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Altered diversity of bacterial communities in two *Drosophila* species under laboratory conditions and lead exposure

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Abstract: The composition of microbiota affects different traits of *Drosophila* throughout its life cycle and represents an important part of the adaptive response to environmental changes, such as increased levels of accumulated heavy metals in their habitats. We investigated the effects of lead added to the feeding substrate on the microbiota diversity in the fruit flies, *Drosophila melanogaster* and *Drosophila subobscura*. We compared the bacterial diversity of wild-caught flies and their progeny reared under standard laboratory conditions. We analyzed the shifts in bacterial diversity in the experimental groups of flies reared for 13 generations on standard and lead(II) acetate ($Pb(CH_3COO)_2$), Pb acetate-saturated substrates. Identification of the main bacterial genera was performed by next-generation sequencing (NGS) of the V3-V4 variable regions of the 16S rRNA gene. Results indicate significant species-specific differences in bacterial composition between natural and laboratory populations, and between the substrates. Diversity increased in both species under prolonged exposure to lead-polluted substrate, suggesting a potential adaptive response to an environmental stress.

Keywords: *Drosophila melanogaster; Drosophila subobscura;* lead exposure; microbiota diversity; next-generation sequencing

INTRODUCTION

Understanding the mechanisms and processes by which organisms adapt to environmental changes is a central issue in evolutionary biology. One of the most important interactions among entities in a particular habitat is that between the host and its microbial community, which depends on species biology and environmental conditions [1].

Insects, like all metazoans, have a variable microbial community, which consists mostly of saprophytic and nonpathogenic bacteria, including bacteria found in the gut, as well as symbiotic bacteria. Invertebrate-associated gut microorganisms are less abundant and less diverse than in vertebrates. Dominant bacterial taxa are widespread and found in host species that have diverse taxonomy, ecology and geography. Competition for an ecological niche among microbes and interactions with hosts can lead to replacement of microbe species within the host [1,2]. Thus, better identification of the factors that shape the diversity of microbe communities and their interactions with hosts is a prerequisite in understanding the role of microbiota in the physiology, ecology and evolution of insects.

Microbiota affect host fitness under changing environmental conditions [3,4]. Climate and nutrition changes and increased pollution affect the presence of certain microbial species [5]. Sex, age and nutrition have proven to be important factors that affect the diversity of microbiota [6,7], and the increased concentration of heavy metals is among the most influential environmental factors. Ingested heavy metals have been found to cause significant changes in the microbiota and, consequently, in various physiological statuses

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[8-10]. Lead (Pb) is one of the most widely spread heavy metals in nature; it has a significant effect on different life-history traits of *Drosophila* species [11-13]. Increased concentrations of Pb were observed to significantly affect *D. melanogaster* in their developmental stages, causing a decrease in the percentage of hatching [14]. It remains to be seen to what extent the interaction between microorganisms and their host is part of an adaptive response to lead pollution.

According to their feeding and breeding substrates, *Drosophila* species range from cosmopolitan generalists to species with a specialized diet. Due to the breadth of environmental conditions they can tolerate and the possibility of maintaining some of the species in the laboratory, fruit flies present an informative model in the study of microbial dynamics throughout the life span of its host under different stress conditions.

Herein we focused on the microbiomes of two species, *D. melanogaster* and *D. subobscura,* that have wide but distinct geographic distributions, genetics and ecology. *D. melanogaster* is a cosmopolitan species, while *D. subobscura* has a broad Palearctic distribution that rapidly expanded to South and North America in the late 1970s [15]. With spatially and temporally variable inversion polymorphism, *D. subobscura* has proven to be a good model species for studying the effects of environmental change, but its microbiome has not been explored thus far. We first determined the difference in bacterial diversity between wild-caught samples of *D. melanogaster* and *D. subobscura* and their laboratory-reared progeny under the same conditions (the "Wild_Lab" experiment). Secondly, the bacterial diversity in both species was estimated after generations of laboratory-controlled rearing using a standard substrate and a Pb acetate-saturated substrate. Amplicon sequencing of the variable regions V3-V4 of the bacterial 16S rRNA gene with the MiSeq Illumina next generation sequencing (NGS) platform was used.

MATERIALS AND METHODS

General methods and conditions

Flies of both species were sampled in the same manner, with a sweeping net, using the same type of fermented apple traps. Isofemale lines (IFLs), which represent the progeny of single field-caught gravid females inseminated in the wild, were maintained in 50-mL vials with 15 mL of substrate at 19±0.5°C, and a 12 h light-dark cycle, on a standard molasses corn meal diet (14 g agar, 208 g corn meal, 188 g sugar, 40 g dry active yeast, 5 g Nipagin diluted in 60 mL of 96% ethanol in 2.2 L distilled water). Both species were reared at 19°C. *D melanogaster* is widespread in a wide temperature range in the wild and is successfully reared at 19-25°C, unlike *D. subobscura*, which has a much narrower temperature range in the wild, with 19°C being the optimal temperature. At 19°C, both species successfully develop under laboratory conditions, and importantly for this study of their microbiota, we could maintain experimental cultures of both species for the same number of days under the same conditions. Dry yeast powder was added on the surface of the substrate to both species, as *D. subobscura* generally does not lay eggs without it.

The "Wild_Lab" experiment

For this experiment, *D. melanogaster* and *D. subobscura* were collected from Kalna, Serbia (43.4217 N, 22.4159 E) and Mitrovac, Serbia (43.9217 N, 19.4239 E), respectively. Field- collected males were stored directly in ethanol at -20°C, and the females were used to establish the IFLs for each species and population. After 10 generations of rearing under standard laboratory conditions, individuals of adult males were sampled randomly from the IFLs (five males per IFL) of each species and stored at -20°C in ethanol for NGS.

The "St_Pb" experiment

In this experiment, the flies of both species were from the same locality, Kalna, Serbia (43.4217 N, 22.4159 E). After maintaining IFLs under standard laboratory conditions for 45 generations for *D. subobscura*, and 30 generations for *D. melanogaster*, 9 pairs of flies from each of 10 randomly chosen IFLs were transferred to 240-mL bottles with 40 mL of Pb acetate-saturated substrate (labeled as C3), and a control substrate (standard – St) for 7 days to lay eggs; they were then stored in ethanol for NGS (parental, both sexes) at -20°C. The Pb acetate-saturated substrate contained 1000 μg/mL of Pb acetate. Their progeny was transferred and reared on lead-polluted and control substrates under the same conditions and controlled population density

(by transferring 20 random pairs in each bottle) for 13 generations (Progeny F13, both sexes).

DNA isolation and amplicon library preparation

For the "Wild_Lab" experiment, total DNA was extracted from pools of 30 males from wild and lab-reared *D. melanogaster* (labeled as D-mel-Wild and D-mel-Lab, respectively) and 30 males from wild and lab-reared *D. subobscura* (labeled as D-sub-Wild and D-sub-Lab, respectively). DNA isolation was performed according to the modified protocol [16]. The samples were homogenized using a handheld motor homogenizer in a 1.5-mL Eppendorf tube (2 tubes of 15 individuals each) in 320 μL of 10 mM Tris HCl, 60 mM NaCl, 5% (wt/vol) sucrose, 10 mM EDTA, pH 7.8. Then, 400 μL of 1.25% sodium dodecyl sulfate (SDS), 300 mM Tris HCl, 5% sucrose, 10 mM EDTA, pH 9.0, were added and gently mixed. The mixture was incubated for 30 min at 65°C, after which 120 μL of 3 M potassium acetate, pH 4.8, was added and the mixture was kept in the freezer for 15 min. The supernatant was collected after centrifugation at 20000 × *g*. One volume of isopropanol was added, and after 5 min at room temperature, the mixture was centrifuged for 5 min to pellet the DNA. The pellet was washed with 70% ethanol, centrifuged at 20000 × *g* for 2.5 min and the supernatant was collected again. The pellet was dried for 30 min resuspended in 100 μL nuclease-free water. Sequencing was performed by Macrogen, South Korea.

For the "St_Pb" experiment, total DNA was extracted from 16 samples (2 species × 2 generations × 2 substrates × 2 sexes) containing pools of about 10 individuals, reared on the standard (St) and Pb acetate-saturated substrate (C3), and separated by sex (F for female and M for male). DNA isolation was performed according to the modified protocol [17]. The samples were homogenized using a handheld motor homogenizer in a 1.5-mL Eppendorf tube in 200 μL of Solution A (1 M Tris HCl, pH 9, 0.5 M EDTA, pH 8 and 1% SDS). Then, 3 μL of proteinase K (20 mg/mL) were added, the samples were vortexed and incubated for 30 min at 56°C, and incubated for another 30 min at 70°C and, finally, for a few minutes at 37°C. Afterwards, 2 μL of RN*ase* A (10 mg/mL) were added and incubated for 30 min at 37°C. Next, 28 μL 8 M potassium acetate were added and the mixture was kept in the freezer for 30 min. The supernatant was collected after centrifugation at 16000 × *g* for 15 min. One volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, and the mixture was centrifuged for 5 min at 16000 × *g*. After the supernatant was collected, the previous step was repeated with 0.75 volumes of pure chloroform. Afterwards, 2.5 volumes of 95% ice-cold ethanol were added, centrifuged at 9500 × *g* for 5 min to pellet the DNA. The pellet was washed with 1 mL of 70% ethanol, centrifuged at 16000 × *g* for 5 min and the supernatant was collected again. The pellet was dried for 30 min and resuspended in 50 μL of TE buffer (10 mM Tris HCl and 1 mM EDTA). The sequencing was performed by Fisabio (Spain).

The sequences of primers targeting the V3-V4 region of the 16S rRNA gene were as follows: 16S forward primer 5'-CCTACGGGNGGCWGCAG-3', reverse primer 5'-GACTACHVGGGTATCTAATCC-3'. The primers were obtained from [18]. Briefly, samples were first amplified in a limited-cycle PCR, using the Kapa 2 G HiFi Hot-start ready mix 2 × (Kapa Biosystems, Massachusetts, USA), with the following temperature cycling conditions: 5 min at 95 °C, 30 s at 95 °C, 30 s at 53 °C, 45 s at 72 °C, and a final elongation step at 72 °C for 10 min, followed by an 1X AMPure XP bead clean-up (Beckman Coulter Inc., Brea, CA, USA). The amplicon libraries were prepared using a Nextera XT Index Kit as per the 16S Metagenomic Sequencing Library preparation protocol (Part #15044223 Rev. B) using i5 and i7 primers that add multiplexing index sequences, as well as common adapters required for cluster generation, followed by a final AMPure XP bead clean-up. The amplicon libraries were checked with an Agilent High Sensitivity (HS) chip on a Bioanalyzer 2100 and quantified with NanoPhotometer® N60 (IMPLEN, CA, USA). The Illumina 16S V3-V4 amplicon library preparation and MiSeq 300 bp paired-end sequencing was performed using an MiSeq Reagent Kit (Illumina). The samples passed a quality check and FASTQ data were delivered for further processing. All sequence data have been submitted to the GenBank (SRA) database under accession number SRP254478.

Metagenomic analysis, bioinformatics

The NGS facility provided demultiplexed sorted reads with adapters and linkers removed. NGS analysis was performed following the DADA2 pipeline [19,20]. Filtering, trimming, error rate estimation, sample

inference, paired-end merger and chimera depletion were performed, starting from paired-end data [21]. Prior to quality control and filtering, primer sequences were removed using BBDuk software [22]. For the "Wild_Lab" experiment, primers were removed using the literal primer sequences mentioned previously using kmer length 15 and a Hamming distance of 2 (2 mismatches were allowed). As regards the "St_Pb" experiment, primers were removed using kmer length 15 and a Hamming distance of 1 for parental, and kmer length 14 and a Hamming distance of 2 for F13 progeny. After primer removal, sequences were right-trimmed to 255 bases for the forward reads, and 195 bases for the reverse reads ("Wild_Lab" experiment), and 250 bases for the forward reads and 200 bases for the reverse reads ("St_Pb" experiment), based on the distribution of FASTQ quality scores. Reads were truncated at the first instance of a Phred quality score less than or equal to 6. After truncation, reads with the expected errors (sum(10^(-Q/10)), where Q is the quality score) greater than 2 and 6 for forward reads ("Wild_Lab", "St_Pb" experiment, respectively) and greater than 4 for all reverse reads were discarded. Additionally, reads with ambiguous bases (N) and reads shorter than 50 bases were discarded. After DADA2 error estimation and sequence inference, sequence pairs were merged using a minimum overlap of 17 bases ("Wild_Lab" experiment) and 20 bases ("St_Pb" experiment) without mismatches, and all sequences shorter than 400 ("Wild_Lab" experiment) and shorter than 402 or longer than 427 ("St_Pb" experiment) were discarded, based on the sequence length distribution. Finally, chimeric sequences were removed using the "consensus" method in DADA2, resulting in 61-69% of the initial sequenced reads being retained in the "Wild_Lab" experiment, 89-93% for parental, and 65-83% for F13 progeny in the "St_Pb" experiment.

Microbial community composition and taxonomic analysis

To establish which bacterial taxa are present, a representative sequence of each operational taxonomic unit (OTU) was identified at different classification levels. The OTUs were aggregated at the phylum, family and genus levels, and those with a normalized abundance <1% or 2% (depending on the number of identified classes), were labeled as "genus/family/phylum represented at <1% or 2%". Taxonomy assignment was performed using the Silva v132 database [23] with the RDP Naïve Bayesian Classifier algorithm [24] using kmer size 8 and 100 bootstrap replicates. The minimum bootstrap confidence for assigning a taxonomic level was set to 50. Prior to further analysis, all sequences with undetermined phylum or that were categorized as eukaryotic (kingdom), chloroplast (order), mitochondrial (family) or Ralstonia (genus) (a frequent contaminant in MiSeq) were removed. Although these OTUs made up only a small proportion of the total reads, their share in the total number of OTUs was not negligible.

SILVA 16S [23] training sets were used as reference taxonomy databases. Additionally, species assignment was performed by exact matching of OTUs against reference FASTA sequences [25] to identify the genus-species classification of the input sequences. The analysis was performed on the SILVA taxonomy-assigned OTUs. The normalized abundance of each OTU was calculated in each sample by dividing the OTU read count by the total read count for each sample. Bacterial diversity within communities (alpha diversity) was determined by a sampling-based analysis of OTUs, and was shown through estimators Shannon, compliment Simpson (1-D) and invSimpson alpha indices. The observed and estimated richness was determined according to the number of observations (OBS), with Chao1, and Chao1 standard error. We used the Man Whitney Wilcoxon (MWW) test for alpha diversity and found no statistical significance, except one close to $P<0.05$ for the Shannon estimator in F13 Progeny between species. Additionally, rarefaction analysis was performed in order to estimate the overall diversity. Beta-diversity or diversity shared across sample communities was determined using multidimensional scaling (MDS) performed on the weighted UniFrac distance matrix calculated using log(x+1)-transformed abundances rarefied to even depth.

RESULTS

16S rRNA metagenomics data and diversity of microbial communities

The number of sequences obtained before and after filtering is summarized in Supplementary Table S1. Additionally, species richness through rarefaction curves at phylum, family and genus taxonomic levels

Table 1. Richness and evenness of bacterial communities of two *Drosophila* species presented through alpha diversity indices in the "Wild_Lab" experiment.

Sample	Shannon	Simpson	invSimpson	**OBS**	CHAO1	CHAO1.SE	ACE	ACE.SE
D-mel-Wild	2.97	0.89	9.43	**476**	476.00	0.00	476.00	7.63
D-mel-Lab	2.45	0.71	3.39	**414**	414.00	0.08	414.39	4.26
D-sub-Wild	4.01	0.83	5.99	**629**	629.20	0.62	629.85	3.82
D-sub-Lab	4.35	0.95	18.28	**489**	489.17	0.54	489.66	4.42

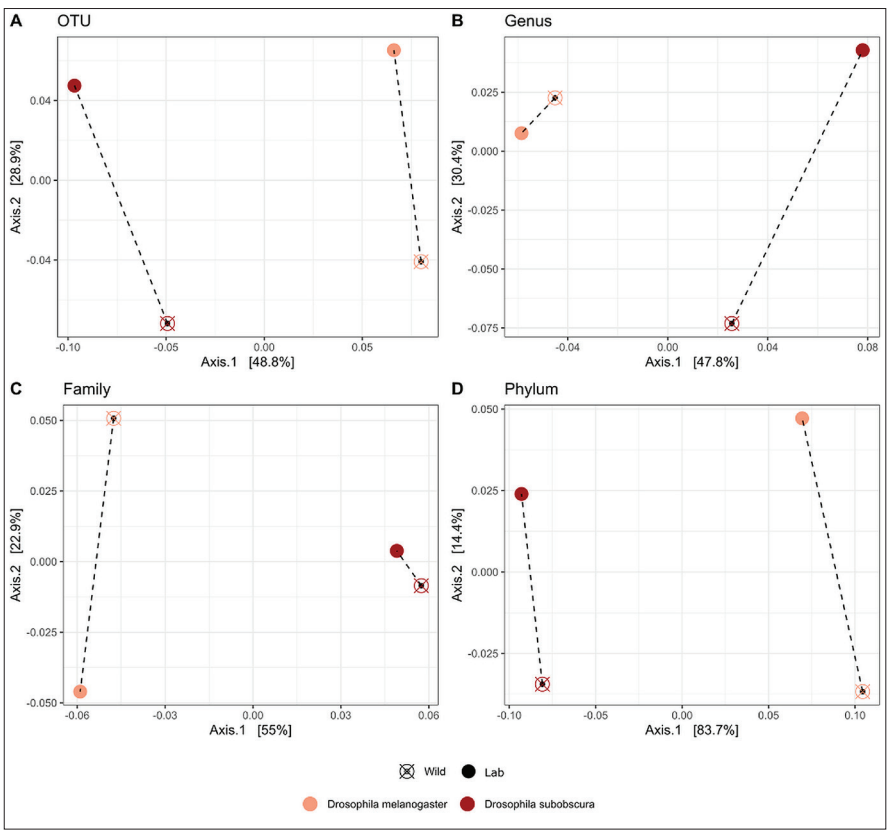

Fig. 1. Multidimensional scaling (MDS) performed on the weighted UniFrac distance matrix calculated using log(x+1)-transformed abundances rarefied to an even depth of bacterial composition in different *Drosophila* samples (*D. melanogaster* and *D. subobscura*; wild and lab) at (**A**) OTU, (**B**) genus, (**C**) family and (**D**) phylum taxonomic levels in the "Wild_Lab" experiment. The dashed line refers to differences in treatments within species.

are plotted in Supplementary Figs. S1 and S2. According to the rarefaction curves, species saturation was achieved, and the sampling size was sufficient to estimate the bacterial diversity.

The "Wild_Lab" experiment

The microbial alpha diversity indices for each sample at the OTU level are presented in Table 1, indicating that bacterial communities were relatively rich and homogeneous in diversity across samples. Alpha diversity values at the genus, family and phylum levels are shown in Supplementary Table S2. Alpha diversity was higher in both *D. subobscura* samples compared to *D. melanogaster*, with slightly higher levels in lab-reared than in wild-caught flies, according to Shannon's and Simpson's diversity indices. Conversely, the observed richness according to OBS showed the highest bacterial richness in *D. subobscura* wild-caught flies. However, all indices showed lower diversity and evenness in both *D. melanogaster* samples than in *D. subobscura* samples. The differences between the observed and estimated richness are in positive correlation according to the estimated Chao1 and ACE indices. The lowest richness was observed in the *D. melanogaster* lab-reared samples, which was positively correlated with alpha diversity indices.

Multidimensional scaling (MDS) was plotted as a summary of the beta diversity relationships of the compositional differences among the samples of the microbial communities in order to represent the original position of the communities in a multidimensional space (Fig. 1). The microbiota from the two *Drosophila* species were separated by the first MDS axis at all taxonomic levels examined, while separation between wild-caught and lab-reared flies bacterial communities was observed by the second MDS axis at the OTU and phylum levels.

The "St_Pb" experiment

The Chao1 and ACE indices showed that in both species and substrates (standard and polluted) there was a higher richness in OTUs in the F13 progeny compared

Table 2. Richness and evenness of bacterial communities of two *Drosophila* species presented through alpha diversity indices in the "St_Pb" experiment.

Sample	Shannon	Simpson	InvSimpson	**OBS**	CHAO1	CHAO1.SE	ACE	ACE.SE
Parental								
D. mel_St_F	0.36	0.16	1.18	**14**	14.00	0.16	14.49	1.87
D. mel_C3_F	0.39	0.19	1.24	**12**	13.00	2.28	14.36	1.52
D. mel_St_M	0.33	0.15	1.18	**16**	16.00	0.00	16.00	1.98
D. mel_C3_M	0.28	0.10	1.11	**17**	17.00	0.24	17.31	1.97
D. sub_St_F	0.08	0.02	1.02	**16**	16.00	0.00	16.00	2.00
D. sub_C3_F	0.17	0.06	1.07	**8**	8.00	0.00	8.00	1.22
D. sub_St_M	0.08	0.02	1.02	**16**	17.00	2.29	16.92	1.93
D. sub_C3_M	0.56	0.32	1.48	**9**	9.00	0.00	9.00	1.25
Progeny								
D. mel_St_F	1.42	0.68	3.15	**25**	25.00	0.00	25.00	2.45
D. mel_C3_F	1.24	0.63	2.73	**16**	16.00	0.00	16.00	1.32
D. mel_St_M	0.98	0.43	1.76	**19**	19.00	0.00	19.00	2.10
D. mel_C3_M	0.62	0.27	1.38	**18**	18.00	0.00	18.00	2.00
D. sub_St_F	0.61	0.33	1.49	**10**	10.00	0.00	10.00	1.45
D. sub_C3_F	0.29	0.11	1.12	**17**	17.00	0.00	17.00	1.88
D. sub_St_M	0.64	0.34	1.52	**14**	14.00	0.00	14.00	1.69
D. sub_ C3_M	0.35	0.13	1.15	**22**	22.00	0.00	22.00	2.31

Note: St – standard substrate; C3 – lead-saturated substrate; F – female; M – male

to the parental generation, i.e. the richness increased over the number of generations in the lab (Table 2).

The Shannon and Simpson diversity indices supported these observations in both sexes, with higher values observed mostly in females. *D. melanogaster* had higher bacterial diversity compared to *D. subobscura*, with a higher diversity in F13 progeny than the parental generation, showing the increase in diversity over the generations, especially on the Pb-polluted substrate, according to Shannon's and Simpson's diversity indices. The observed results according to OBS showed the highest bacterial richness in *D. melanogaster* F13 progeny on a standard substrate and the lowest one was in parental *D. subobscura* on Pb. However, all indices showed lower diversity and evenness in both generations of *D. subobscura* than in *D. melanogaster*. Alpha diversity at the genus, family and phylum levels are shown in Supplementary Table S3.

MDS analysis showed global microbiota differences between substrates, species and genders (Fig. 2). The plot revealed trends of sample separation by fly species and by substrate in the parental generation, and lower multivariate dispersion of samples from Pb-polluted substrate as compared to control substrate in F13 progeny. Sex separation was observed to some extent on both substrates and in both species, notably at the phylum level in F13. The results on the bacterial phylum, family and genus levels are summarized in Figs. 3 and 4 and Supplementary Tables S4-S9 (the data are available at: figshare.com/s/57e9909c4f76af5a3e90).

In the "Wild_Lab" experiment, 30 phyla were detected in all samples and the most dominant were Proteobacteria, Firmicutes and Bacteroidetes. Proteobacteria were dominant in *D. melanogaster* while Firmicutes, Bacteroidetes and Actinobacteria had higher relative abundance in *D. subobscura*. Firmicutes, Actinobacteria, Fusobacteria and Acidobacteria were more abundant in laboratory-reared *D. subobscura* samples than in the other ones (Fig. 3A). At the family level, in the *D. subobscura* wild-caught flies, Acetobacteraceae, Weeksellaceae, Prevotellaceae, Ruminococcaceae and Rikenellaceae were predominant (>2%, Fig. 3B). In comparison to other samples in wild-caught *D. melanogaster* flies, the dominant families were Enterobacteriaceae, Leuconostocaceae, Wohlfahrtiimonadaceae (only detected within this sample, >7%) and Enterococcaceae. In *D. melanogaster* lab-reared flies the presence of the family Anaplasmataceae had the highest relative amount (>48%). Interestingly, different families, such as Lactobacillaceae and Propionibacteriaceae, predominated in *D. subobscura* lab-reared flies, as well as Bacteroidaceae, Staphylococcaceae, Burkholderiaceae, Pseudomonadaceae, Flavobacteriaceae, Rhizobiaceae, Leptotrichiaceae, Caulobacteraceae, Paludibacteraceae and Succinivibrionaceae, although in lower percentages (0.5-2%).

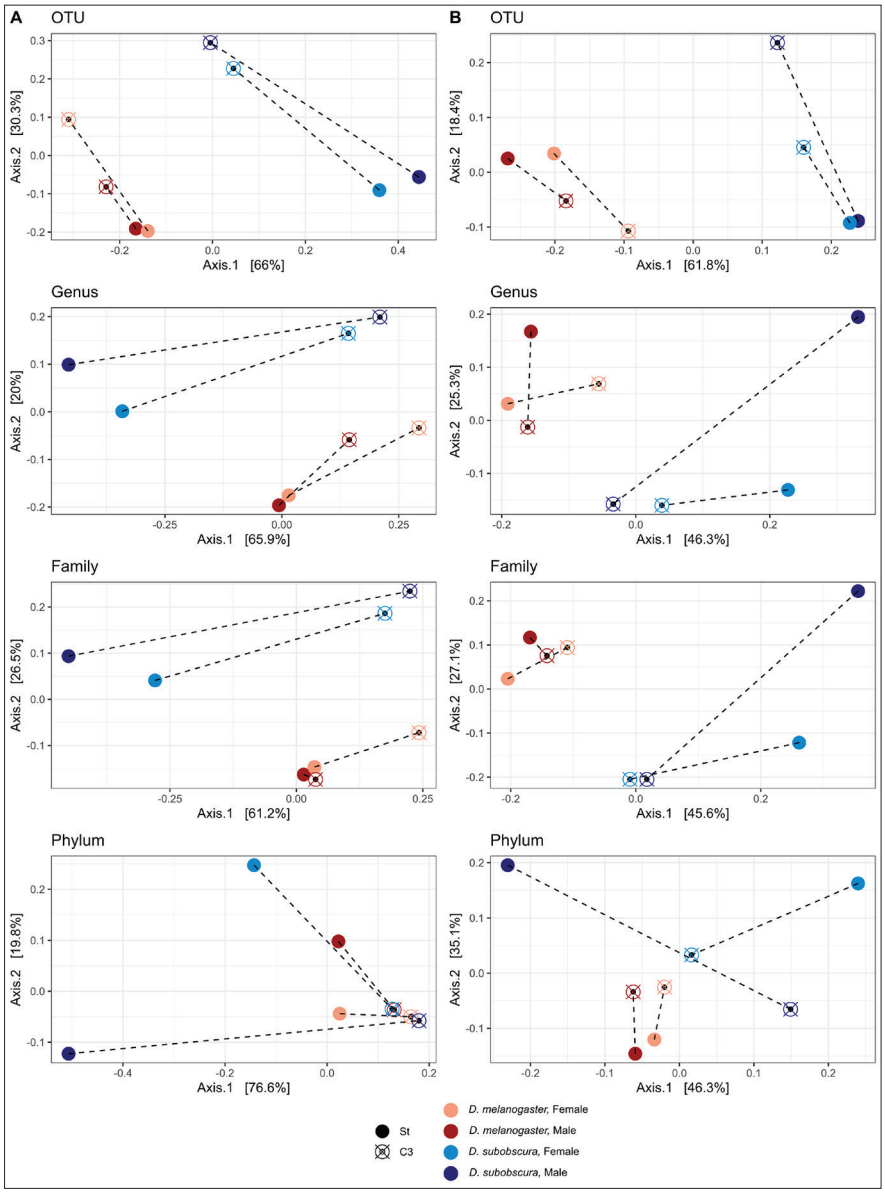

Fig. 2. Multidimensional scaling (MDS) performed on the weighted UniFrac distance matrix calculated using log(x+1)-transformed abundances rarefied to an even depth of bacterial composition in different *Drosophila* samples (*D. melanogaster* and *D. subobscura*) in the parental (**A**) and progeny F13 (**B**) generation at OTU, genus, family and phylum taxonomic levels in the "St_Pb" experiment. The dashed line refers to the differences in treatments within species; St – standard substrate and C3 – lead-saturated substrate.

Within the family Anaplasmataceae, the genus *Wolbachia* was present in the highest percentages in both samples of *D. melanogaster* flies, while it was minor in the *D. subobscura* wild-caught sample (0.05%) and absent in the lab-reared one (Fig. 3C). This could be a novel indication for further research on *D. subobscura*, as we excluded any biological or technical contamination or "cross-contamination" due to index hopping. The genus *Acetobacter* was highly prevalent in *D. melanogaster*, both lab-reared and wild-caught (26.17% and 13.04%, respectively), while it was almost absent in the *D. subobscura* wild-caught sample (0.02%) and completely absent in the lab-reared one. In contrast, *Gluconobacter* was the predominant genus in the wild-caught *D. subobscura* (41%) and less in the lab-reared flies (8%), while it was present in *D. melanogaster* in negligible amounts (1% and 0.2 % in the wild-caught and lab-reared samples, respectively). Additionally, *Chishuiella* (6.3%) and *Prevotella* (3.3%) were predominant in wild-caught *D. subobscura*, while *Providencia* (30.4%), *Fructobacillus* and *Wohlfahrtiimonas* (only detected in these samples, with 17.3% and 7.4%, respectively), and *Pantoea* (2.1%) were the most abundant genera in wild-caught *D. melanogaster* flies. Remarkably, completely different genera, such as *Lactobacillus, Leuconostoc, Cutibacterium* and *Bacteroides,* predominated in the lab-reared *D. subobscura.* When compared to the wild-caught flies, *Lactobacillus* was less abundant in the natural samples (*D. subobscura*, 3.1% and *D. melanogaster* 0.08%). A similar observation was noticed for the genera *Leuconostoc* and *Bacteroides.*

In the "St_Pb" experiment, seven phyla were detected in all samples and the most predominant were Proteobacteria and Firmicutes, where Proteobacteria was present with 95% in the parental and 97% in the F13 generation (Fig. 4A). The phylum Firmicutes was present at a higher percentage in the parental generation (>4%), with higher abundance in *D. subobscura* and

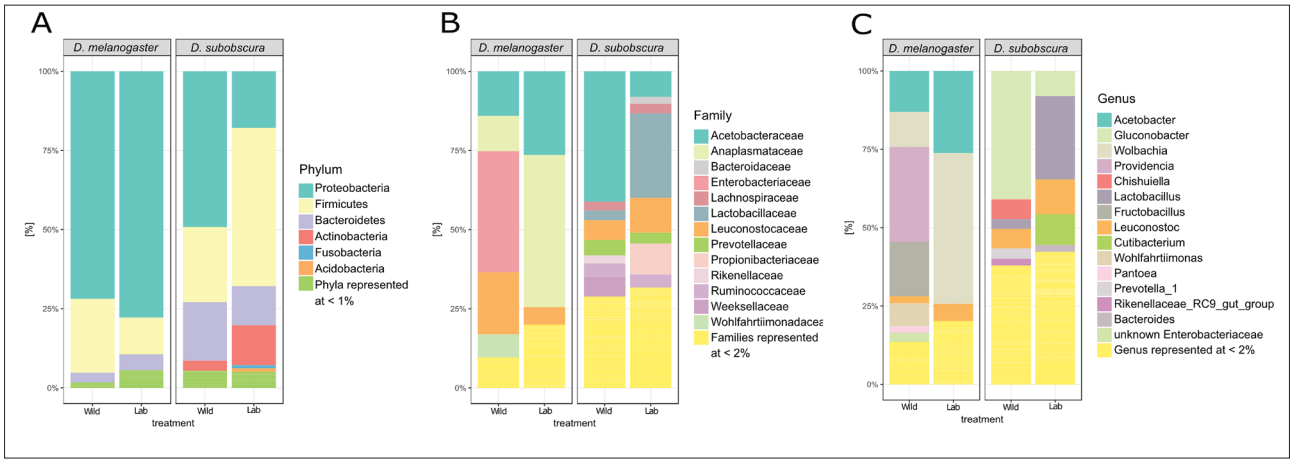

Fig. 3. Relative abundance of bacterial taxa in different *Drosophila* samples (*D. melanogaster* and *D. subobscura*; wild and lab) as assessed by 16S rRNA gene sequences at the phylum (**A**), family (**B**) and genus (**C**) levels in the "Wild_Lab" experiment.

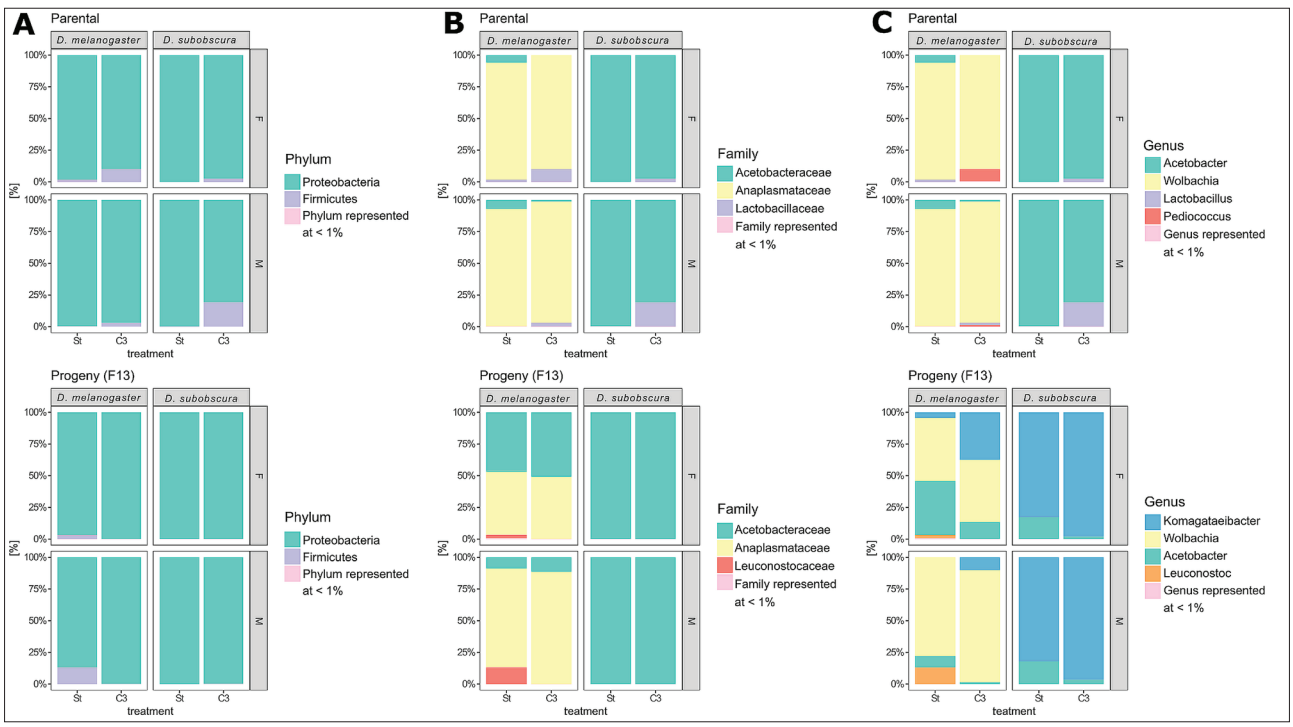

Fig. 4. Relative abundance of bacterial taxa in different *Drosophila* samples (*D. melanogaster* and *D. subobscura*) as assessed by 16S rRNA gene sequences at the phylum (**A**), family (**B**) and genus (**C**) levels in the parental and F13 generation in the "St_Pb" experiment; St – standard substrate and C3 – lead-saturated substrate.

the polluted substrate, but decreased in F13 progeny (>2%), showing opposite results (higher abundance in *D. melanogaster* samples and the control substrate). Other phyla, Bacteroidetes, Actinobacteria, Thermotogae, Planctomycetes and Verrucomicrobia, represented less than 1% (Supplementary Tables S5 and S6).

At the family level, the most abundant families in the parental generation were Anaplasmataceae, Acetobacteraceae and Lactobacillaceae, at 49%, 46% and >4%, respectively (Fig. 4B). Regarding the F13 generation, the families Acetobacteraceae, Anaplasmataceae and Leuconostocaceae were the most abundant at >64%, 33% and 2%, respectively. Other families were less than 1% abundant. When it comes to species, the predominant family in the parental *D. subobscura* was Acetobacteraceae, and in *D. melanogaster,* Anaplasmataceae (94% and 92%, respectively). The second most

abundant family for both species was Lactobacillaceae. The most prevalent family on the control substrate was Acetobacteraceae, while Anaplasmataceae were predominant on the polluted substrate. Interestingly, the percentage of the family Lactobacillaceae on the Pb-polluted substrate was >8%, whereas on the control substrate it was lower than 0.5% and favored by male flies. The F13 progeny of *D. subobscura* flies were dominated by the family Acetobacteraceae (>99%). *D. melanogaster* flies showed greater microbial diversity regarding family, where they had three families with more than 1% (Anaplasmataceae 66%, Acetobacteraceae 29% and Leuconostocaceae 4%). Both substrates had a similar abundance of Anaplasmataceae and Acetobacteraceae, but over generations females accumulated greater differences in abundance of these two families than male flies. Regarding species, substrate and sex, the family Leuconostocaceae was favored by *D. melanogaster*, control substrate and male flies.

The most abundant genera in the parental generation were *Acetobacter*, *Wolbachia*, *Lactobacillus* and *Pediococcus* (>99.9%) (Fig. 4C). *Acetobacter* (94%) and *Lactobacillus* (5%) were the only genera represented by more than 1% in *D. subobscura*. *D. melanogaster* had all four predominant genera (*Wolbachia* 92%, *Acetobacter* 4%, *Pediococcus* >2% and *Lactobacillus* 1%). *Acetobacter* was highly abundant in the control samples. *Pediococcus* was the most abundant in female flies of *D. melanogaster* on Pb-polluted substrate (>2%). The most prevalent genera in the progeny after 13 generations were *Komagataeibacter*, *Wolbachia*, *Acetobacter* and *Leuconostoc* (51%, 33%, 13% and 2%, respectively). All four genera were represented in *D. melanogaster* microbiota, with a prevalence of *Wolbachia* (66%), followed by *Acetobacter* (16%), *Komagataeibacter* (13%) and *Leuconostoc* (4%). *D. subobscura* samples contained only two genera with >1% abundance, *Komagataeibacter* with more than 89% and *Acetobacter* (>9%). Both substrates and sexes showed the same order of abundance of the genera: *Komagataeibacter* >*Wolbachia*>*Acetobacter*>*Leuconostoc.*

DISCUSSION

Our primary focus was to ascertain the diversity of *Drosophila*-associated bacterial communities in two host species sampled from natural and laboratory environments, and also to examine the influence of externally added contaminants (Pb) to microbial diversity. We investigated, for the first time, the bacterial communities associated with *D. subobscura* in comparison to the well-studied *D. melanogaster*, reared under the same conditions.

The results of the metagenomic analysis in the first experiment ("Wild_Lab") revealed that the two species exhibited different directions of change in diversity of the bacterial community between the wild-caught and laboratory samples reared on standard substrate. Namely, while an increase was observed in *D. subobscura*, bacterial richness was consistent when compared to *D. melanogaster*, especially at the genus and OTU levels. The results of the second experiment ("St_Pb") showed a different pattern, with bacterial diversity increasing in both species after 13 generations, but more so in *D. melanogaster*. Since the *D. subobscura* samples from our two experiments were of different origin, the inconsistency of the results could be due to an initial population-specific response of *D. subobscura* to lab-rearing conditions in the first experiment, whereas it decreased over 45 generations in the lab. The overall bacterial diversity observed in the "St_Pb" experiment was reduced compared to "Wild_Lab" experiment.

Analysis of the composition of bacterial communities in *D. melanogaster* revealed a significant predominance of the families Acetobacteraceae and Anaplasmataceae, while other families such as Lactobacillaceae and Leuconostocaceae were less abundant. The family Anaplasmataceae was represented by only one genus, *Wolbachia*. For several years we have run regular tests for the presence of the genus *Wolbachia* prior to all laboratory experiments with *D. subobscura* samples from different populations, and it has never been detected in this species thus far [26,27]. A similar study confirmed that these families, and in particular the bacterial genera *Lactobacillus*, *Acetobacter, Gluconobacter* and *Leuconostoc*, are common gut symbionts of *D. melanogaster* and *D. simulans* [28]. They also reported *Gluconobacter* as the most prevalent genus in wild-caught *D. melanogaster* and *D. simulans*, which are sibling cosmopolitan species sharing similar habitats but with different seasonal population dynamics [29]. However, another study examined 14 different *Drosophila* species and reported almost complete deficiency of *Gluconobacter* (Acetobacteraceae) in

their samples [30]. Our findings support their results, since its presence in the *D. melanogaster* population ("Wild_Lab" experiment) was negligible, but the opposite was found in wild-caught *D. subobscura* where the most prevalent genus was *Gluconobacter*. Our experiments supported the observation that lab-reared flies are deficient in *Gluconobacter* since it has not been found either in parental or in progeny generations. According to our findings, *Acetobacter*, which belongs to the same family Acetobacteraceae, is the most prevalent genus within wild-caught *D. melanogaster* flies, but also in lab-reared *D. subobscura* (even though the "Wild_Lab" experiment showed the opposite). Members of the genus *Acetobacter* have the ability to oxidize acetic acid via the tricarboxylic acid cycle (TCA). In contrast, *Gluconobacter* lacks a functional TCA cycle because of deficiencies in the two key enzymes, alpha-ketoglutarate dehydrogenase and succinate dehydrogenase, and cannot oxidize acetic acid and other organic acids. The genus *Gluconobacter* is further characterized by a better ability to use sugars than *Acetobacter* [31]. The "St_Pb" experiment revealed the prevalence of *Acetobacter* in *D. subobscura* on both substrates. Interestingly, over several generations in the laboratory, the abundance of *Acetobacter* decreased in favor of the *Komagataeibacter* genus from the same family. *Komagataeibacter* is not a typical member of the *Drosophila* microbiome, but its presence was reported in aging studies of *D. melanogaster*, in which the authors associated the ability of *Komagataeibacter* to reduce triglyceride and glucose levels in *Drosophila* with the host's lifespan [32,33]. While they reported the presence of *Komagataeibacter* in *D. melanogaster*, we found it in a greater amount in *D. subobscura*, which further increased in specimens from the Pb-polluted substrate. *D. melanogaster* also accumulated this bacterial genus, particularly in females, but at a slower rate. The dominance of the *Komagataeibacter* could be due to their tolerance of increased concentrations of acetic acid, which inhibited the growth of the other genera, especially in *D. subobscura* on both substrates.

Another significant difference in the composition of bacterial communities of *D. subobscura* is the highly prevalent presence of the genus *Lactobacillus* in lab-reared *D. subobscura* flies ("Wild_Lab" experiment), with a lower abundance in the wild population. The presence of this genus is notable also in the parental generation of *D. subobscura* ("St_Pb" experiment), with a higher abundance in males. Over several generations in the lab, *Lactobacillus* disappeared from F13 progeny. Similar results were observed in *D. melanogaster*, where the parental generation (mostly in females) showed the presence of *Pediococcus*, a genus from the Lactobacillaceae family, which however disappeared over several generations in the lab. *Lactobacillus* and *Pediococcus* metabolize glucose via homolactic fermentation, producing lactic acid. *Leuconostoc* species, which appeared in the *D. melanogaster* F13 generation, in addition to lactic acid produced ethanol, CO_2, and occasionally acetic acid [34]. All the abovementioned genera are tolerant to low pH and a high ethanol concentration. They are also sensitive to acetic acid, which is an important stress factor inhibiting the growth of microorganisms. Therefore, *Acetobacter* and *Komagataeibacter* possibly became the dominant microbes in F13 progeny due to their higher tolerance of acetic acid [35].

In the "Wild_Lab" study, *Chishuiella* and *Prevotella* were the prevalent genera in wild-caught *D. subobscura*, while, *Fructobacillus* and *Wohlfahrtiimonas* (exclusively detected in *D. melanogaster*), as well as *Providencia* and *Pantoea*, were the most abundant genera in wild-caught *D. melanogaster*. The genus *Providencia*, which is highly abundant in wild-caught flies, contains species with various pathogenic potentials and an increased mortality rate in *D. melanogaster* [36].

In contrast to other insect groups, only *Wolbachia* and *Spiroplasma* as endosymbionts have been detected in *Drosophila* species, indicating that heritable symbionts are uncommon in this genus, possibly due to a robust innate immune response that eliminates many bacteria [37]. Generally, these symbionts can have both beneficial and harmful effects on host reproduction, biasing of the sex ratio, parthenogenesis, or they can cause incompatibility in crosses with uninfected strains of the same host species [38]; however, in *Drosophila* species they form facultative associations and are maternally transmitted, undergoing occasional horizontal transfer into other hosts. Studies on *Wolbachia* strains in *D. melanogaster* wild populations showed a clinal frequency distribution and association with an environmental temperature gradient [39]. Our results confirm the presence of *Wolbachia* in *D. melanogaster* samples and a negligible presence in *D. subobscura*. *Wolbachia* might also affect the resistance of *D. melanogaster* to lead consumption, limiting survival and

decreasing longevity [40]. The minimal presence of this genus in *D. subobscura* might be advantageous in an environment with elevated concentrations of heavy metals.

Lead exposure has proven to be an important factor in shaping gut microbiota composition in different species [9,41]. The predominance of the genus *Komagataeibacter* in F13 progeny of *D. subobscura* growing on Pb indicates that *Komagataeibacter* tolerates the presence of Pb to a greater extent than any other bacterial genus present in the gut of flies. This genus compensated for the absence of all other genera that were present in the parental generation, pointing to its great importance for intestinal microbiota. *Acetobacter* also proved to be a good Pb-tolerant bacterial genus, sometimes even with the ability to adsorb heavy metals [42]. Contrary to these results, *Lactobacillus* and *Pediococcus* completely disappeared from the Pb-saturated substrate over several generations, indicating a very low tolerance to heavy metals.

Because the microbiome is highly influenced by environmental factors, laboratory lines of both *Drosophila* species used in the present study were maintained under the same controlled conditions as regards temperature, humidity, density, food substrate content and quantity, size of bottles/vials, etc., although these conditions were not equally optimal for both species. Additionally, the dried yeast powder had to be placed on the surface of the substrate to enable *D. subobscura* to lay eggs, which represented another sub-optimal condition imposed on *D. melanogaster*.

CONCLUSIONS

The overall bacterial diversity and richness decreased over generations under standard laboratory conditions. *D. subobscura* displayed higher bacterial diversity and a better response to the lab-rearing conditions than *D. melanogaster*. After 30 and 45 generations in the lab, bacterial diversity was significantly reduced in both species, decreasing more in *D. subobscura*. Interestingly, bacterial diversity increased in both species after prolonged exposure to a substrate containing lead. The increase in bacterial diversity over generations in the lab could be due to the adaptive response of both species to environmental stress. The bacterial community of some *D. subobscura* populations had a lower tolerance to Pb contamination as compared to the *D. melanogaster* community. The lab-reared flies exhibited the predominance of the *Komagataeibacter* genus, which is not a common member of the microbiota of fruit flies. The predominance of this genus in the presence of lead points to its higher tolerance of this heavy metal than other members of the community. Additionally, *Acetobacter* is another candidate for a good lead-tolerant bacterial genus.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/5836_Supplementary%20Material.pdf