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Long lasting effects of antibiotics on bacterial communities of adult flies

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One-sentence summary: Antibiotic treatment reduces but does not destroy fly bacterial diversity and its effects last over several generations in untreated flies.

Abstract

Insect symbionts benefit their host and their study requires large spectrum antibiotic use like tetracycline to weaken or suppress symbiotic communities. While antibiotics have a negative impact on insect fitness, little is known about antibiotic effects on insect microbial communities and how long they last. We characterized the bacterial communities of adult cabbage root fly *Delia radicum* in a *Wolbachia*-free population, evaluated the effect of tetracycline treatment on these communities over several generations.

Three *D. radicum* generations were used: the first and second generation flies either ingested tetracycline or not, while the third generation flies were untreated but differed with their parents and/or grand-parents that had or had not been treated. Fly bacterial communities were sequenced using a 16S rRNA gene.

Tetracycline decreased fly bacterial diversity and induced modifications in both bacterial abundance and relative frequencies, still visible on untreated offspring which parents and/or grandparents had been treated therefore demonstrating long lasting trans-generational effects on animal microbiomes after antibiotic treatment. Flies with an antibiotic history shared bacterial genera, potentially tetracycline-resistant and heritable.

Next, the transmission should be investigated by comparing several insect development stages and plant compartments to assess vertical and horizontal transmissions of *D. radicum* bacterial communities.

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1 Introduction

Insects can harbor a diversity of microbes that can profoundly influence their phenotypes and by extent, their ecology and evolution. To study symbiotic-mediated phenotypes, microbial-colonized hosts are usually compared to microbial-free hosts, which microorganisms were suppressed from the host body. The two commonly used methods are dechorionation and the use of antibiotics. In the first case, the chorion is removed to prevent offspring feeding on it after hatching and consuming the microorganisms left on the chorion by the mother during oviposition (Bakula 1969; Wong, Ng and Douglas 2011). In the second case, symbiotic bacteria are eliminated using antibiotics added to the insect food or water or microinjected in insect body. While their use is controversial as to whether the observed effects come from the method used or the bacterial loss, the study of Heys *et al.* (2018) showed that a low dose of antibiotics in the larval diet of *Drosophila melanogaster* is more effective than dechorionation at removing bacteria forming gut communities and has reduced effects on insect physiology. Treating fly larvae through their diet successfully eliminated bacteria from the gut of adults but only reduced its overall adult microbiota, which comprises both internal (i.e. gut and other organs) and external microorganisms (Heys *et al.* 2018). Lin *et al.* (2015) tested increasing doses of five antibiotics on the larval gut microbiota of *Plutella xylostella*, an aboveground chewing insect. They showed that larval growth and development were negatively affected by all antibiotics but tetracycline was the most toxic, increasing pupal malformations and mortality. Such results raise the question of whether the observed phenotype is due to a direct and toxic effect of the antibiotic or an indirect effect from the bacterial loss. In the latter case, it is necessary to assess whether the microbiote perturbation is temporary or not, because the elimination of antibiotic-sensitive symbionts might lead to the establishment of a new microbiote community, with carry-over effects on the next generations if at least part of the microbiote is vertically transmitted.

Tetracycline is a broad-spectrum antibiotic (Dorosz 2017), which inhibits protein synthesis and is naturally produced by bacteria from the *Streptomyces* genus (Chopra and Roberts 2001). It has a broad spectrum as it can eliminate a wide range of gram-positive and negative bacteria, but also atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae (Chopra and Roberts 2001). In insects, tetracycline is often used to eliminate most prevalent bacterial endosymbionts like *Wolbachia* (Li *et al.* 2014). This bacterial genus is estimated to infest up to 52% of arthropod species (Weinert *et al.* 2015) and has various phenotypic effects on insect hosts such as inducing feminization, parthenogenesis, male killing or cytoplasmic incompatibility (Werren, Baldo and Clark 2008; Zug and Hammerstein 2015).

The presence of *Wolbachia* is inconvenient when studying bacterial communities in general because it tends to largely dominate the community in terms of abundance (Yong *et al.* 2017). It can represent 63% of the community in the common bed bug (Meriweather *et al.* 2013), and over 90% of total bacteria in mosquitoes *Culex pipiens* (Muturi *et al.* 2016). In *Drosophila sp*, *Wolbachia* sequences likewise represented 38% of the total sequences and rise to over 90% in several adult samples (Staubach *et al.* 2013). While tetracycline is used to eliminate *Wolbachia* to compare phenotypes of infected and free lines, how this antibiotic treatment affects the bacterial community is still an open question. Depending on the effect of this antibiotic on other elements of the microbiota consortium, differences between *Wolbachia*-infected and free lines may not be related to *Wolbachia* but rather to its impact on the microbiota. Therefore determining the effect of

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tetracycline on the microbiota of an insect not infected with *Wolbachia* and whether this effect lasts over insect generations is important to understand symbiotic-mediated phenotypes.

The cabbage root fly (*Delia radicum*) is a root herbivore of Brassicaceous species, which can also harbor *Wolbachia* as a facultative symbiont. *Wolbachia* prevalence varies depending on the fly population and it can go down to 0% (Lopez 2018). Bacterial communities have been characterized in adults of two *D. radicum* populations and both populations were dominated by the *Wolbachia* genus that largely overwhelmed the other detected genera by accounting for 80% and 97% of the retrieved sequences (Bili *et al.* 2016). So far however, the effects of an antibiotic treatment on *D. radicum* bacterial communities in the absence of *Wolbachia* and whether these effects last over several generations remain to be unraveled.

Tetracycline being a broad-spectrum antibiotic, it is expected that such treatment greatly disturbs bacterial communities and eliminates most bacteria. Moreover, when species disappear it is usually expected that others arise to recolonize the habitat. After several generations, bacterial recolonization could be due to the transmission of tetracycline-resistant bacteria, not eliminated, from the previous treated generation (i.e. vertical transmission) and/or external contamination (i.e. horizontal transmission). As several studies have shown that bacterial communities of insects differ from males to females (Simhadri *et al.* 2017; Yong *et al.* 2017), it raises the question of whether antibiotics could differently shape fly bacterial communities depending on the insect sex.

Our study aimed first at characterizing *D. radicum* bacterial communities at the adult stage in a *Wolbachia*-free population. Then, we evaluated the direct effect of tetracycline on these communities and assessed if effects lasted after one or two generations of successive antibiotic treatments and to what extent. For that purpose, we conducted an experiment over three generations of cabbage root flies: flies from the first and second generations either ingested tetracycline or not, while flies from the third generation were not in contact with the antibiotic but differed by their family history since their parents and/or grandparents had been treated or not. Bacterial communities of adult flies were identified by high-throughput DNA sequencing (Illumina MiSeq) of a 16S rRNA gene.

2 Materials and Methods

2.1 Fly population and rearing conditions

The cabbage root fly ("*Delia radicum*") population used in our experiment came from 300 adults emerged from pupae collected in experimental broccoli fields in 2015 near Le Rheu (48°07'16"N, 1°47'41"O, Brittany, France) which were reared in the laboratory for several generations. In the laboratory, flies were supplied with unsterilized food (ratio 1:1:1 of sugar, milk powder and yeast) and unsterilized water (cotton moistened with water) and they were reared on rutabaga roots (*Brassica napus* subsp. *rapifera*) in a climatic room (16:8 LD, 21 ± 2°C; 60% ± 10% RH) as described in Neveu Bernard-Griffiths (1998).

2.2 Experimental setup

The experimental design is detailed in figure 1.

Generation 0 (G0) – Two days after emergence from several rutabaga roots, 100 males and 100 females were placed in a control (hereafter called 'C') cage (Bug Dorm-4 Insect Rearing Cage, 47.5 × 47.5 × 47.5cm) and supplied with food and water, while another 100 males and 100 females were placed in an antibiotic (hereafter called 'A') cage with both food and antibiotic-containing water. The antibiotic used was tetracycline hydrochloride powder (Sigma-Aldrich, CAS number: 64-17-5), dissolved in water to a final concentration of 0.5 $mg.mL^{-1}$ because preliminary experiments

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showed that this concentration was the strongest one we could use without increasing the mortality of treated individuals (Lopez, 2018). Treated individuals were given tetracycline continuously during their whole adult lifespan. After 15 days of treatment, a rutabaga was placed in each cage during 48h for egg-laying and after 30 days of treatment, adult flies were captured individually by aspiration, placed in 96% ethanol and stored at –20°C until further analysis.

Generation 1 (G1) – For each G0 cage, the eggs obtained on a piece of rutabaga in each cage were placed in two different cages and developed on fresh rutabaga roots until emergence thus obtaining 4 cages. Emerging flies received either the same treatment as their parents or the alternative one (C or A) for 15 days before reproducing. They were thus exposed to four different treatments: untreated G0 and G1 (hereafter called 'CC' treatment'); untreated G0 but antibiotic-treated G1 (hereafter called 'CA' treatment'); antibiotic-treated G0 and untreated G1 (hereafter called 'AC' treatment'); antibiotic-treated G0 and G1 (hereafter called 'AA' treatment').

For G0 and G1 flies, the food and water were changed every two days.

Generation 2 (G2) – For each of the four previous cages, 10 eggs were placed on turnip roots (*Brassica rapa* L. subsp. *rapa*, N = 40 per cage) and continued their development until adult emergence, where they all underwent the control treatment (i.e. untreated water) without food. At this stage, the four treatments were as follows: untreated G0 and G1 (hereafter called 'CCC' treatment'); untreated G0 but antibiotic-treated G1 (hereafter called 'CAC' treatment'); antibiotic-treated G0 and untreated G1 (hereafter called 'ACC' treatment'); antibiotic-treated G0 and G1 (hereafter called 'AAC' treatment'). G2 flies were sampled once dead and stored in 96% ethanol and –20°C until further analysis.

2.3 Analyses of fly bacterial communities

2.3.1 Molecular analysis

Stored individuals were dried out on a filter paper and individually placed in a well of a semi deep 96 wells plate. DNA was extracted using 300 µL of lysis buffer: 1 M of Tris, 5 M of NaCl, 0.5 M of EDTA, 20% SDS and sterile ultrapure water; 6 µL of proteinase K (5 mg/mL) and 3 sterilized glass beads (3 mm diameter) added to each well. The plate was sealed and samples were ground during 6 min and incubated at 37°C overnight. Then, plates were rapidly centrifuged and 85 µL of NaCl (5 M) were added per well, followed by short vortexing and a 30 min centrifugation at 3,500 rpm and 4°C. Approximately 290 µL of supernatant were then transferred to a new plate containing 280 µL of 100% ice-cold ethanol and samples were homogenized by pipetting and incubated at –20°C for 1h. After a 30 min centrifugation at 3,500 rpm and 4°C, the supernatant was discarded and the pellet was washed with 200 µL of 70% ice-cold ethanol. After another centrifugation and elimination of the supernatant, samples were vacuum-dried 30 min at 30°C. Pellets of DNA were resuspended in 50 µL of sterile ultrapure water and stored at 4°C overnight. Samples were then transferred at –20°C until further analysis.

PCR amplification using the bacterial primers 799F (5'-AACMGGATTAGATACCCKG-3') and 1223R (5'-CCATTGTAGTACGTGTGTA-3') that amplify 16S rDNA genes, library preparation and sequencing were performed at the GenoScreen platform (Lille, France). Sequencing was performed using the Illumina MiSeq platform and a 2 × 300 bases paired-end version. The final library at a concentration of 4 pM and the PhiX control library were loaded onto the flow cell. Sample demultiplexing and barcode suppression were performed by the GenoScreen platform using the Illumina CASAVA software and PERL script "ConfigureBclToFastq.pl", before suppressing the primers with "FLASH" tool (Magoc and Salzberg 2011).

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Raw data sets were deposited on the European Nucleotide Archive database system under the project number PRJEB36052. Accession numbers of fly samples range from ERS4217587 to ERS4217762.

2.3.2 Bioinformatics analysis

The dada2 workflow, based on Divisive Amplicon Denoising Algorithm ("DADA") was used with the "dada2" R package on our samples to obtain an amplicon sequence variant (ASV) table, which identified fine-scale variations compared to the operational taxonomic unit (OTU) table (Callahan *et al.* 2016).

We made the following modifications to the default functions proposed by the dada2 workflow and package. After inspecting the quality profiles of reads 1 and 2 through plotting, trimming was performed at 250 reads for both read 1 and read 2 respectively where the quality score started to drop below than 30. To learn the error rates, we increased the number of samples, bases and reads taken into account by the machine-learning algorithm, with the arguments "nbases = 1e+09" and "randomize = TRUE". Then, the dereplication, sample inference and merging steps were performed as proposed in the workflow. Lastly, the sequence lengths were inspected and only sequences which length ranged from 454 and 472 nucleotides were kept, then chimeric sequences were removed.

Taxonomic affiliations were performed using the Silva reference database, version 132.

2.3.3 Statistical analyses

Analyses were performed using the R software (R Core Team 2018) and a 5% threshold for statistical significance.

For data manipulation, we used the "phyloseq" and "microbiome" packages (Lahti *et al.* 2012; McMurdie and Holmes 2013). First step, we compared A and C at G0 in order to assess the effect of tetracycline on the bacterial communities. Second step, we compared CCC, CAC, ACC and AAC in order to evaluate whether the effect of tetracycline lasted after two generations. For each step, we used the same analytical methods.

Rarefaction curves were obtained using the "ggrare" function from the "ranacapa" package (Kandlikar 2019) to make sure that most of the species richness was assessed in every sample. Then, samples were rarefied using the "rarefy_even_depth" function and the setting "set.seed (400)" with a sample size of 3500, corresponding to the sequence sample size where species richness reached a plateau in all our samples. Samples were expressed in per mille proportions (i.e. the sums of reads per sample transformed in 1000) instead of percentage as many ASVs had a very low abundance, and then filtered by removing ASV which proportions were lower than 1/1000.

Using rarefied, proportion-expressed and filtered data, we plotted the relative abundance of bacterial phyla and classes, obtained with the "tax_glom" function ("phyloseq" package), to visually identify the dominant phyla and classes in each treatment, using the "ggplot" function from the "ggplot2" package (Wickham 2016).

Alpha diversity analysis was performed on proportion-expressed samples and both richness and Shannon indices were calculated using the "estimate_richness" function from the "phyloseq" package. To respect residues normality and homoscedasticity, each index was squared-root

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transformed for G0 samples but it was not necessary for G2, and was tested against the antibiotic treatment, the sex of individuals and interaction between these both factors using a linear model. When normality and homoscedasticity of the model residues were achieved, the significance of each term in the model was determined by a F-test as a type II analysis of variance to respect the principle of marginality ("Anova" function, "car" package, Fox and Weisberg, 2011). When a factor was significant, the estimated marginal means ("emmeans" function, the "emmeans" package, Lenth, 2019) were calculated and a Tukey test was applied to perform pairwise comparisons using the "CLD" function ("emmeans" package), thus to assess the differences between modalities within this factor. P values were corrected using the "False Discovery Rate" (FDR) as multiple comparisons were performed ("p.adjust" function). Plotting required the "ggplot" function.

Beta diversity or community structure analysis was performed on proportion-expressed and filtered samples. To assess whether antibiotic treatment eliminates bacteria, it was necessary for ASV proportions to be turned into a 0/1 matrix, thus presence-absence table. Then, data were transformed using the Hellinger distance as it gives low weights to variables with many zeros. A transformation-based redundancy analysis (tb-RDA) was applied on these data ("rda" function from the "vegan" package) using the antibiotic treatment, the sex of individuals and interaction between these both factors to build the model (Oksanen *et al.* 2016). The "RVaideMemoire" package (Hervé 2016) was used to perform a type II permutation F-test for constrained multivariate analyses to test the significance of each term in the model ("MVA.anova" function), to perform pairwise comparisons when a factor was significant to assess the differences between modalities within this factor (pairwise.factorfit" function) and to plot the data ("MVA.plot" function). The matrix used to perform the tb-RDA was also used to determine indicator ASVs for each treatment ("indval" function, "labdsv" package; Roberts, 2019) and p values were FDR-corrected.

By using generalized linear model (GLM) with binomial error and a logit link function, the presence of a given ASV was tested against antibiotic treatment, individual sex and interaction between both factors. A likelihood-ratio test was performed on the model to test the significance of each term in the model and then pairwise comparisons when a factor was significant to evaluate the differences between the modalities within this factor ("Anova", "emmeans", "CLD" functions), followed by FDR-corrected P values as multiple comparisons were performed.

In order to visualize community data at the different taxonomical level and map statistic using colors, we realized heat trees with the "heat_tree" or "heat_tree_matrix" functions from the "metacoder" package developed by Foster *et al.* (2017). Different heat trees were realized in order i) to assess significant variations of bacterial relative abundance between treatments, using proportion-expressed and filtered data and the default statistical test (i.e. Wilcoxon rank-sum test, followed by FDR correction), and ii) to determine whether taxa could be specific or shared between treatments, using proportion-expressed and filtered data, that were transformed into presence/absence data with an adapted function, replacing the default one.

3 Results

3.1 Effects of antibiotic on the fly bacterial communities (A vs C at G0)

A total of 118,000 and 120,000 reads were detected in individuals of the control (C) and antibiotic (A) treatments respectively after the rarefying step (figure S1). Following the different cleaning steps, the most identified taxon was Proteobacteria (≈ 970-900‰, respectively for C and A), more precisely α-, δ- and γ-Proteobacteria (figure 2A). The second phylum represented was

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Bacteroidetes (≈ 25-90‰, for C and A), with the Bacteroidia class, while Firmicutes was the third and less abundant phylum (≈ 5-10‰, for C and A), with the Bacilli class.

Concerning the alpha diversity (figure 3A), the number of observed ASVs was reduced by the treatment ($F_{1,38}$=9.45; P=0.004) but did not differ between sexes ($F_{1,38}$=0.04; P=0.85). Control flies (C treatment) had 47-100 (minimum-maximum) ASVs while treated flies (A treatment) had only 5-141 ASVs. Similarly, the Shannon index mean value was also reduced by the treatment ($F_{1,38}$=26.18; P<0.001) but not by sex ($F_{1,38}$=0.03; P=0.85). For both dependent variables, the treatment effect did not vary according to the individual sex (interaction term non significant). Globally, the addition of tetracycline to drinking water decreased microbial diversity by 1.6 fold in the host.

Beta diversity was significantly driven by the treatment (F=4.24; P=0.001) but not sex (F=0.76; P=0.696, figure 3B). The interaction between the treatment and the sex of individuals was not significant. Our model explained 11.63% of the total variance, with the treatment explaining 8.09% and sex 1.46% of the variance. ASVs were dispatched in two groups according to their calculated indicator values (table S1).

A total of 592 ASVs was detected and corresponded to 87 genera (table S1). *Pseudomonas* was the most assigned genus (65 ASVs), followed by *Sphingobacterium* (34), *Acinetobacter* (27) and *Flavobacterium* (22) while 81 ASVs were not assigned at the genus level, but at the family level. Among the 87 detected genera, there were 61 and 71 in the flies of treatments C and A respectively (table S2). In treated flies, 16 genera were eliminated and 26 new genera appeared compared to control flies but these genera had a very low relative abundance, while 45 genera remained present in both treatments. When looking at the relative abundance, *Pseudomonas*, *Providencia*, *Serratia* and *Acinetobacter* were the most abundant genera in the C treatment in a decreasing order and accounted for 707‰ of the total relative abundance (table S1). Regarding the A treatment, *Serratia* was the most abundant and accounted for 546‰.

Binomial GLM was performed on the 592 detected ASVs (table S1). For all ASVs, the sex factor and the interaction between the treatment and the individual sex had no effect on ASV occurrence. Among the 592 ASVs, 71 were significantly influenced by the treatment (table 1) and among the first 100 dominant ASVs, only 7 were influenced by the treatment starting at ASV 40, 55 and 56 (*Acinetobacter*), followed by ASV 76, 85, 94 (*Providencia*) and ASV 98 (*Comamonas*). Most treatment-influenced ASVs were more frequent in the C treatment and not present or very scarcely in the A treatment. Interestingly, 2 ASVs from the *Gibbsiella* genus, 2 non-assigned from the Chitinophagaceae family and another 2 non-assigned ASVs from the Enterobacteriaceae family were more frequent in the A treatment and completely absent from the C treatment. The Enterobacteriaceae (i.e. *Gibbsiella*, *Providencia*, *Rahnella*, *Serratia* and non-assigned genera) and Pseudomonadaceae (i.e. *Pseudomonas* genus) families had the most ASVs impacted by the treatment. It should be noted that the frequency of ASV in a given treatment never exceeded 55%, meaning that each ASV was present in at most about half of the replicates for a given treatment.

Significant variations of taxa relative abundance were observed in both C and A treatments (figure 4A). Relative abundances of nine genera were significantly higher in the C treatment: *Chryseobacterium* from the Bacteroidetes; *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* from the α-Proteobacteria class; *Peredibacter* from the δ-Proteobacteria class; *Acinetobacter*, *Delftia*, *Providencia*, *Pseudomonas*, *Rahnella*, *Stenotrophomonas* from the γ-Proteobacteria class. However, the relative abundance of three genera was higher in the A than C treatment: *Gibbsiella* and *Serratia*

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from the Enterobacteriaceae family (class of γ-Proteobacteria) and *Fluviicola* from the Crocinitomicaceae family (phylum of Bacteroidetes).

We identified several taxa that were specific to the C treatment (figure 4B): the six γ-Proteobacterial genera from above (*Acinetobacter*, *Delftia*, *Providencia*, *Pseudomonas*, *Rahnella*, *Stenotrophomonas)* and also *Trabulsiella*; were present in all flies (i.e. replicates) of only the C treatment. However, no taxa was present in all flies of the A treatment only, which explains the absence of blue branches on the plot. Interestingly, *Serratia* was the only genus to be present in all flies of both treatments.

3.2 Bacterial community after two generations of antibiotics

After the rarefying step, we detected 116,000 reads in the CCC treatment and 119,000 reads in each of the other three treatments CAC, ACC and AAC (figure S2). Like in G0 flies, we found mainly Proteobacteria (ranged from 813 to 931‰), Bacteroidetes (ranged from 45 to 123‰) and Firmicutes (barely detectable) in a decreasing order (figure 2B). However, only α- and γ-Proteobacteria (no δ-Proteobacteria) were detected in the dominant phylum.

Alpha diversity of the G2 flies also varied according to the antibiotic treatment (figure 5A). The number of observed ASVs was influenced by the treatment ($F_{3,130}$=3.10; P=0.03) but not by sex ($F_{1,130}$=4.72; P=0.062). Treatment AAC had the significantly highest number of observed ASVs while ACC had the lowest. The treatments CAC and CCC were similar and not different from the other two treatments (ACC and AAC). The Shannon index was impacted by the treatment as well ($F_{3,130}$=3.08; P=0.03), but not by sex ($F_{1,130}$=1.23; P=0.268). As previously, the Shannon index was higher in AAC and lower in ACC treatments. Again, CAC and CCC treatments were not statistically different and were similar to both ACC and AAC treatments. For both diversity index, the treatment effect did not vary according to the individual sex (interaction term non significant).

Beta diversity was also driven by the treatment (F=5.84; P=0.001) and not by sex (F=1.91; P=0.051, figure 5B). The interaction between the treatment and the sex of individuals was not significant. Our model explained 12.95% of the total variance, with the treatment explaining 9.91% and sex 1.08% of the variance. While all treatments were significantly different from one another as their community structure differed, profiles of CAC female and male tended to separate.

Moreover, 82 treatment-influenced ASVs were significant indicator ASVs and they were classified between three clusters (figure 5B, table 2). Cluster 1 corresponded to the AAC treatment and aggregated 9 indicator ASVs, associated to the genera of *Sphingobacterium*, *Pseudochrobactrum*, *Pseudomonas* and *Serratia*. Cluster 2 corresponded to the ACC treatment and aggregated 4 indicator ASVs from the *Falsochrobactrum*, *Comamonas* and *Gibbsiella*. While there was no significant indicator ASV for the cluster 3 (i.e. CAC treatment), cluster 4 corresponding to the CCC treatment had the highest number of significant indicator ASVs. Among the 69 indicator ASVs of cluster 4, 47 belonged to the γ-Proteobacteria and mostly to the genera of *Pseudomonas*, *Comamonas*, *Stenotrophomonas*, *Acinetobacter* and non-assigned genera; 8 to the α-Proteobacteria (*Falsochrobactrum*, *Pseudochrobactrum*) and 14 to the Bacteroidia, including 12 assigned to the *Sphingobacterium* genus.

A total of 489 ASVs was detected and corresponded to 44 genera (table S3). The most assigned genus was *Pseudomonas* (177 ASVs), followed by *Sphingobacterium* (77), *Stenotrophomonas* (25) and *Falsochrobactrum* (25). This time, *Acinetobacter* and *Flavobacterium* accounted for 21 and 6 ASVs respectively. Fifty-three ASVs were however non-assigned. Among the

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44 genera, 30 of them were detected in flies of the CCC treatment, 32 in the CAC, 26 in the ACC and 30 in the AAC (table S4). While 19 genera were shared between the four treatments, 6 were still exclusively present in CCC and 3 in CAC, ACC and AAC, but these 6 and 3 genera had a very low relative abundance. For the four G2 treatments, *Pseudomonas*, *Serratia*, *Sphingobacterium* and *Comamonas* were the most abundant genera in a decreasing order and they accounted for 557‰, 654‰, 657‰ and 744‰ of the total relative abundance, respectively associated with the CCC, CAC, ACC and AAC treatments (table S3).

A binomial GLM was performed on the 489 detected ASVs (table S3). For all ASVs, the sex factor and the interaction between the treatment and the individual sex had no effect on ASV occurrence. Among the 489 ASVs, 153 were significantly influenced by the treatment, among which 31 led to significant pairwise comparisons (table 3). Among the first 100 dominant ASVs, 18 were significantly influenced by the treatment, starting at ASV 4, and belonging to the following genera: *Sphingobacterium*, *Falsochrobactrum*, *Serratia*, *Acinetobacter*, *Pseudomonas* and *Stenotrophomonas*.

Interestingly, 13 ASVs were significantly more frequent in the AAC, ACC and CAC treatments and barely present in the CCC treatments while 18 ASVs were more frequent in the CCC, intermediate in CAC and barely present in the AAC and ACC treatments. These two situations occurred in most genera: for the same genus, some ASVs such as *Falsochrobactrum* (from the α-Proteobacteria class) and *Comamonas*, *Serratia*, *Acinetobacter*, *Pseudomonas* (from the γ-Proteobacteria class) were more frequent in AAC, ACC and CAC while other ASVs from the same latter genera were more frequent in the CCC treatment. We observed that frequency did not exceed 51%, meaning that none of the ASVs were present in all flies of a treatment. Among all ASVs analyses, only the ASV 99 (*Pseudomonas*) presented a frequency varying according to the fly sex.

While we found no significant variation of taxa relative abundance between the four treatments (figure S3), we noted that *Comamonas* was present in all flies of ACC and that *Pseudomonas* was present only in the CAC and ACC treatments (figure 6). Lastly, we observed that the family of Enterobacteriaceae was present in all flies of all treatments.

4 Discussion

Our study showed that tetracycline decreased microbiota diversity but did not suppress all bacteria. While some bacteria were eliminated, others appeared and several genera were found to be shared between control and treated flies. We also showed that effects of the antibiotic were still visible after two generations of treatment, demonstrating that antibiotic treatments can have trans-generational effects on insect microbiote communities.

4.1 *Wolbachia*-free *D. radicum* show a higher bacterial diversity

The *D. radicum* population used in our experiment was *Wolbachia*-free. Only 4 ASVs (ASV 15, 17, 20 and 23) that could putatively corresponded to this genus were found but did not pass the cleaning steps (i.e. rarefying and filtering steps). The bacterial communities we detected were highly diverse. Bacterial communities of the G0 control treatment had a mean Shannon index of 3.12, which is twice higher than the value found in the *D. radicum* population used by Bili *et al.* (2016) where *Wolbachia* was the dominant and most abundant genus in adult flies. In this study, α-Proteobacteria was the most dominant phylum only because of *Wolbachia,* whereas here γ-Proteobacteria largely dominated the communities. Moreover, up to 88 bacterial genera were detected here (table 1 and figure 3) compared to the 10 genera identified by Bili *et al.* (2016). Interestingly, our results showed

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the presence of many genera that were not described so far in the studies using *Wolbachia*-infected lines and very few of the genera we detected were also found in these studies (Lukwinski *et al.* 2006; Bili *et al.* 2016; Welte *et al.* 2016). Still, comparisons between studies are to be made carefully as different fly populations and protocols were used. Although not interacting directly, intracellular endosymbiont such as *Wolbachia*, could have a major impact on extracellular bacteria such as bacteria from the gut, genital organs or cuticle. Indeed, these bacteria could compete for resources with *Wolbachia* and display anti-pathogenic potential to overcome the competition (Zug and Hammerstein 2015). Our study seems to be the first one highlighting the richness of bacterial communities in a *Wolbachia*-free population of *D. radicum*. We are currently conducting a study using free and *Wolbachia*-infected lines obtained through breeding from the same initial population in order to confirm the influence of an endosymbiont like *Wolbachia* on extracellular bacterial communities.

4.2 Tetracycline reshapes bacterial communities and does not eliminate them

The daily ingestion of tetracycline in drinking water during the whole adult life of individuals decreased the diversity by 37% (i.e. Shannon index, from 3.12 to 1.96). Rosas *et al.* (2018) also observed a 45% decrease of the bacterial diversity of German cockroach (*Blattella germanica*) treated with another antibiotic (i.e. rifampicin) and a shift in the composition, indicating that several bacteria were still present despite the treatment.

Tetracycline also modified the bacterial community structure and we observed that 16 genera were suppressed by the antibiotic treatment out of the 71 genera detected in treated-flies (i.e. G0 A treatment). Despite inducing compositional changes in bacterial community, tetracycline would have a partial effect. This may be explained by the antibiotic property, as tetracycline has bacteriostatic but not bactericidal activities, hence it inhibits bacterial growth and does not kill bacteria (Jones and Morrison 1962). As reviewed by Li *et al.* (2014), various doses of tetracycline have been applied on insects to remove their symbionts. Lin *et al.* (2015) treated *P. xylostella* larvae with a 3 mg/mL dose of tetracycline and still detected bacteria in the gut. Similarly, whitefly (*Bemisia tabaci*) treated to rifampicin still harbored traces of several symbionts (Shan *et al.* 2016) while the symbiont *Burkholderia* was not completely removed from its southern chinch bug (*Blissus insularis*) host after an oxytetracycline treatment of 1.4 mM or 0.6 mg/mL (Xu, Buss and Boucias 2016). In our study, a 0.5 mg/mL dose of tetracycline was given to the flies as preliminary experiments showed that this concentration was the strongest one that could be used without increasing the mortality of treated individuals. However, as indicated by previous studies, using a stronger dose, at the expense of insect viability, may not guarantee the elimination of all bacteria.

Tetracycline treatment revealed the presence of 26 new genera and three genera had their relative abundance increased: *Gibbsiella*, *Serratia* and *Fluviicola*. It may be either new acquisition or remaining bacterial that were detected due to titer increase. Bansal *et al.* (2011) also observed that the proportion of β-Proteobacteria, *Paenibacillus* and *Stenotrophomonas* increased in treated Hessian flies (*Mayetiola destructor*) larvae compared to control ones, while it was the proportion of α-Proteobacteria and of *Paenibacillus* that increased in treated pupae. It could be suggested that tetracycline would hinder some very competitive bacteria, which would be taken over by newly acquired and less competitive bacteria. A first explanation to such bacteria apparition and increase after ingesting tetracycline is that flies did not develop in a sterile environment and were not surface-sterilized as we wanted to have access to both external and internal microbial diversity. Thus, flies were most likely to have been subjected to environmental contamination or horizontal transmission while feeding or moving around during the experiment, hence potentially accumulating bacteria on their cuticle. A second explanation is that apparition of new genera and increase in abundance of the remaining bacteria following the treatment may be due to resistance to tetracycline (Chopra and

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Roberts 2001). For instance, Vazirianzadeh *et al.* (2014) identified 59.4% of gram-negative bacilli and 63.4% of gram-positive cocci isolated from brown-banded cockroaches (*Supella longipalpa*) that were resistant to tetracycline. Regarding the bacteria in our study that increased following the tetracycline treatment, *Serratia* was described as having a natural resistance to tetracycline (Dorosz 2017) and interestingly this genus was the only one to be present in both C and A treatments. As for *Fluviivola*, the study of Wang *et al.* (2019) showed that its abundance increased in the presence of oxytetracycline while the study of Han *et al.* (2018) did not find any relationship between this genus and tetracycline resistant genes from animal manures in soil microcosms. To our knowledge, there are few records of *Gibbsiella* in insects, as this genus was mainly detected in diseased oak trees and oral cavity of bears and only *G. papilionis* was isolated from a butterfly intestinal tract (Kim *et al.* 2013), but there is no information about this genus being tetracycline resistant.

4.3 Tetracycline has trans-generational consequences on bacterial communities

Overall, effects of tetracycline were still observable after two generations of antibiotic treatment, demonstrating the trans-generational effect of the antibiotic treatment.

Bacterial communities of flies with different antibiotic history tended to be similar between each other but distinct from the ones with no antibiotic history. After two generations of treatment, *Serratia* was still more frequent in flies which parents and/or grandparents were tetracycline-treated (i.e. G2 AAC, ACC, CAC), and so were other genera like *Falsochrobactrum*, *Comamonas*, *Acinetobacter*, *Pseudomonas* and *Stenotrophomonas*. These genera could contaminate flies with an antibiotic history as they could be tolerating and/or overcoming the antibiotic (i.e. show resistance to tetracycline). According to Han *et al.* (2018), *Pseudomonas* and *Acinetobacter* seem to have significant relationships with tetracycline resistant genes while Chen *et al.* (2019) mentioned that *Comamonas* was resistant to antibiotics and even suggested that this genus could contribute to oxytetracycline biodegradation. However, studies focusing on the genus *Falsochrobactrum* are scarce. It was isolated from sheep and soil but so far, no study has shown a resistance to antibiotics (Sun *et al.* 2019). We also noted that several ASVs of these genera (i.e. *Falsochrobactrum*, *Comamonas*, *Serratia*, *Acinetobacter*, *Pseudomonas* and *Stenotrophomonas*) were more frequent in flies with an antibiotic history, while other ASVs from these same genera were more frequent in the control flies. A potential explanation for such results is that these ASVs could correspond to different species but, as our data went down only to the genus, we lacked taxonomical precision to confirm such hypothesis. Another explanation could be that the antibiotic treatment induces mutations, which eventually lead to antibiotic resistance (Martinez 2014). For instance, certain ASVs could have been subjected to such mutations and promoted acquisition of tetracycline resistance as previously discussed, while others have not. Simultaneously, these mutations could potentially explain the variability we observed when flies ingested tetracycline (i.e. G0 flies) by introducing, replacing or suppressing one or several nucleotides in the sequenced fragments. A single change in nucleotides leads to the identification of another ASV but ASVs that vary by one nucleotide can eventually belong to the same species.

Interestingly, the Enterobacteriaceae family was shared among the four treatments. The Enterobacteriaceae family has been largely detected in insects. For instance, Enterobacteriaceae was the most commonly found and abundant bacterial family in both Lepidoptera (Paniagua Voirol *et al.* 2018; González-Serrano *et al.* 2019) and mosquitoes (Muturi *et al.* 2016), and *Serratia* was the most abundant order and genus (Heise *et al.* 2019). In *D. radicum*, it was found that *Serratia* present in the larval gut had the ability to degrade isothiocyanates, a chemical defense emitted by the plant, so that its insect host could keep developing without being harmed (Welte *et al.* 2016). To explain the persistence of *Serratia*, and more broadly Enterobacteriaceae in all treatments of our study, we could emit two hypotheses: i) Enterobacteriaceae bacteria were already present in control flies and they

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could overcome the antibiotic treatment by being tetracycline resistant and/or ii) Enterobacteriaceae bacteria are important for fly survival, and thus transmitted to the offspring.

Vertical transmission occurs when microorganisms are passed down from the parents to their offspring. Therefore, potential tetracycline-resistant bacteria could have been vertically transmitted, which might explain the similarly shared bacteria between the three G2 batches with antibiotic history. Indeed, maternal transmission can occur when the female contaminate the egg shell with its reproductive organ (Moran and Dunbar 2006) and, upon hatching, larvae ingest bacteria from the shell (Bakula 1969). As our experiment was not conducted under sterile conditions and our samples were not surface-sterilized prior to DNA extraction, it is highly possible for flies to have acquired bacteria through vertical transmission. For the same reasons, horizontal transmissions might also have occurred with flies acquiring bacteria from their environment and host plant. For example, the bean bug *Riptortus pedestris* acquires its symbiotic bacteria of the genus *Burkholderia* from the soil during its development (Kikuchi *et al.* 2012) while the microbiota of the caterpillar *Mamestra brassicae* seems to be have similarities with leaf and soil microbiota (Hannula *et al.* 2019). Pons *et al.* (2019) showed that host plant could mediate the circulation of *Serratia symbiotica* between aphids, as uninfected aphids acquired the bacteria after feeding on a plant, previously attacked by infected aphids. In our study, G0 and G1 flies were reared on rutabaga roots (*B. napus* subsp. *rapifera*) and G2 flies developed from turnip roots (*B. rapa* L. subsp. *rapa*). According to Card *et al.* (2015), there were several *Pseudomonas*, *Serratia* and *Stenotrophomonas* species that were recorded as being beneficial endophytic bacteria associated with *B. napus* roots while *Streptomyces*, and *Pseudomonas* species were associated to *B. rapa* roots. Therefore, such bacteria could be acquired by the insects from the plants.

4.4 Fly microbiota and sex

In our study, we differentiated males from females and observed that both control and tetracycline treatments had similar effects on alpha and beta diversity, as well as ASVs of G0 male and female flies. However, male bacterial communities of the Solanum fruit fly were richer and more diverse than female ones according to the study of Yong *et al.* (2017) while Simhadri *et al.* (2017) pointed out compositional differences between insect sex. In our study, we could suggest that tetracycline would have a “clean sweep” effect that would erase bacterial differences between insect sex, but the absence of difference in control flies prevent us from further hypothesizing. Still, we observed a slight difference between male and female after two generations of treatments: bacterial community structure of CAC tended to separate between insect sex and a single ASV identified as a *Pseudomonas* was influenced by the sex. Both results can be difficultly linked and using precise taxonomy at the species level may provide the missing information to explain our observations.

5 Conclusion

Our study showed that tetracycline decreased bacterial diversity in flies but not to the point of turning them into sterilized (i.e. aposymbiotic) insects. The application of antibiotic also induced shifts in the bacterial composition, in terms of both abundance and frequency, and this shift were still visible on untreated offspring, which parents and/or grandparents were treated. This study therefore demonstrates the potential long-ranging effects antibiotic treatments, trans-generational effect being especially noteworthy considering the pervasive use of such treatments in scientific experiments as well as in veterinary and medical applications. Flies with antibiotic history shared bacterial genera, potentially tetracycline-resistant and transmissible. While our study identified changes at taxonomic level, a metagenomics experiment would now be desirable to assess changes at the functional level.

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As microbial transmission has not been studied in *D. radicum*, we can only hypothesize that vertical and/or horizontal transmission occurred, but it would be nice to study by comparing several insect development stages and plant compartments and by using finer taxonomic tools to identify bacteria to the species level. As the flies were viable despite these shifts, it also raises the questions of functional redundancy between the original bacterial communities of untreated flies and bacteria remaining after treatment with tetracycline and of microorganism role in influencing insect life history traits. The long lasting modifications in fly bacterial communities induced by tetracycline could potentially have repercussions on the fly development and fitness. In a recent study, Lopez (2018) found that life history traits of *D. radicum* can be affected by tetracycline treatment one or even two generations after treatment, but did not quantify changes to the microbiote. Linking such host phenotype changes to specific microbial modifications is a difficult task, but would allow to identify which symbionts most influence the insect phenotype.

6 Author Contributions

VL, DP and AMC conceived and designed research. VL carried out the experiment. MO did the bioinformatical and statistical analyses, advised by MH, LL and YO, and wrote the manuscript that was commented and approved by all authors.

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The authors declare no conflict of interest.

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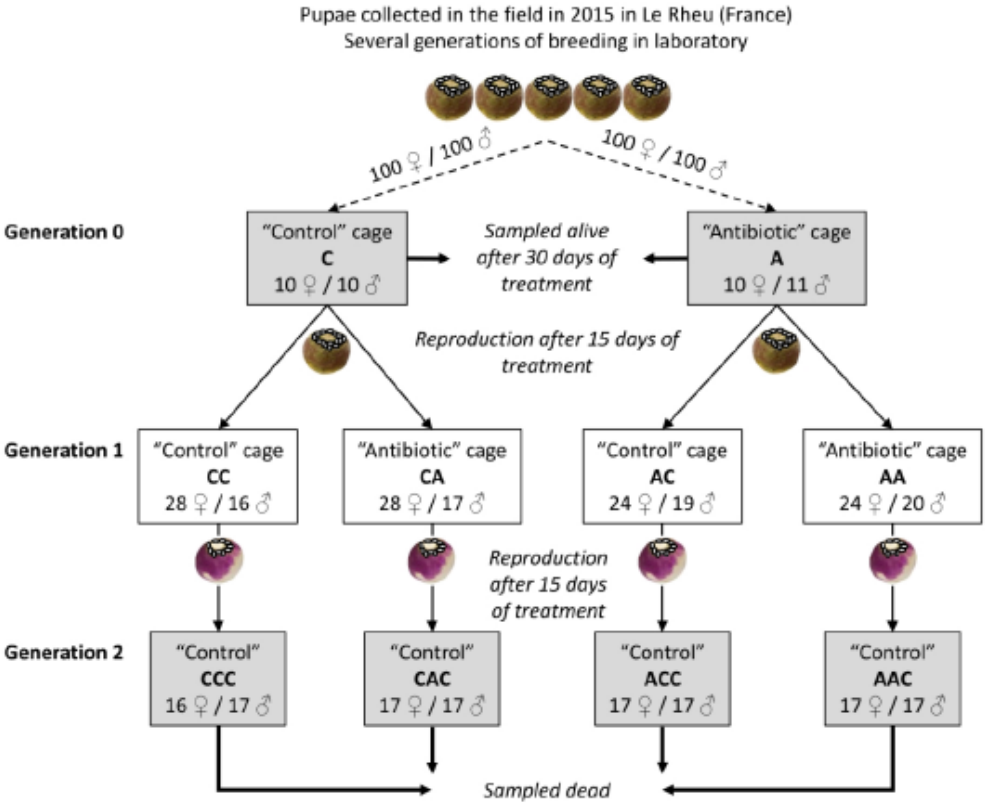


Figure 1 Experimental design used to create four lines with crossed treatments between antibiotic "A" and control "C" treatments. Grey boxes indicate the treatments and the number of samples per treatment that were sequenced for bacterial community analysis.

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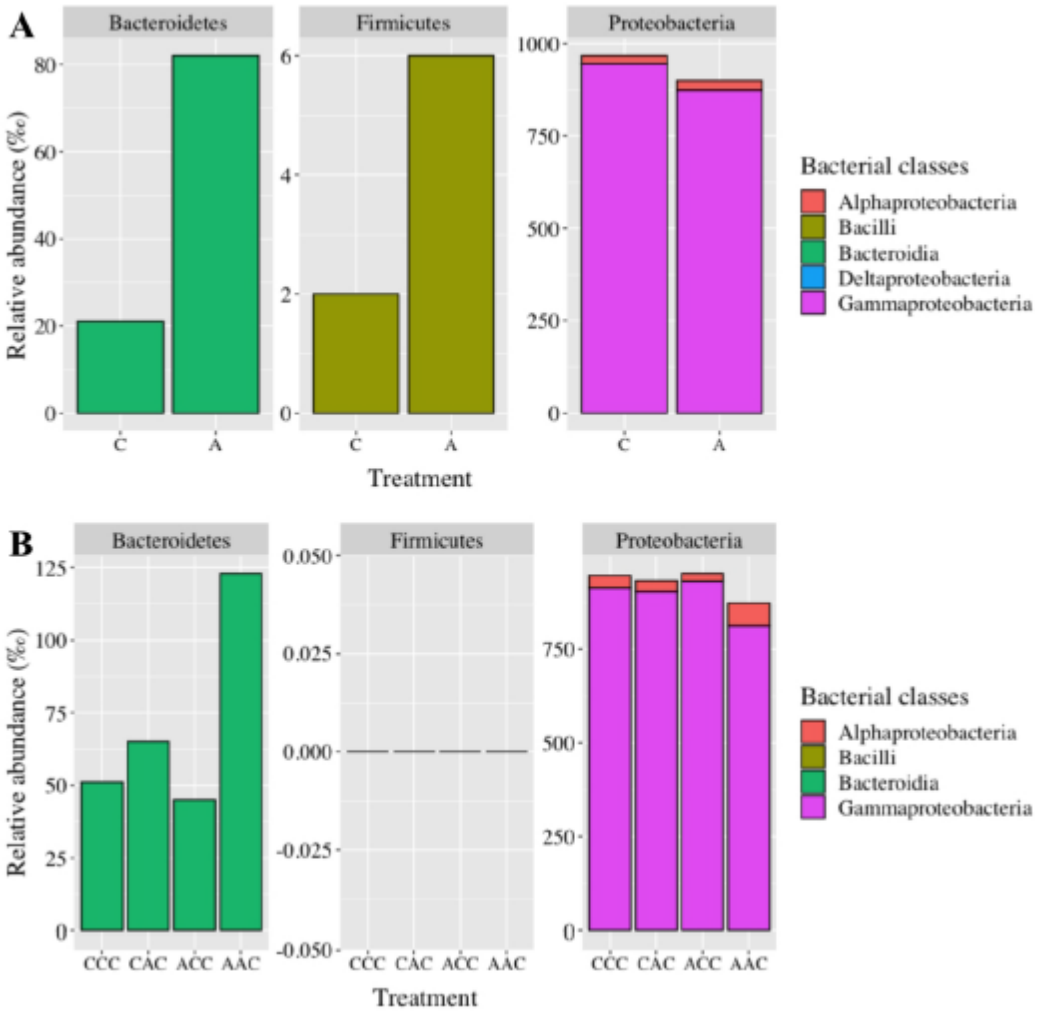


Figure 2 Dominant bacterial phyla and classes of G0 (A) and G2 (B) flies. “C” and “A” stand for control and antibiotic treatments respectively.

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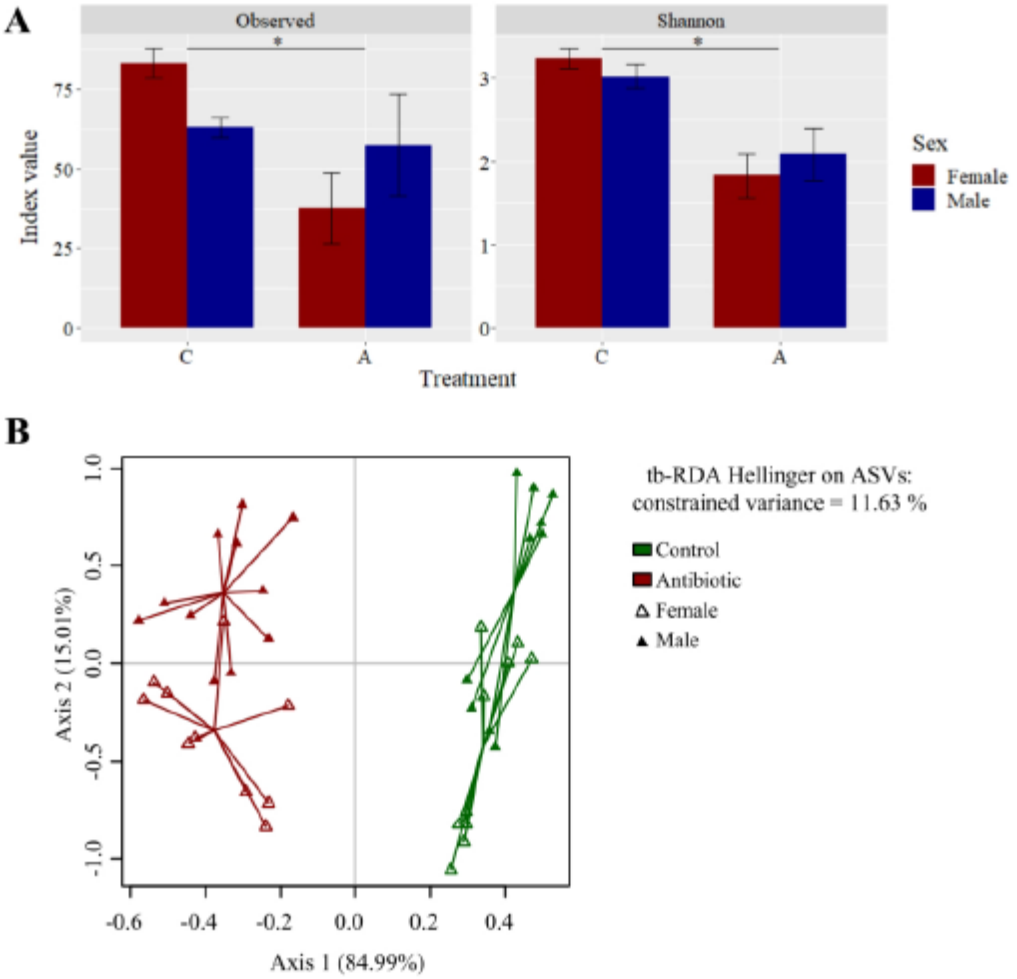


Figure 3 Bacterial diversity of G0 flies treated with tetracycline “A” or not “C”. (A) Alpha diversity is represented by the number of observed ASVs and the Shannon index (mean ± se). An asterisk represents a significant difference between the two treatments. (B) Community structure is represented by beta diversity analyzed using a tb-RDA, which sample projection is plotted. The variances explained by the tb-RDA axes are given in parenthesis.

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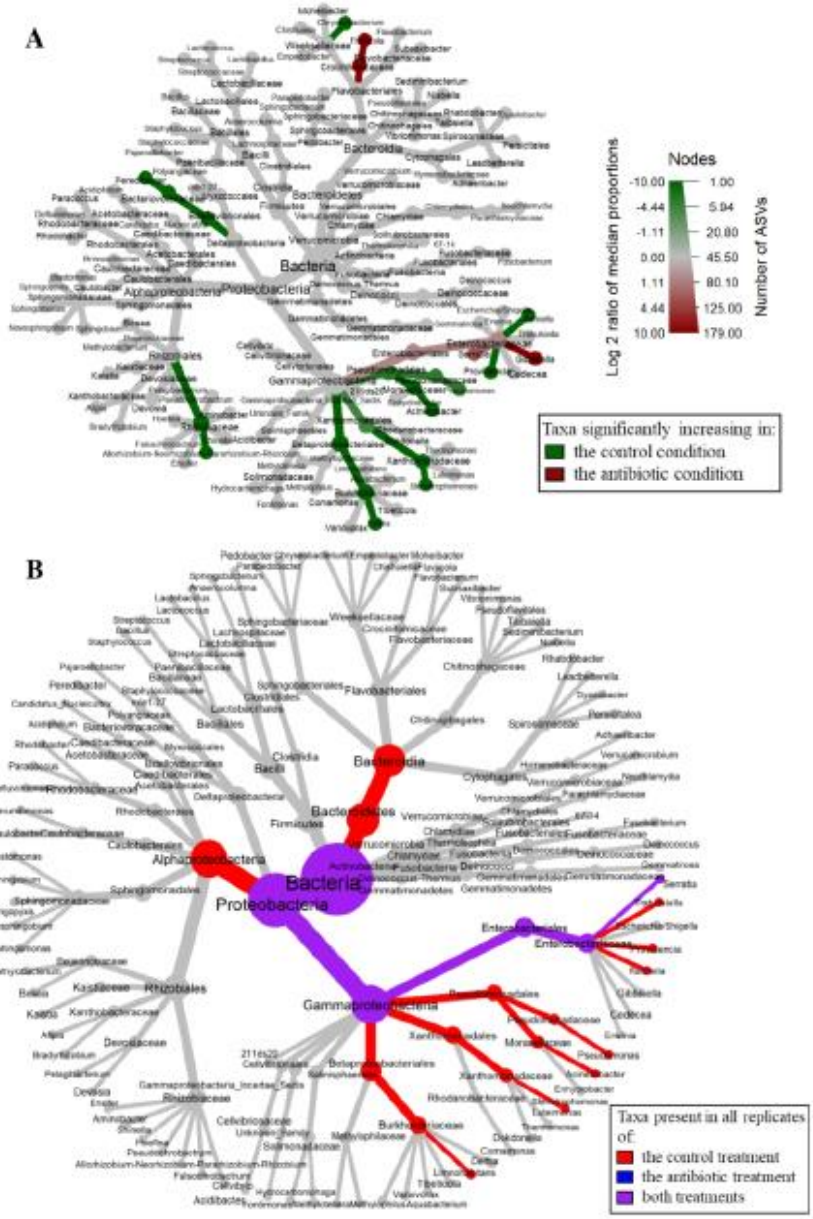


Figure 4 Heat trees comparing taxa between the antibiotic and control treatment of G0 flies. (A) The color of each taxon represents the log-2 ratio of median relative abundances observed for each treatment (control or antibiotic) and only significant differences are colored. (B) The color of each taxon represents the taxa presence in all flies (i.e. replicates) of a given treatment.

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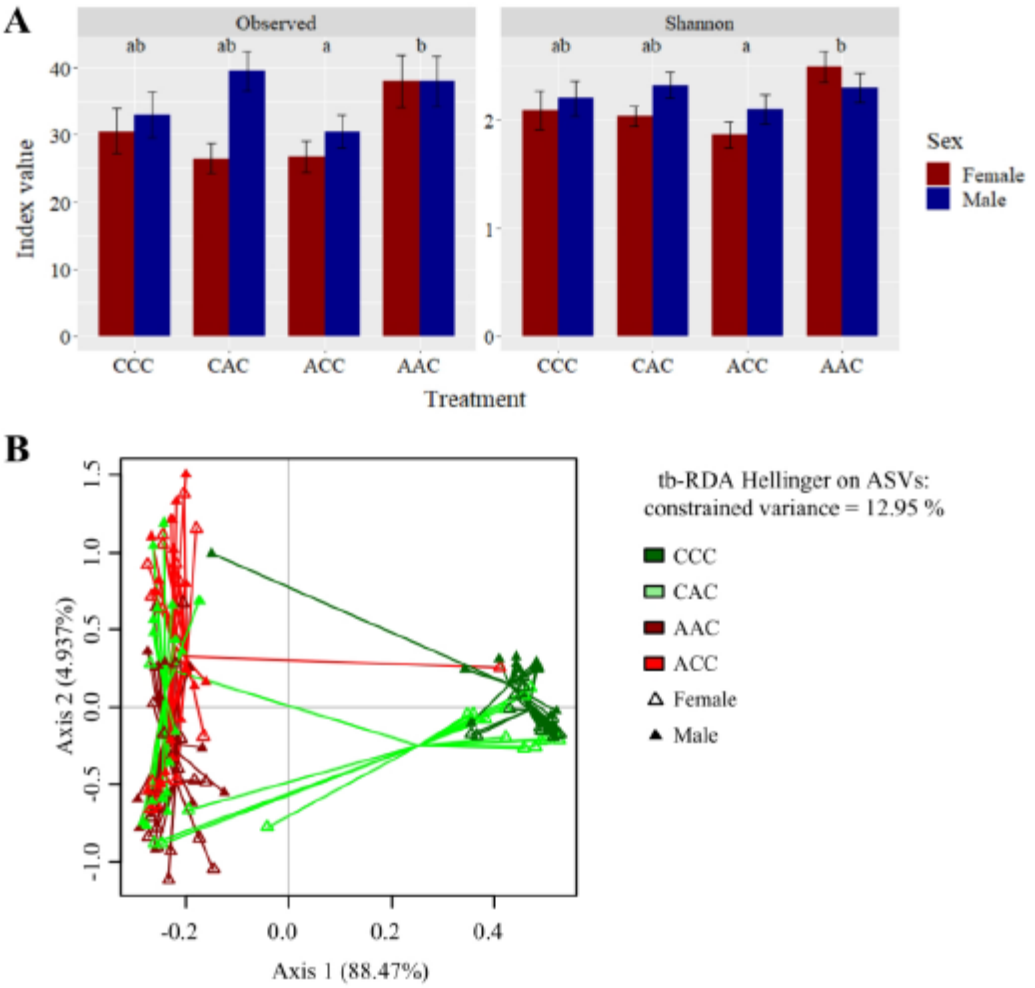


Figure 5 Bacterial diversity of G2 flies treated with tetracycline or not. (A) Alpha diversity is represented by the number of observed ASVs and the Shannon index (mean ± se). Treatments (AAC, ACC, CAC, CCC) sharing at least one lowercase letter (a, b, ab) are not significantly different. (B) Community structure is represented by beta diversity analyzed using a tb-RDA, which sample project is plotted. The variances explained by the tb-RDA axes are given in parenthesis.

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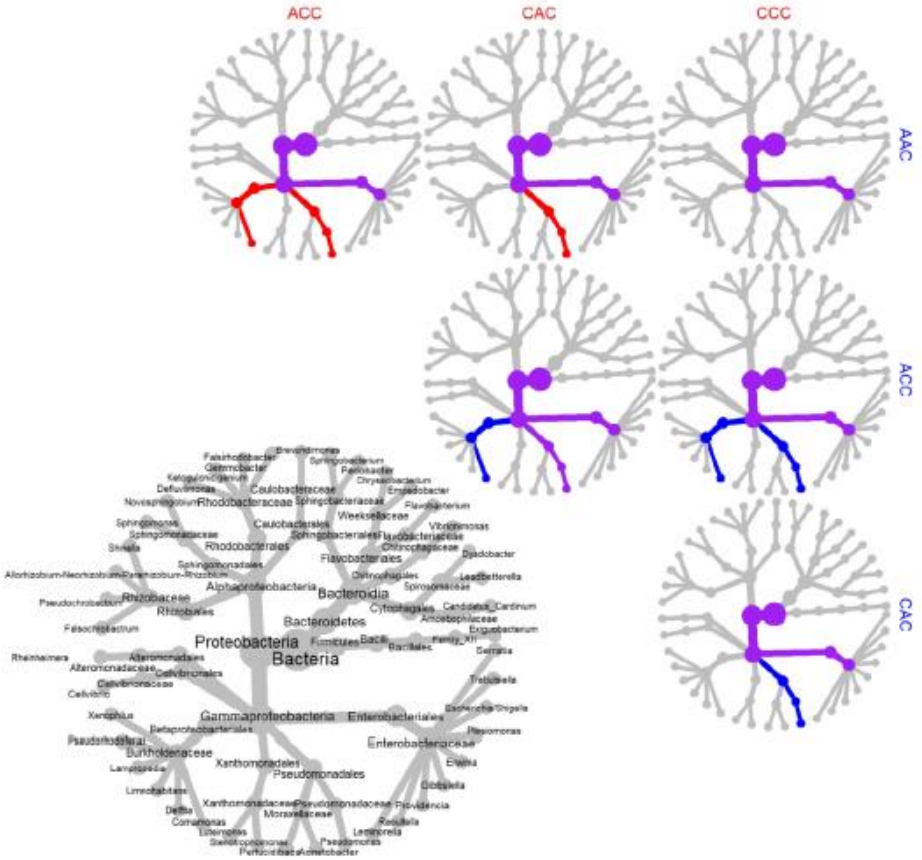


Figure 6 Heat trees comparing taxa between the antibiotic and control treatment of G2 flies. The color of each taxon represents the taxa presence in all flies (i.e. replicates) of a given treatment (AAC, ACC, CAC, CCC). Taxa colored in red are present in all flies of only the treatment shown in the column above heat trees, taxa colored in blue are present for only the treatment shown in the row on the right of heat trees and taxa colored in purple are present for both treatments. The grey tree on the lower left functions as a key for the smaller unlabeled trees.

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Table 1 Statistical outputs of ASVs with significant pairwise comparisons within the treatment ("C" and "A" stand for control and antibiotic treatment), associated to the G0 flies. This table compiles taxonomic, beta diversity, frequencies and statistical information. Clusters 1 and 2 refer as to A and C respectively and probability values in bold indicate a significant indicator distribution.

Taxonomy				**Indicator ASV**			**Frequency**		**Statistics**
Order	Family	Genus	ASV	Cluster	Value	Probability	A	C	Treatment P value
†Chitinophagales	Chitinophagaceae	NA	316	1	0.286	0.091.	6/21	0/20	0.032*
			327	1	0.286	0.208	6/21	0/20	0.032*
†Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	554	2	0.25	0.135	0/21	5/20	0.042*
			564	2	0.3	0.106	0/21	6/20	0.022*
			576	2	0.25	0.148	0/21	5/20	0.042*
			580	2	0.25	0.128	0/21	5/20	0.042*
†Sphingobacteriales	Sphingobacteriaceae	*Sphingobacterium*	603	2	0.45	**0.041***	0/21	9/20	0***
			629	2	0.35	0.07.	0/21	7/20	0.016*
			832	2	0.3	0.122	0/21	6/20	0.022*
			865	2	0.25	0.195	0/21	5/20	0.042*
αRhizobiales	Rhizobiaceae	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	588	2	0.35	0.074.	0/21	7/20	0.016*
			593	2	0.3	0.112	0/21	6/20	0.022*
			676	2	0.3	0.074.	0/21	6/20	0.022*
			695	2	0.25	0.189	0/21	5/20	0.042*
δBdellovibrionales	Bacteriovoracaceae	*Peredibacter*	646	2	0.25	0.189	0/21	5/20	0.042*
			726	2	0.3	0.122	0/21	6/20	0.022*
γBetaproteobacteriales	Burkholderiaceae	*Comamonas*	98	2	0.3	0.099.	0/21	6/20	0.022*
		Delftia	220	2	0.55	**0.024***	0/21	11/20	0***
			236	2	0.55	**0.024***	0/21	11/20	0***
			260	2	0.417	**0.024***	1/21	9/20	0.022*
			276	2	0.45	0.056.	0/21	9/20	0***

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Taxonomy				Indicator ASV			Frequency		Statistics
Order	Family	Genus	ASV	Cluster	Value	Probability	A	C	Treatment P value
			597	2	0.3	0.086.	0/21	6/20	0.022*
			611	2	0.35	0.086.	0/21	7/20	0.016*
			725	2	0.3	0.122	0/21	6/20	0.022*
			780	2	0.3	0.099.	0/21	6/20	0.022*
[y]Enterobacteriales	Enterobacteriaceae	*Gibbsiella*	320	1	0.524	**0.024***	11/21	0/20	0***
			466	1	0.333	0.106	7/21	0/20	0.016*
		NA	455	1	0.333	0.091.	7/21	0/20	0.016*
			522	1	0.286	0.187	6/21	0/20	0.032*
			242	2	0.4	**0.024***	0/21	8/20	0***
			251	2	0.3	0.122	0/21	6/20	0.022*
			381	2	0.25	0.228	0/21	5/20	0.042*
			387	2	0.25	0.187	0/21	5/20	0.042*
			398	2	0.25	0.148	0/21	5/20	0.042*
			400	2	0.25	0.158	0/21	5/20	0.042*
		Providencia	108	2	0.55	**0.024***	0/21	11/20	0***
			76	2	0.55	**0.024***	0/21	11/20	0***
			85	2	0.55	**0.024***	0/21	11/20	0***
			94	2	0.55	**0.024***	0/21	11/20	0***
		Rahnella	119	2	0.45	**0.041***	0/21	9/20	0***
			123	2	0.502	**0.024***	1/21	11/20	0***
			131	2	0.45	**0.024***	0/21	9/20	0***
			141	2	0.5	**0.024***	0/21	10/20	0***
		Serratia	159	2	0.355	0.112	1/21	8/20	0.041*
			170	2	0.35	0.074.	0/21	7/20	0.016*
			172	2	0.3	0.091.	0/21	6/20	0.022*
			177	2	0.4	**0.024***	0/21	8/20	0***
			262	2	0.3	0.091.	0/21	6/20	0.022*
			271	2	0.35	0.079.	0/21	7/20	0.016*
[y]Pseudomonada	Moraxellaceae	*Acinetobacter*	40	2	0.514	**0.024***	1/21	11/20	0***

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Taxonomy				Indicator ASV			Frequency		Statistics
Order	Family	Genus	ASV	Cluster	Value	Probability	A	C	Treatment P value
les									
			55	2	0.514	**0.024***	1/21	11/20	0***
			56	2	0.417	**0.024***	1/21	9/20	0.022*
			909	2	0.25	0.158	0/21	5/20	0.042*
	Pseudomonadaceae	*Pseudomonas*	105	2	0.502	**0.024***	1/21	11/20	0***
			122	2	0.502	**0.024***	1/21	11/20	0***
			134	2	0.55	**0.024***	0/21	11/20	0***
			137	2	0.45	**0.024***	0/21	9/20	0***
			144	2	0.463	0.074.	2/21	11/20	0.016*
			147	2	0.55	**0.024***	0/21	11/20	0***
			152	2	0.45	**0.024***	0/21	9/20	0***
			158	2	0.55	**0.024***	0/21	11/20	0***
			176	2	0.472	**0.041***	2/21	11/20	0.016*
			186	2	0.472	0.056.	2/21	11/20	0.016*
			239	2	0.413	**0.041***	1/21	9/20	0.022*
			252	2	0.356	0.079.	1/21	8/20	0.041*
			363	2	0.5	**0.024***	0/21	10/20	0***
			371	2	0.5	**0.024***	0/21	10/20	0***
			394	2	0.35	0.056.	0/21	7/20	0.016*
			401	2	0.35	0.079.	0/21	7/20	0.016*
			405	2	0.25	0.158	0/21	5/20	0.042*
			545	2	0.25	0.122	0/21	5/20	0.042*

.: P < 0.1, *: P < 0.05, **: P < 0.01, ***: P < 0.001

[†]Bacteroidetes – Bacteroidia; [α]Proteobacteria – alphaproteobacteria; [δ]Proteobacteria – deltaproteobacteria; [γ]Proteobacteria – gammaproteobacteria (phylum – class).

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Table 2 Significant indicator ASVs of G2 flies influenced by the treatment.

Phylum	**Class** - Genus	Number of indicator ASVs in each cluster		
		1 = AAC	2 = ACC	4 = CCC
Bacteroidetes	**Bacteroidia**	**5**	**0**	**14**
	Chryseobacterium	0	0	2
	NA	1	0	0
	Sphingobacterium	4	0	12
Proteobacteria	**Alphaproteobacteria**	**1**	**2**	**8**
	Falsochrobactrum	0	2	4
	Pseudochrobactrum	1	0	4
	Gammaproteobacteria	**3**	**2**	**47**
	Acinetobacter	0	0	4
	Comamonas	0	1	8
	Erwinia	0	0	1
	Gibbsiella	0	1	0
	NA	0	0	13
	Pseudomonas	2	0	14
	Serratia	1	0	2
	Stenotrophomonas	0	0	5
Total		**9**	**4**	**69**

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Table 3 Statistical outputs of ASVs with significant pairwise comparisons within the treatment, associated to the G2 flies. This table compiles taxonomic, indicator ASV, frequencies and statistical information. In the frequency columns, treatments sharing at least one lowercase letter are not significantly different. Clusters 1, 2 and 4 refer as to AAC, ACC and CCC respectively and probability values in bold indicate a significant indicator distribution.

Taxonomy				**Indicator ASVs**			**Frequency**				**Statistics**
Order	Family	Genus	ASV	Cluster	Value	Probability	AAC	ACC	CAC	CCC	Treatment P value
†Sphingobacteriales	Sphingobacteriaceae	*Sphingobacterium*	91	4	0.252	**0.008****	0/34 ab	1/34 a	4/34 ab	12/33 b	0***
αRhizobiales	Rhizobiaceae	*Falsochrobactrum*	125	2	0.209	**0.038***	13/34 b	16/34 b	10/34 b	1/33 a	0***
			140	2	0.245	**0.008****	13/34 b	17/34 b	8/34 ab	1/33 a	0***
			101	4	0.268	**0.008****	0/34 ab	1/34 a	3/34 a	12/33 b	0***
			88	4	0.328	**0.008****	0/34 ab	0/34 ab	4/34 a	14/33 b	0***
		Pseudochrobactrum	166	4	0.263	**0.008****	0/34 ab	1/34 a	5/34 ab	13/33 b	0***
			171	4	0.328	**0.008****	0/34 ab	0/34 ab	4/34 a	14/33 b	0***
γBetaproteobacteriales	Burkholderiaceae	*Comamonas*	150	1	0.16	0.212	15/34 b	12/34 b	10/34 b	1/33 a	0***
			110	2	0.228	**0.014***	14/34 b	17/34 b	10/34 b	1/33 a	0***
			120	2	0.191	0.139	16/34 b	16/34 b	10/34 b	1/33 a	0***
			160	2	0.133	0.419	13/34 b	12/34 b	9/34 b	1/33 a	0.004**
			178	4	0.318	**0.008****	0/34 ab	1/34 a	5/34 a	15/33 b	0***
			198	4	0.306	**0.008****	0/34 ab	1/34 a	2/34 a	13/33 b	0***
γEnterobacteriales	Enterobacteriaceae	NA	107	4	0.261	**0.008****	0/34 ab	1/34 a	2/34 a	11/33 b	0***

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Taxonomy				Indicator ASVs			Frequency				Statistics
Order	Family	Genus	ASV	Cluster	Value	Probability	AAC	ACC	CAC	CCC	Treatment P value
			45	4	0.247	**0.008****	0/34 ab	1/34 a	6/34 ab	13/33 b	0***
			5	4	0.302	**0.008****	0/34 ab	1/34 a	6/34 ab	15/33 b	0***
			6	4	0.391	**0.008****	0/34 ab	0/34 ab	6/34 a	17/33 b	0***
			61	4	0.302	**0.008****	0/34 ab	1/34 a	6/34 ab	15/33 b	0***
		Serratia	4	2	0.193	0.1	15/34 b	16/34 b	11/34 b	1/33 a	0***
			8	4	0.302	**0.008****	0/34 ab	1/34 a	6/34 ab	15/33 b	0***
[Y]Pseudomonadales	Moraxellaceae	*Acinetobacter*	48	2	0.221	0.065.	16/34 b	17/34 b	9/34 b	1/33 a	0***
			56	2	0.214	0.057.	13/34 b	16/34 b	9/34 b	1/33 a	0***
			13	4	0.314	**0.008****	0/34 ab	1/34 a	2/34 a	13/33 b	0***
			18	4	0.268	**0.008****	0/34 ab	1/34 a	3/34 a	12/33 b	0***
Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	113	1	0.13	0.286	12/34 b	9/34 ab	7/34 ab	1/33 a	0.015*
			14	1	0.192	0.1	16/34 b	11/34 b	9/34 b	1/33 a	0***
			27	4	0.256	**0.008****	0/34 ab	1/34 a	5/34 ab	13/33 b	0***
			35	4	0.259	**0.008****	0/34 ab	1/34 a	5/34 ab	13/33 b	0***
			68	4	0.367	**0.008****	0/34 ab	0/34 ab	1/34 a	13/33 b	0***
[Y]Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*	74	2	0.15	0.309	13/34 b	13/34 b	10/34 b	1/33 a	0***
			77	2	0.12	0.437	10/34 b	11/34 b	10/34 b	1/33 a	0.012*

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.: P < 0.1, *: P < 0.05, **: P < 0.01, ***: P < 0.001

[†]Bacteroidetes – Bacteroidia; [α]Proteobacteria – alphaproteobacteria; [γ]Proteobacteria – gammaproteobacteria (phylum – class).

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