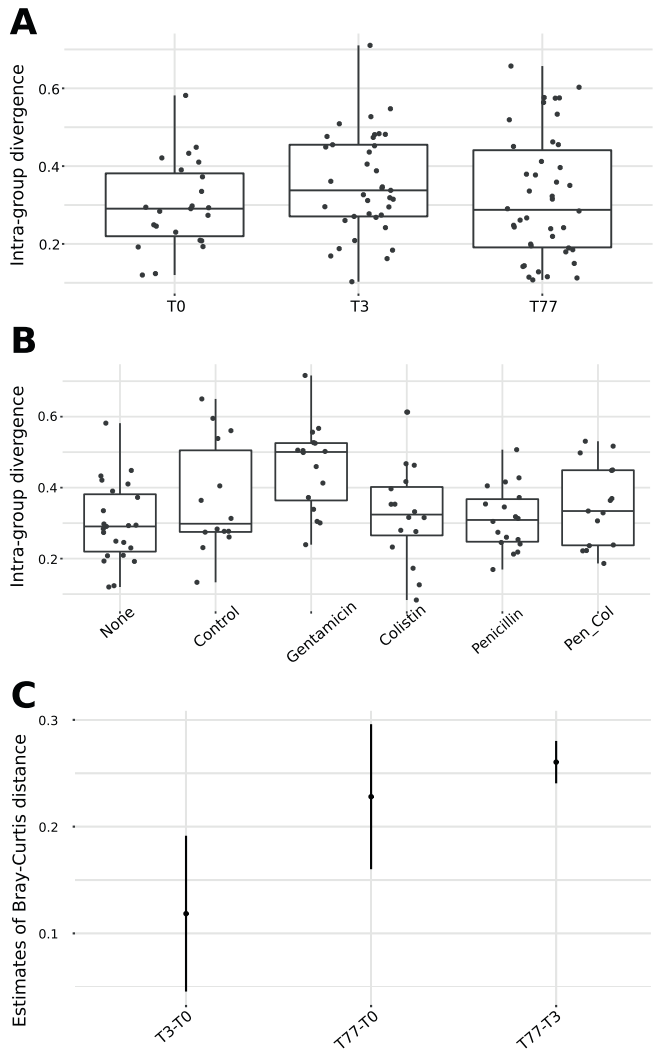

Fig. 3. Parasite bacterial community shifting to an alternative state. Divergence of bacterial communities at the phylum level as a measure of the average dissimilarity of each sample from the group mean for the three time points (A), and treatment groups (B). T0, pre-antibiotic treatment; T3, after antibiotic exposure; T77, 77 days after the beginning of the experiment. pen_col, penicillin and colistin. (C) Bray-Curtis dissimilarity estimates for time points-level comparisons at phylum level. Error bars represent the 95% confidence interval for the estimated means.

(Supplementary Tables S1–S7). At genus and class levels, differences between bacterial communities at T77 and T3 were less pronounced than between T77 and T0.

3.5. Can we detect similar bacterial community dynamics in snail hosts?

Due to sequencing deficiencies, our data included a low number of samples from the snail host (T0 = 9, T3 = 8, T77 = 12), limiting our power to infer changes in community composition among treatments. We did, however, conduct analyses among the different time points to determine whether, as observed in the parasites, bacterial communities found in the snail host tissue were also affected by the antibiotic treatments. Similar to what was found for the parasite, antibiotic exposure led to an increase in divergence among the different samples, but only when evaluated at family level and not at class and phylum levels, where no difference was found. Samples from post-antibiotic treatment presented the highest bacterial community divergence (Fig. 4, T3 versus T0: $P < 0.0001$, T3 versus T77: $P < 0.0001$). For the snail host tissue, we also found marginal support for an increase in bacterial community divergence at T77 when compared with T0 ($P = 0.049$).

Comparisons of bacterial community composition among the three time points supported the fact that communities were most dissimilar at T77 when compared with T0 (Fig. 4, Supplementary Tables S1–S7), in line with the fact that the communities changed from the initial baseline status as observed for the parasite.

4. Discussion

The growing recognition that microbes harboured by both hosts and parasites are associated with multiple facets of the host-parasite interaction (Hooper et al., 2012; Koch and Schmid-Hempel, 2012; Dheilly et al., 2015, 2019; Martinson et al., 2020) is driving the need to develop approaches to experimentally manipulate their microbiota to establish its causal role. Here, we have shown that exposure of *P. attenuatus*–infected snails to antibiotics results in changes in the microbiota of the trematode, thus validating indirect, within-host antibacterial treatment as a means to alter the parasite's microbial communities. However, as we discuss below, the impact of antibiotic treatment is not easily predictable, and the altered microbiota is subject to dynamic changes in the weeks following treatment.

One of the main aims of this study was to determine whether we could manipulate the bacterial community of the parasite by exposing it indirectly to antibiotics solutions while it was still inside its living snail host. To achieve that, we targeted the successful elimination of specific components of the microbiota. Antibiotics have different activity spectra; they have been widely used in therapeutic treatment of infections, and more recently to investigate the role of healthy microbiomes versus disrupted ones (e.g., Cox et al., 2014; Dogra et al., 2020; Strati et al., 2021). In the context of microbial dynamics, antibiotics can in fact lead to unforeseen changes in bacterial community composition by directly killing the target bacteria, thereby indirectly providing both opportunities for remaining community members to increase in abundance, and empty niches for new colonisers (Costello et al., 2012). As a result of antibiotic treatment, the microbiota can shift to a different state depending on the community's ability to resist the antibiotics, and its ability to return to its previous baseline composition.

In our study, indirect exposure to antibiotics resulted in changes in the microbiota of the parasitic trematode within its snail host (Fig. 2). For instance, the diversity of bacterial taxa in parasites exposed to antibiotic treatments differed from those in the control group, although the direction and significance of these effects depended on the taxonomic level considered in estimates of diversity. Furthermore, the abundance of certain bacterial taxa was changed by the treatments; for example, the abundance of Bacteroidetes was lower in parasites exposed to most antibiotics than in control parasites. Interestingly, the treatment combining two antibiotics, penicillin and colistin, resulted in parasite microbiota that generally showed the greatest dissimilarity from those of other treatments or the control group. Therefore, antibiotic exposure of parasites within snails succeeded in modifying their microbiota.

We were able to successfully eliminate fractions of the parasite's bacterial communities that were targeted by antibiotics. Namely, parasites exposed to the gentamicin solution, which has an activity spectrum against both Gram-positive and Gram-negative bacteria as well as Mycoplasma, were found to harbour no bacteria belonging to the family Mycoplasmataceae after the 3 day experimental exposure, while parasites in all other treatments and the control did. However, we have also detected more complex dynamics of antibiotic susceptibility. While penicillin

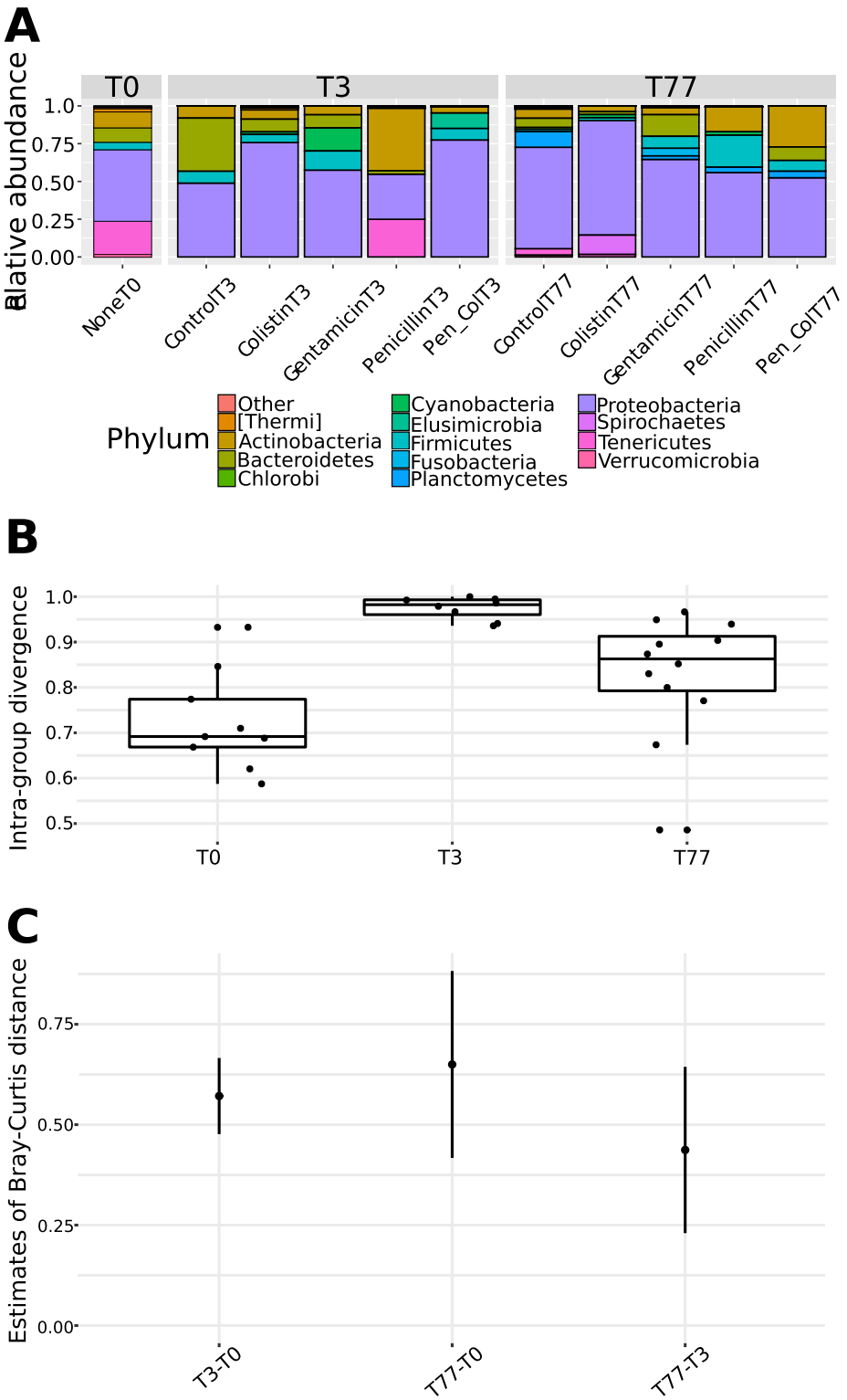

Fig. 4. Snail host bacterial community transitioning to a new composition state. (A) Relative abundance of phyla at the three sampling points. 'Other' represents all taxa whose relative abundances are less than 1% of the total abundance. T0, pre-antibiotic treatment; T3, after antibiotic exposure; T77, 77 days after the beginning of the experiment. pen_col, penicillin and colistin. (B) Divergence of bacterial communities at family level as a measure of the average dissimilarity of each sample from the group mean for the three time points. (C) Bray-Curtis dissimilarity estimates for time points-level comparisons at family level. Error bars represent the 95% confidence interval for the estimated means.

has an activity spectrum targeting mainly Gram-positive bacteria, and has a limited activity against Gram-negative bacteria due to differences in structures of their respective cells, we observed an increase in the relative abundance of Gram-positive bacteria following exposure to penicillin, mainly due to a decrease in Gram-negative abundance. This may have resulted from differences in susceptibility to the antibiotics among the existing bacterial taxa, as the exact composition of the community is likely a greater

determinant of bacterial succession than the antibiotic's spectrum. The disturbance due to penicillin exposure created an opportunity for other members of the community to increase in abundance and for new bacterial colonists to occupy the available niche. Other studies have shown that bacterial taxa recover differently from an initial disturbance induced by antibiotic treatments, or may not even recover, depending on an animal's age (Laubitz et al., 2021), and on the type of antibiotic administered (Strati et al., 2021). The bottom line is that although antibiotic treatment altered the microbiota of parasites, the nature of these changes could not be predicted in a straightforward manner based on the activity spectra of the antibiotics used.

Although we observed shifts in bacterial community composition which can be attributed to the antibiotic exposure, we also observed shifts in community composition of the parasites' microbiota over time in our controls compared with what we defined as the baseline state at T0. And we observed temporal changes too, in the bacterial communities of water samples compared with water from the natural environment. Previous studies investigating the sources of the bacterial taxa making up trematodes' microbiota did not find that the environment was the main contributor of community composition (Jorge et al., 2020, 2021). Thus, while we observed a decrease in bacterial diversity in water samples over the course of the experiment, as well as several bacterial phyla with differential abundances over time, we do not believe that this was the main factor explaining the shifts in bacterial community composition observed in the parasites. One exception, however, might be the increase in Proteobacteria, which was in fact detected in both parasite and water samples (Fig. 1). These bacteria may have proliferated in the water over time, and subsequently colonised the parasite after niches were left vacant due to the action of antibiotics.

The sampling design followed in our study allowed us to assess the temporal stability of the bacterial community composition following the antibiotic treatments, but also whether it recovered, and how it did so. The timing of the last sampling point (T > 70 days) following treatment fits with recommendations from the microbiome quantitative stability landscape framework to investigate transition in bacterial community states (Shaw et al., 2019). Our results suggest that the bacterial communities within the parasites experienced dysbiosis, i.e. a disruption in their composition following the perturbation associated with antibiotic exposure, but subsequently continued to change and ended up in a new state (Fig. 5). Two results support this conclusion. First, although divergence in composition among replicate communities increased 3 days post-exposure compared with pre-exposure (T0), it then decreased over the following weeks until T77 (Fig. 3). Some antibiotics had a greater impact, for instance gentamicin caused the greatest post-exposure divergence among replicate communities. Regardless of these differences among treatments, different bacterial communities subjected to the same antibiotic treatment followed multiple trajectories afterwards in the short term, thus diverging in composition, but thereafter converged again toward a similar new state. Second, across treatments, bacterial communities in parasites at the end of the experiment were clearly different from those at the beginning, based on Bray-Curtis dissimilarity estimates. It seems that all communities have undergone a long-term transition to an alternative community state dominated by Proteobacteria. The latter may have been recruited from the snail host or external water environment, where this bacterial taxon was also highly abundant.

Overall, our findings indicate that the indirect exposure of trematodes to antibiotics, while they are infecting their snail host, can result in changes in their microbiota. This opens the door to experimental manipulation of trematode microbiota, allowing the use of cercariae inheriting the altered microbial communities

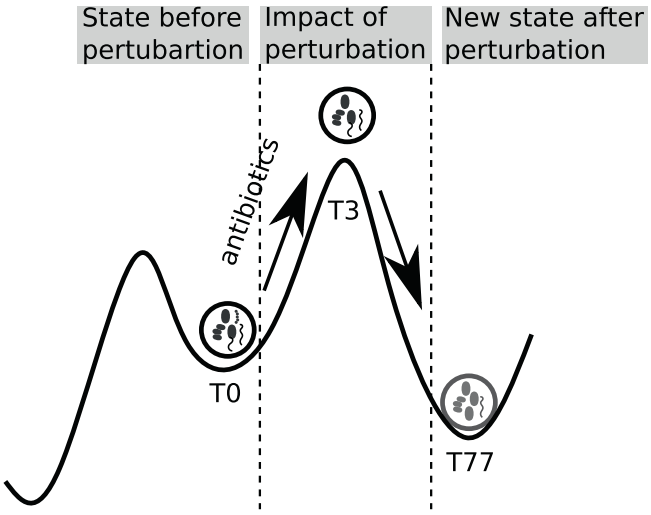

Fig. 5. Antibiotic-induced perturbation leads to an altered bacterial community state. Conceptual illustration depicting the impact of antibiotics on the parasite bacterial community composition, where the solid line represents the temporal transitions of the community through periods of stability (troughs) and challenges (peaks). Beginning with the bacterial community within a parasite at an initial baseline state, it passes across a transitional state after the antibiotic-induced perturbation, and then moves toward a new "stable" state. T0, pre-antibiotic treatment; T3, after antibiotic exposure; T77, 77 days after the beginning of the experiment.

of their parent rediae to be used for further studies. However, our results also indicate that the actual changes in the composition of trematode microbial communities achieved this way are variable and difficult to anticipate, and also that they are dynamic, with the community unlikely to revert to its original state but instead progressing toward a new community state over time. We conclude that this approach allows for perturbation of the microbiota at a coarse level, providing a hit-and-miss strategy rather than a precise deletion of selected taxa. Nevertheless, it can prove useful to quantify the impact of bacterial dysbiosis in helminths on their interactions with the host, and paves the way for further experimental research on parasite microbiomes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2021.11.006.

References

Ben-Yosef, M., Zaada, D.S.Y., Dudaniec, R.Y., Pasternak, Z., Jurkevitch, E., Smith, R.J., Causton, C.E., Lincango, M.P., Tobe, S.S., Mitchell, J.G., Kleindorfer, S., Yuval, B., 2017. Host-specific associations affect the microbiome of *Philornis downsi*, an introduced parasite to the Galápagos Islands. Mol. Ecol. 26, 4644–4656.

Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G.A., Caporaso, J.G., 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6, 90. https://doi.org/10.1186/s40168-018-0470-z.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodriguez, A. M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M.,

Durall, Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J. M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson II, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vazquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., . Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583.

Cao, Y., Zhang, A., Li, H., 2019. Large covariance estimation for compositional data via composition-adjusted thresholding. J. Am. Stat. Assoc. 44, 1–14.

Costello, E.K., Stagaman, K., Dethlefsen, L., Bohannan, B.J., Relman, D.A., 2012. The application of ecological theory toward an understanding of the human microbiome. Science 336 (6086), 1255–1262.

Cox, L., Yamanishi, S., Sohn, J., Alekseyenko, A., Leung, J., Cho, I., Kim, S., Li, H., Gao, Z., Mahana, D., Zárate Rodriguez, J., Rogers, A., Robine, N., Loke, P'ng, Blaser, M., 2014. Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. Cell 158 (4), 705–721. https://doi.org/10.1016/j.cell.2014.05.052.

Dheilly, N.M., Poulin, R., Thomas, F., 2015. Biological warfare: microorganisms as drivers of host-parasite interactions. Infect. Genet. Evol. 34, 251–259.

Dheilly, N.M., Martinez, J.M., Rosario, K., Brindley, P.J., Fichorova, R.N., Kaye, J.Z., Kohl, K.D., Knoll, L.J., Lukeš, J., Perkins, S.L., Poulin, R., Schriml, L., Thompson, L.R., 2019. Parasite Microbiome Project: grand challenges. PLoS Pathogens 15, e1008028.

Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A., et al., 2011. Normal gut microbiota modulates brain development and behavior. Proc. Nat. Acad. Sci. USA 108, 3047–3052.

Dogra, S.K., Doré, J., Damak, S., 2020. Gut microbiota resilience definition, link to health and strategies for intervention. Front. Microbiol. 11,. https://doi.org/10.3389/fmicb.2020.572921 572921.

Ezenwa, V.O., Gerardo, N.M., Inouye, D.W., Medina, M., Xavier, J.B., 2012. Animal behavior and the microbiome. Science 338, 198–199.

Feldhaar, H., 2011. Bacterial symbionts as mediators of ecologically important traits of insect hosts. Ecol. Entomol. 36, 533–543.

Galaktionov, K.V., Dobrovolskij, A.A. 2003. The Biology and Evolution of Trematodes. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Gould, A.L., Zhang, V., Lamberti, L., Jones, E.W., Obadia, B., Korasidis, N., Gavryushkin, A., Carlson, J.M., Beerenwinkel, N., Ludington, W.B., 2018. Microbiome interactions shape host fitness. Proc. Natl. Acad. Sci. USA 115 (51), E11951–E11960. https://doi.org/10.1073/pnas.1809349115.

Hahn, M.A., Rosario, K., Lucas, P., Dheilly, N.M., 2020. Characterization of viruses in a tapeworm: phylogenetic position, vertical transmission, and transmission to the parasitized host. ISME J. 14 (7), 1755–1767. https://doi.org/10.1038/s41396-020-0642-2.

Hooper, L.V., Littman, D.R., Macpherson, A.J., 2012. Interactions between the microbiota and the immune system. Science 336, 1268–1273.

Jenkins, T.P., Brindley, P.J., Gasser, R.B., Cantacessi, C., 2019. Helminth microbiomes: a hidden treasure trove? Trends Parasitol. 35, 13–22.

Jorge, F., Dheilly, N.M., Poulin, R., 2020. Persistence of a core microbiome through the ontogeny of a multi-host parasite. Front. Microbiol. 11, 954. https://doi.org/10.3389/fmicb.2020.00954.

Jorge, F., Dheilly, N.M., Froissard, C., Wainwright, E., Poulin, R., 2021. Consistency of bacterial communities in a parasitic worm: variation throughout the life cycle and across geographic space. Microb. Ecol. in press. https://doi.org/10.1007/s00248-021-01774-z.

Koch, H., Schmid-Hempel, P., 2012. Gut microbiota instead of host genotype drive the specificity in the interaction of a natural host-parasite system. Ecol. Lett. 15, 1095–1103.

Laubitz, D., Typpo, K., Midura-Kiela, M., Brown, C., Barberán, A., Ghishan, F.K., Kiela, P.R., 2021. Dynamics of gut microbiota recovery after antibiotic exposure in young and old mice (A Pilot Study). Microorganisms 9, 647. https://doi.org/10.3390/microorganisms9030647.

Leung, T.L.F., Poulin, R., 2011. Small worms, big appetites: ratios of different functional morphs in relation to interspecific competition in trematode parasites. Int. J. Parasitol. 41 (10), 1063–1068.

Marotz, C., Amir, A., Humphrey, G., Gaffney, J., Gogul, G., Knight, R., 2017. DNA extraction for streamlined metagenomics of diverse environmental samples. BioTechniques 62, 290–293.

Martin, B.D., Witten, D., Willis, A.D., 2020. Modeling microbial abundances and dysbiosis with beta-binomial regression. Ann. Appl. Stat. 14, 94–115. https://doi.org/10.1214/19-aoas1283.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 17 (1), 10. https://doi.org/10.14806/ej.17.1.200.

Martinson, V.G., Gawryluk, R.M.R., Gowen, B.E., Curtis, C.I., Jaenike, J., Perlman, S.J., 2020. Multiple origins of obligate nematode and insect symbionts by a clade of bacteria closely related to plant pathogens. Proc. Nat. Acad. Sci. USA 117, 31979–31986.

McFall-Ngai, M., Hadfield, M.G., Bosch, T.C.G., Carey, H.V., Domazet-Loso, T., Douglas, A.E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S.F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A.H., Kremer, N., Mazmanian, S.K., Metcalf, J.L., Nealson, K., Pierce, N.E., Rawsl, J.F., Reid, A., Ruby, E.G., Rumpho, M., Sanders, J. G., Tautz, D., Wernegreen, J.J., 2013. Animals in a bacterial world, a new imperative for the life sciences. Proc. Nat. Acad. Sci. USA 110, 3229–3236.

Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., Blondel, M., Prettenhofer, P., Weiss, R., Dubourg, V., Vanderplas, J., Passos, A., Cournapeau, D., Brucher, M., Perrot, M., Duchesnay, E., 2011. Scikit-learn: machine learning in Python. J. Mach. Learn. Res. 12, 2825–2830.

R Core Team, 2021. R: a Language and environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12 (6), R60. https://doi.org/10.1186/gb-2011-12-6-r60.

Shaw, L.P., Bassam, H., Barnes, C.P., Walker, A.S., Klein, N., Balloux, F., 2019. Modelling microbiome recovery after antibiotics using a stability landscape framework. ISME J. 13 (7), 1845–1856. https://doi.org/10.1038/s41396-019-0392-1.

Sinnathamby, G., Henderson, G., Umair, S., Janssen, P., Bland, R., Simpson, H., 2018. The bacterial community associated with the sheep gastrointestinal nematode parasite *Haemonchus contortus*. PLoS ONE 13, e0192164.

Strati, F., Pujolassos, M., Burrello, C., Giuffrè, M.R., Lattanzi, G., Caprioli, F., Troisi, J., Facciotti, F., 2021. Antibiotic-associated dysbiosis affects the ability of the gut microbiota to control intestinal inflammation upon fecal microbiota transplantation in experimental colitis models. Microbiome 9, 39. https://doi.org/10.1186/s40168-020-00991-x.

Wilkinson, D.A., Duron, O., Cordonin, C., Gomard, Y., Ramasindrazana, B., Mavingui, P., Goodman, S.M., Tortosa, P., Goodrich-Blair, H., 2016. The bacteriome of bat flies (Nycteribiidae) from the Malagasy region: a community shaped by host ecology, bacterial transmission mode, and host-vector specificity. Appl. Environ. Microbiol. 82 (6), 1778–1788.

Willis, A., Bunge, J., Whitman, T., 2017. Improved detection of changes in species richness in high diversity microbial communities. J. R. Stat. Soc.: Ser. C. (Appl. Stat.) 66 (5), 963–977. https://doi.org/10.1111/rssc.12206.

Willis, A.D., Martin, B.D., 2020. Estimating diversity in networked ecological communities. Biostatistics kxaa015. https://doi.org/10.1093/biostatistics/kxaa015.