



Gut microbial community response to herbicide exposure in a ground beetle

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Abstract

Gut microbiota plays a key role in physiological processes of insects, including nutritional metabolism, development, immunity and detoxification. Environmental stressors such as herbicides, used to optimize and improve crop yields, may interfere with the mutualistic relationships causing negative consequences for the host health. Dinitroaniline herbicides, for example pendimethalin, are used worldwide in pre-emergence application to control grass and some broadleaf weeds. They target microtubules to arrest cell division and inhibit the development of roots and shoots. Effects of a pendimethalin-based herbicide were assessed on the gut microbial community of *Pterostichus melas italicus* Dejean, 1828 (Coleoptera, Carabidae). The exposure effect was tested in vivo by using a recommended field rate (4 L per ha, 330 gL⁻¹ of active ingredient) and evaluating the variability of responses in 21 days, corresponding to the half-life of pendimethalin. The 16S rRNA sequencing data showed that the gut lumen was dominated by Proteobacteria, Firmicutes, Fusobacteria, Tenericutes and Bacteroidetes. The exposure interfered with the bacterial community richness and diversity associated with the gut from 2 days after the treatment. The differential abundance analyses highlighted a shift involving Lactobacillaceae, Streptomyetaceae, Neisseriaceae, Ruminococcaceae and Enterobacteriaceae. An increase in species such as *Enterobacter* sp., *Pseudomonas* sp., *Pantoea* sp and *Paracoccus* sp. involved in the herbicide degradation was also recorded after 21 days of exposure. Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) analysis indicated that the exposure has effects on the most predicted functional categories of gut microbiota related to metabolic function including carbohydrate, amino acid and lipid metabolism. These results demonstrate that pendimethalin can impact microbial communities associated with generalist predators inhabiting croplands leading to severe implications for the species' ecological role. Understanding the effects of herbicides such as pendimethalin on ground beetles may help to protect beneficial soil insects that have a crucial role in the ecosystem services.

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KEYWORDS

agroecosystem, carabid beetles, dinitroaniline, ecotoxicology, microbiota, symbiosis

1 | INTRODUCTION

The gut microbiota is a complex community of obligate or facultative symbiotic bacteria that profoundly influences the host's fitness and life span interacting with its biological (Engel & Moran, 2013) and behavioural (Hosokawa & Fukatsu, 2020; Yuval, 2017) traits. Beneficial gut bacteria show a wide range in their degree of action on metabolic activities, including nutrition, xenobiotic detoxification (Engel & Moran, 2013; Itoh et al., 2018), and physiological processes of insects (tolerance and resistance to pathogens, modulation of innate immune responses and immune priming) (Dillon & Dillon, 2004; Engel & Moran, 2013; Futo et al. 2016). The diversity of bacterial communities varies according to the host species and its ecological niche (Bonilla-Rosso & Engel, 2018; Yun et al. 2014), developmental stage (Cini et al. 2020; Kim et al. 2017), diet and environmental conditions (Colman et al. 2012; Jones et al. 2018; Kolasa et al. 2019). This is the result of selection pressures that they impose on each other, promoting continuous co-adaptation (Oliver & Martinez, 2014).

The direct or indirect exposure to agrochemicals (insecticides and fungicides), used for pest control in conventional agriculture, can significantly affect the structure and function of the gut microbiome in beneficial insects (Kakumanu et al. 2016; Syromyatnikov et al. 2020) and humans through the trophic web (Yuan et al. 2019). The alteration of the gut microbial climax community results in physiological disorders and has consequences on survival and disease susceptibility of the host (Botina et al. 2019; Xia et al. 2018; Zeng et al. 2020), compromising its ability to respond to environmental stressors. There is growing evidence that also herbicides have adverse effects on the gut microbiota. Recent toxicological studies have been linked alterations of the gut microbiota to the glyphosate exposure in honey bee *Apis mellifera* Linneus, 1758, (Blot et al. 2019; Dai et al. 2018; Motta et al. 2018) and the Colorado potato beetle *Leptinotarsa decemlineata* Say, 1824 (Gómez-Gallego et al. 2020). The atrazine had been tested to reduce the gut microbial diversity of house (*Culex pipiens*) and tiger (*Aedes albopictus*) mosquitos (Juma et al. 2020).

Pendimethalin is a dinitroaniline herbicide used worldwide to control weeds. It inhibits the development of roots and shoots in seedlings, interrupting the polymerization of microtubules and arresting cell division (mitosis) (Rose et al. 2016; Vighi et al. 2017). Its half-life can vary according to weather conditions and soil pH, from 24–39 to 76–98 days in aerobic soil (Strandberg & Scott-Fordsmand, 2004; Vighi et al. 2017). The residual dose (10%–15%), tested to remain in the soil until 300–400 days after physical, chemical or microbiological transformations, is harmful to non-target species (Strandberg & Scott-Fordsmand, 2004). Previous studies have been highlighted the potential toxicity of the pendimethalin sublethal dose on non-target organisms at different levels of the trophic web in both terrestrial (Belden et al. 2005; Oliver et al. 2009; Traoré et al. 2018) and aquatic

(Ahmad & Ahmad, 2016; Tab assum et al. 2015; Traoré et al. 2018; Vighi et al. 2017) environments. Furthermore, the persistence of pendimethalin residues in the environment significantly impacts on the soil bacterial (Strandberg & Scott-Fordsmand, 2004) and fungal (Roca et al. 2009) community richness reducing its growth (Kocárek et al. 2016; Singh et al. 2002) up to 61% starting from 25 days of treatment (Nayak et al. 1994) and consequently affecting the soil biodiversity and fertility.

Ground beetles are an essential group of beneficial insects of particular interest as indicators in the environmental quality assessment (Avgin & Luff, 2010; Ghannem et al. 2018; Rainio & Niemelä, 2003) and useful for their contribution in agroecosystems to promote biological control services (De Heij & Willenborg, 2020; Holland, 2002; Koivula, 2011). Carabids act in the soil food web on pests including aphids, beetles, lepidopterans, slugs and dipterans as generalist or specialist predators (Ferrante et al. 2017; Giglio et al. 2012; Holland, 2002; Roubinet et al. 2017) and consumers of weed seeds (Hana et al. 2020; Honek et al. 2003; Kulkarni et al. 2015; Martinková et al. 2019; Talarico et al. 2016). However, carabids inhabiting agricultural landscapes are exposed, by direct contact or consuming contaminated food, to residual doses of agrochemicals that have sublethal effects on morphology, physiology and behaviour of organisms (Benitez et al. 2018; Giglio et al. 2017; Kunkel et al. 2001; Tooming et al. 2017; Van Toor, 2006) and consequently considerable effects on the diversity and abundance of species (De Heij & Willenborg, 2020; Holland & Luff, 2000). Some studies have also been provided evidence that the exposure to herbicides causes mortality or sublethal effects in carabids (Brust, 1990; Cavaliere et al. 2019; Cobb et al. 2007; Giglio et al. 2019; Kegel, 1989; Michalková & Pekár, 2009; Prosser et al. 2016).

Despite the ecological and agricultural interest of carabids, little is known about the composition and diversity of bacterial communities associated with their gut systems. Previous metagenomic analysis has been highlighted that the biodiversity of the gut bacterial communities depends on the feeding habits and habitat of the host (Kudo et al. 2019) and facilitates seed consumption in omnivorous species (Lundgren & Lehman, 2010; Schmid et al. 2014). In this context, knowledge of the herbicide effects on the carabid microbiota is lacking. This study aimed to investigate effects that a commonly used pendimethalin-based commercial formulation have on the structure and potential function of the gut microbiome of beneficial species inhabiting the soil in agroecosystems. We choose as a model *Pterostichus melas italicus* Dejean, 1828 (Coleoptera, Carabidae), an eurytopic and thermophilous clay soil species. In Central and Southern Europe, this species inhabits pastures, open forests and forest edges, and agricultural lands where it acts as a natural enemy of pests including aphids, lepidopterans and dipterans (Sunderland, 2002). The experiment was designed to test in vivo a recommended field rate and evaluate the variability of responses in

a time of 21 days corresponding to the half-life of the pendimethalin. We hypothesized exposure effects on the bacterial community associated with the gut of *P. melas*. We also expected the possible effects of PND on gut microbiota to vary over time. This study will make a significant contribution to optimizing the use of this agrochemical in the agroecosystems to reduce sublethal effects on non-target organisms.

2 | MATERIALS AND METHODS

2.1 | Species collection and treatments

Adults of *P. melas* ($n = 90$) were collected in an organic olive grove (39°59'27.56"N, 16°15'32.64"E, 1,202 m a. s. l. San Marco Argentano, Calabria, Southern Italy) in October 2019 by using in vivo pitfall traps (plastic jars 9 cm in diameter) containing fruit as an attractant. In the laboratory, the beetles were identified using a dichotomous key, separated by gender and kept in 5 L plastic boxes that were filled to a depth of 6 cm with soil from the capture site, held at 60% relative humidity (rh), had a natural photoperiod and were at room temperature. They were fed with mealworms and fruit (organic apples) ad libitum.

To evaluate exposure effects of a pendimethalin-based commercial formulation (PND; Activus EC, product n° HRB00858-39; active ingredient pendimethalin 330g L⁻¹), males were exposed for 21 days to the recommended field rate (4 L per ha, for cereal and vegetable cultures) taking into account the pendimethalin half-life ranged from 24.4 to 34.4 days in sandy acid soil (Kocárek et al. 2016; Strandberg & Scott-Fordsmand, 2004). The experimental design included three control and six exposure plastic boxes (180.5 cm²) filled with the clean sandy soil (pH 5 approximately) from the capture site. The exposure was performed by spraying the PND solution (7.2 µl of Activus in 14 ml of distilled water) with a pipette onto the soil surface of each box of the treated groups to simulate the field exposure by contact with the contaminated soil. Boxes for control groups were sprayed with distilled water. Males (10 for each box) were introduced 15 min after the PND solution has been sprayed.

To remove the digestive tract (foregut, midgut and hindgut), five beetles for control and treated groups were randomly chosen at 2, 7 and 21 days after the initial exposure. Beetles were anaesthetized in a cold chamber at 0°C for 3 min, gently cleaned in 70% ethanol and dissected under a stereo microscope Zeiss using sterile equipment. The guts, removed from beetles, were individually stored in 2 ml microcentrifuge tubes containing absolute ethanol until DNA extraction.

2.2 | DNA isolation and sequencing

Library preparation and sequencing were performed at the DNA sequencing facility of the Life Sciences Department of Trieste University, Italy. Samples were preliminary washed with phosphate

buffer (PBS) to remove the storage ethanol. Genomic DNA was extracted using the E.Z.N.A.[®] Soil DNA kit (Omega Bio-Tek) following the manufacturer's instructions. DNA quality and quantity were assessed with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). An extraction blank was performed as a control to monitor for contamination of environmental bacteria DNA. The extracted DNA was used as a template for the amplification of V4 hypervariable region of the 16S rRNA by PCR primers 515F (Caporaso et al. 2011), and a mix of 802R (Claesson et al. 2009) and 806R (Caporaso et al., 2011). Primers were tailed with two different GC rich sequences enabling barcoding with a second amplification. For each sample, three technical replicates were performed in 20 µl of volume reaction containing 10 µl AccuStartII PCR ToughMix 2X (Quanta Bio), 1 µl EvaGreen™ 20X (Biotium), 0.8 µl 515 F (10 µM—5' modified with unital 1-CAGGACCAGGGTACGGTG-), 0.4 µl 802 R (10 µM—5' modified with unital 2-CGCAGAGAGGCTCCGTG-), 0.4 µl 806 R (10 µM—5' modified with initial 2-CGCAGAGAGGCTCCGTG-), and 50 ng of DNA template. The amplification was performed in a CFX 96™ PCR System (Bio-Rad) with a real-time limited number of cycles (94°C for 20 s, 55°C for 20 s, 72°C for 60 s). The second PCR amplification (outer PCR) is required to label each sample uniquely and was performed using a forward primer composed of the 'A' adaptor, a sample-specific 10 bp barcode and the tail 1 of the primary PCR primers, and a reverse primer composed of the P1 adaptor sequence and the tail 2. The reactions were performed in 25 µl volume containing 12.5 µl AccuStartII PCR ToughMix 2X (Quanta Bio), 1.25 µl EvaGreen™ 20X (Biotium), 1.5 µl barcoded primer (10 µM), 1 µl of the first PCR product (pool of the three technical replicates) with the following conditions: 8 cycles of 94°C for 10 s, 60°C for 10 s, 65°C for 30 s and a final extension of 72°C for 2 min. All the amplicons were checked for their quality and size by agarose gel electrophoresis, purified by Mag-Bind[®]TotalPure NGS (Omega Bio-Tek), quantified with the Qubit Fluorometer (Thermo Fisher Scientific) and pooled together in equimolar amounts. The library was finally checked by agarose gel electrophoresis and quantified in the Qubit Fluorometer. For sequencing, the library was first subjected to emulsion PCR on the Ion OneTouch™ 2 system using the Ion PGM™ Template Hi-Q OT2 View (Life Technologies) according to the manufacturer's instructions. Then, Ion sphere particles (ISP) were enriched using the E/S module. Resultant live ISPs were loaded and sequenced on an Ion 316 chip (Life Technologies) in the Ion Torrent PGM System.

2.3 | Data analyses

The CLC Microbial Genomics Module as a part of the CLC Genomics Workbench 20.0 (QIAGEN Digital Insights, Aarhus, Denmark) was used to analyse alpha and beta diversity, and the composition of the bacterial community. Raw sequencing reads were imported into the CLC environment, and we perform quality control, primers and adapter sequences removal and minimum size cut-off of 150 bp. The OTUs were picked by mapping sequences against the SILVA 16S v132 97% database (Quast et al. 2013) at the same identity percentage to

observe OTU at the species level. Next, the OTUs were aligned using multiple sequence comparison by log-expectation and constructing a 'maximum likelihood phylogenetic tree' followed by alpha and beta diversity analyses. We estimated the effect size and significance on beta diversity for grouping variables with pairwise permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001). A principal coordinate analysis (PCoA) was carried out of the beta diversity distance matrices to better visualize similarities and differences. We choose the Bray–Curtis distance because it better depicts differences in OTUs representation between the different time points. Differential abundance analysis was performed modelling each OTU as a separate generalized linear model (GLM), where it is assumed that abundances follow a negative binomial distribution. The Wald test was used to determine the significance of group pairs. Finally, for the functional analysis of microbiota, we export the OTU table from the CLC Genomics Workbench environment, and we use PICRUST2 (Douglas et al. 2020) to infer the minimal biological pathways (Ye & Doak, 2009). Using STAMP software (Parks et al. 2014), two-sided Welch's *t* test with Benjamini–Hochberg multiple testing correction was performed to identify the metabolic pathways in the Level-3KEGG (Kyoto Encyclopedia of Genes and Genomes) database that were significantly different ($q < 0.05$) between groups.

3 | RESULTS

3.1 | Sequencing data

The bacterial community in the gut of *P. melas* was analysed using 16S rRNA gene amplicon sequencing and produced a total of 3,278,520 reads with an average of $109,284.00 \pm 12,572.53$ reads per sample. Raw sequences (reads) were trimmed, and the remaining sequences were reference-based clustered against SILVA 16S v132 database with a 97% sequence similarity accounting for

1.480 OTUs and 1.167 de novo OTUs from the 30 assayed samples (5 beetles at each time point per control and treated groups, respectively) for a total of 2.647 OTUs. The mean number of reads in OTUs for all the guts was $72,956.00 \pm 16,589.67$ for control and $73,718.13 \pm 10,936.65$ for PND exposed samples. Rarefaction curves (Figure 1a,b) calculated for total OTU abundance aggregate at the family level and assessed using the maximum likelihood phylogeny analysis always reached the plateau indicating adequate sequencing deepness to analyse the majority of phylotypes in most of the samples.

3.2 | Composition of the gut microbial community

On the basis of the average relative abundance, the main phyla in *P. melas* were Proteobacteria (54%), Firmicutes (17%), Fusobacteria (9.6%), Tenericutes (9%) and Bacteroidetes (9%), including almost 90% of the bacterial community (control groups in Table S1). The predominant families were Enterobacteriaceae (38.42%), Enterococcaceae (10.46%), Leptotrichiaceae (9.6%), Spiroplasmataceae (9.3%), Dysgonomonadaceae (8.8%) and Orbaceae (6.13%), representing altogether more than 80% of the recorded families. These are followed by Lactobacillaceae (3.13%), Wohlfahrtiimonadaceae (3.85%), Neisseriaceae (3%), Pseudomonadaceae (1.3%), Leuconostocaceae (1.2%), Moraxellaceae (1%), Carnobacteriaceae (1%) and Streptococcaceae (1%), while rest of the families were 1.71% cumulatively. At the genus level, the most abundant groups were *Enterococcus* (9.70%), *Sebaldella* (9.60%), *Spiroplasma* (9.28%), *Dysgonomonas* (8.81%), *Pragia* (8.41%), *Serratia* (7.95%), *Pseudocitrobacter* (6.67%) and *Gilliamella* (6.13%), reaching the 60% of the represented genera. Also *Wohlfahrtiimonas* (3.78%), *Hafnia-Obesumbacterium* (3.66%), *Lactobacillus* (3.11%), *Vitreoscilla* (2.98%), *Morganella* (2.72%), *Enterobacter* (2.48%), *uncultured-25* (1.96%), *Citrobacter* (1.96%), *Pseudomonas* (1.27%), *Weissella* (1.20%),

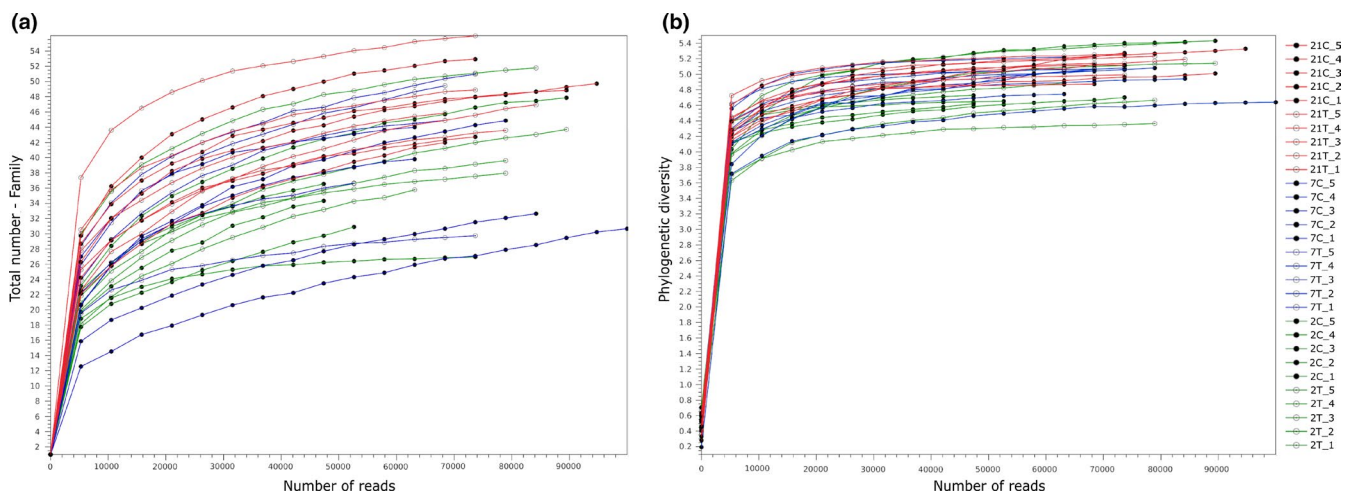


FIGURE 1 Rarefaction curves depicted from randomly subsampled data sets with the same number of 16S sequences were done for both the total number of OTUs aggregate at the family level (a) and the phylogenetic diversity (b). The near-saturated rarefaction curve indicates that the vastness of microbial diversity was retrieved from each sample of *P. melas*

Acinetobacter (1.02%) and *Lactococcus* (1%) were recorded, and the remaining genera were 6.27% cumulatively.

3.3 | Effects of the PND exposure

To investigate potential changes in microbial community diversity between control and PND-treated groups, we used the relative abundance profiles obtained by 16S rRNA amplicon sequencing. The alpha diversity of the bacterial community within the treated and control group across different time points was characterized by the mean of Shannon index. Bacterial phylogenetic diversity significantly increases in the control group from 2 to 21 days (Kruskal–Wallis, p -value = 0.02) of the experiment (Figure 2a), while no significant differences were recorded among Shannon indices of treated groups at the different time points (Figure 2b) after the initial exposure to PND.

To compare the community structure between control and PND-treated samples at each time point, the distance matrices were calculated using Bray–Curtis dissimilarities and visualized using the principal coordinate analysis (PCoA) (Figure 3a–c), taking into account the abundance of each OTU balanced with the cladistic information (weighted UniFrac index). The clustering observed between control and treated group indicated significant differences in the gut bacterial communities at 2d (PERMANOVA, pseudo- F statistic = 3.856, $p < .001$) (Figure 3a) and 21d (PERMANOVA, pseudo- F statistic = 2.053, $p < .05$) (Figure 3c) after the initial exposure to PND. No significant differences in beta-diversity were found at 7 days (PERMANOVA, pseudo- F statistic = 1.162, $p > .05$) in PND-treated beetles compared with the control ones (Figure 3b).

The differential abundance analysis was performed to identify taxa affected by PND exposure at different time points (Figure 4; PND-treated groups in Table S1). At the family level (Figure 4a), statistical analyses (Wald test with Bonferroni adjusted p -value) showed that the relative abundance of Neisseriaceae ($p < .0001$), in the PND-treated group at 2 days of exposure, was significantly lower than in the control group. The relative abundance of Lactobacillaceae ($p \leq .001$) and Streptomycetaceae ($p < .05$) increased in the PND-treated group after 7 days from the initial exposure, while at 21 days of exposure the relative abundance of Ruminococcaceae ($p = .01$) significantly decrease in the PND-treated group.

The exposure to PND had a significant effect on the relative abundances of several bacterial genera (Figure 4b) belonging to Enterobacteriaceae. A significant reduction was recorded in the relative abundance of *Hafnia-Obesumbacterium* ($p < .05$), *Klebsiella* ($p < .05$) and *Enterobacter* ($p \leq .0001$) at 2 days, and *Morganella* ($p \leq .0001$), *Acetobacter* ($p \leq .001$), *Escherichia-Shigella* ($p \leq .001$), *Pseudocitrobacter* ($p \leq .0001$), *Kluyvera* ($p < .05$) and *Erwinia* ($p < .05$) at 21 days. The relative abundance of amplicons derived from *Pragia* was significantly lower in PND-treated group than control at 7 ($p \leq .0001$) and 21 days ($p < .05$) after the initial exposure. A higher relative abundance of *Pantoea* ($p < .0001$), *Pectobacterium* ($p \leq .001$) and *Tatumella* ($p < .05$) was recorded in treated group at 2 days of

exposure. We also checked changes in the relative abundance of the following less abundant genera: *Alkanindiges* (Moraxellaceae) ($p < .05$) and *Vitreoscilla* (Neisseriaceae) ($p < .0001$) significantly increased in PND-treated samples at 2 days of exposure, while *Lactobacillus* (Lactobacillaceae) ($p < .0001$), *Comamonas* (Burkholderiaceae) ($p = 1.27 \times 10^{-3}$) and *Empedobacter* (Weeksellaceae) ($p < .05$) increased at 7 days. *Pseudoxanthomona* (Xanthomonadaceae) ($p < .05$), *Clostridium sensu stricto* 13 (Clostridiaceae) ($p < .0001$) and *Paracoccus* (Rhodobacteraceae) ($p < .05$) were higher in treated than control at 21 days of exposure.

3.4 | Functional prediction

PICRUSt2 analyses categorized 399 KEGG pathways associated with metabolic functions including carbohydrate, amino acid and lipid metabolism (Figure 5). The functional profile was observed to change under PND exposure (Figure 6). The principal component analyses (PCA) explained 72.5% of the total variation clustering samples in two characteristic groups. A clear separation occurred between control and PND-treated samples at 2 days along the x-axis (48.6% of variability) and at 21 days along the y-axis (23.9%). Significant functional differences were predicted at 2 days between the microbiota of PND-treated and control beetles (Figure 7). In PND-treated beetles, it was observed a significant increase ($q < .001$) of the tricarboxylic acid (TCA) super pathway, responsible for the total oxidation of acetyl-CoA under aerobic conditions and the biosynthesis of several amino acids to bypassing the loss of CO_2 . The sulphate assimilation and L-methionine biosynthesis pathways were significantly higher ($q < .001$) in the PND-treated group than the control ones. A significant reduction in amino acid biosynthesis and tRNA charging ($q < .001$) was observed in the microbiota of PND-treated beetles.

4 | DISCUSSION

This study first described the microbial community in *P. melas* by the analysis of 16S rRNA gene. In field-collected beetles, Proteobacteria and Firmicutes were predominant phyla representing 71% of the total sequences. This result is consistent with previous studies showing these taxa to be commonly associated with the insect's gut (Colman et al. 2012; Yun et al. 2014). Seven bacterial families including Enterobacteriaceae, Enterococcaceae, Spiroplasmataceae, Orbaceae, Pseudomonadaceae, Moraxellaceae and Streptococcaceae (58% of the total OTUs) with high relative abundance (58% in adults of *P. melas* are found to be also abundant in other carabid species previously described acting as predators in their habitat (Kolasa et al. 2019; Kudo et al. 2019; Lehman et al. 2009; McManus et al. 2018). However, Dysgonomadaceae, Leptotrichiaceae, Lactobacillaceae, Wohlfahrtiimonadaceae and Neisseriaceae (36% of the total OTUs) recorded in the microbiota of *P. melas* are not present in other carabid species so far described (Kolasa et al. 2019; Lundgren & Lehman, 2010; Lundgren et al. 2007)., The symbiotic

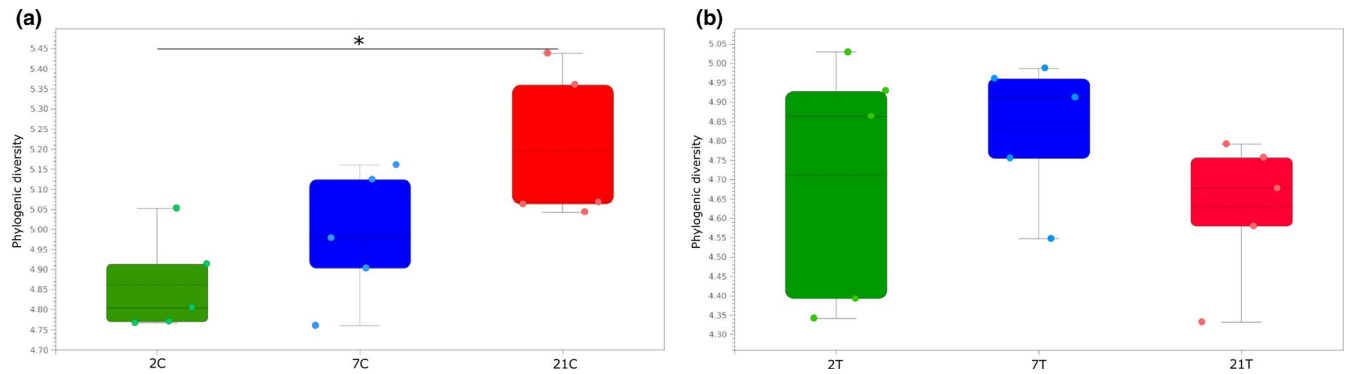


FIGURE 2 Box plots of microbiome alpha diversity metrics at the family level presented for *P. melas* males from control (a) and pendimethalin-treated (b) groups at days 2, 7, 21 from the initial exposure. An asterisk indicates significant differences based on Kruskal–Wallis test ($p < .05$)

relationship with bacteria in wild carabid beetles varies according to the host's habitat and facilitates the capability to digest food (Schmid et al. 2014) influencing their ecological role in the trophic web. Differences can occur at the species level in the same genus as observed among four species belonging to *Bembidion* sharing the same habitat and diet (Kolasa et al. 2019).

Pendimethalin has been well known to reduce the microbial biochemical activity in the soil, acting on carbon dioxide evolution and dehydrogenase activity of enzymes responsible for the oxidation of organic compounds with effects on the pH level (Strandberg & Scott-Fordsmand, 2004). Our findings first showed that PND could affect the gut microbiota in insects. Adults of *P. meals* exposed to a recommended field rate of PND for 21 days hold a lower diversity and species richness of the bacterial community, while a positive shift of the alpha-diversity was observed in the control group over time. Moreover, variations of the OTU abundance at the family level were recorded between PND-treated and control groups at different time points. The relative abundances of Neisseriaceae (neutrophiles and aerobics) and Ruminococcaceae (acidophiles and anaerobics) reduce after 2d and 21d from the initial exposure to PND, respectively. The exposure to PND for 7d positively acted on the abundances of facultative aerobic Lactobacillaceae and Streptomycetaceae. In Lactobacillaceae, the increase is related mainly to the abundance of *Lactobacillus*, involved in the sugar fermentation leading to lactic and acetic acid and CO_2 production, as observed in *Apis mellifera* (Vásquez et al. 2012). Streptomycetaceae secretes a variety of enzymes that hydrolyse complex macromolecules (i.e. degradation-products of chitin), and the resulting compounds can frequently serve as carbon or nitrogen sources (Glaeser & Kämpfer, 2016). They have also been found to produce antifungal compounds in a mutualistic association with termites (Chouvenc et al. 2018).

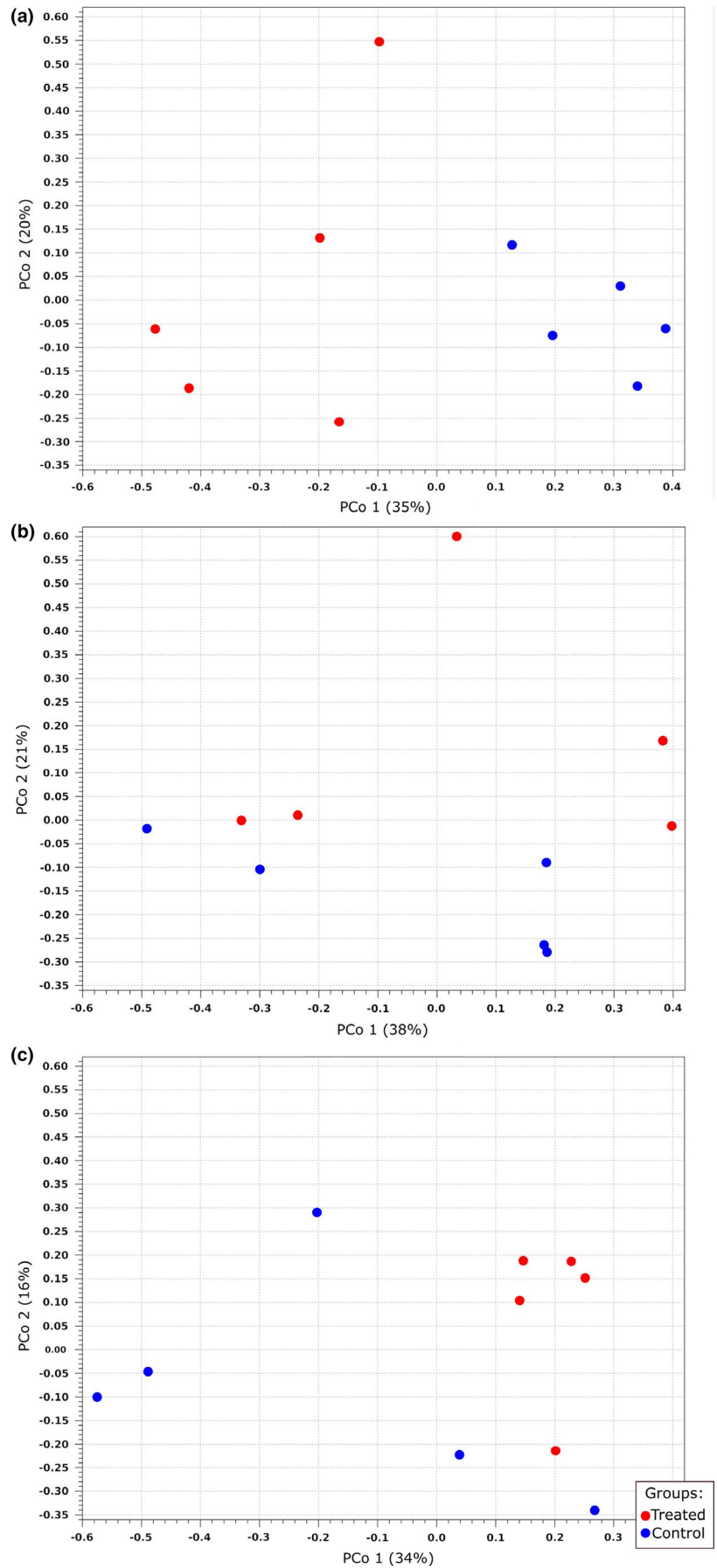
The role of PND in shaping microbial communities is also evident at the genus level. In PND-treated beetles, 22 genera shifted their relative abundance at different time point. The higher variability of responses was recorded for 14 genera belonging to facultative anaerobic Enterobacteriaceae involved in the metabolic pathway of carbohydrates. *Hafnia-Obesumbacterium*, *Klebsiella*, *Enterobacter*, *Pantoea*, *Pectobacterium* and *Tatumella* underwent a shift of relative

abundance after 2d from the initial exposure, while *Morganella*, *Acetobacter*, *Escherichia-Shigella*, *Pseudocitrobacter*, *Kluyvera* and *Erwinia* lowered after 21 days. In addition, species belonging to *Hafnia*, *Enterobacter* and *Serratia* genera are known to be able to produce chitinase (Ruiz-Sánchez et al. 2005; Whitaker et al. 2004), an enzyme required for prey digestion in insectivorous hosts. So that the alteration due to PND exposure could also temporary impair the nutrition.

The genus *Pragia* decreased in PND-treated beetles after 7 days of exposure. This genus belongs to a relatively small group of hydrogen sulphide-producing enterobacteria, including also *Budvicia* spp. and *Leminorella* spp., and it contains only one species, *P. fontium*, a free-living bacterium isolated from an environment that under anaerobic conditions utilizes monosaccharides and their derivatives but lacks fatty acid degradation pathways (Snopková et al. 2017). The genus *Budvicia* has been identified in the red palm weevil (Tagliavia et al. 2014), while *Pragia* was previously found to be associated with the gut of *Poecilus chalcites* (Lehman et al. 2009). Other shifts were observed for genera involved in different metabolic pathways such as pyrimidine (*Alkanindiges* and *Empedobacter*), aromatic compound (*Comamonas*) and amino acid (*Pseudoxanthomonas*) metabolism, hydrogen production (*Clostridium sensu stricto* 13) (Rosenberg et al. 2013) and detoxification of NO by its conversion to nitrate (*Vitreoscilla*) (Stark et al. 2012).

Pendimethalin, acting as a microtubule polymerization inhibitor, has no direct adverse effects on bacteria where the tubulin system is absent. Nevertheless, our results showed a variation of the microbiota structure in beetles exposed to the herbicide likely related to the physiochemical changes that occur during the treatment. In insects, the digestive system is part of a network of reactions and physiological processes that includes respiratory, circulatory, excretory and metabolic functions, so alterations in any of the network components cause changes in the other ones (Chapman, 2012). Previous studies have been indicated PND to have detrimental effects on physiological processes of metazoan organisms. In vertebrates, PND sublethal effects have been measured in the zebrafish *Danio rerio* Hamilton, 1882 (Park et al. 2021) and the teleost *Clarias batrachus* (Linnaeus 1758)(Gupta &

FIGURE 3 Similarity analysis of microbial communities. All principal coordinates analysis (PCoA) were based on weighted UniFrac distances (Bray–Curtis distance) showing the distribution of the bacterial community composition in *P. melas* males exposed to pendimethalin-based herbicide and control ones at days 2 (a), 7 (b) and 21 (c) after the start of treatment. Percentages on the axes represent the proportion of explained variation of each component of the PCoA



Verma, 2020). It has been demonstrated to cause mortality in the wasp *Tiphia vernalis* Rohwer 1924 (Oliver et al. 2009), disruption of the springtail *Folsomia candida* Willem 1902 reproduction,

reduction in the earthworm *Eisenia fetida* (Savigny, 1826) growth (Belden et al. 2005) and negatively affect the humoral and cellular immune response in the ground beetle *Harpalus rufipes* (De Geer,

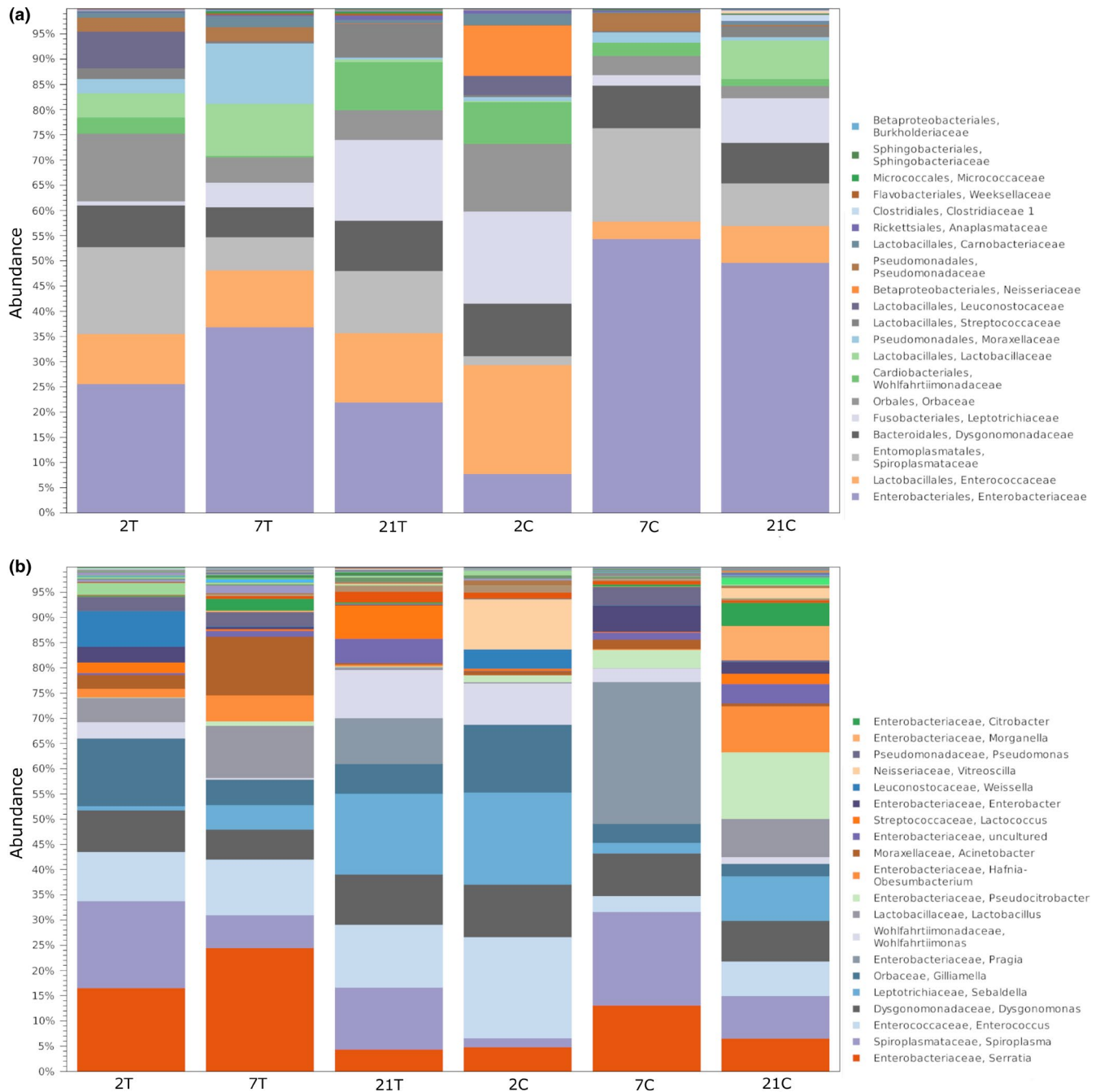
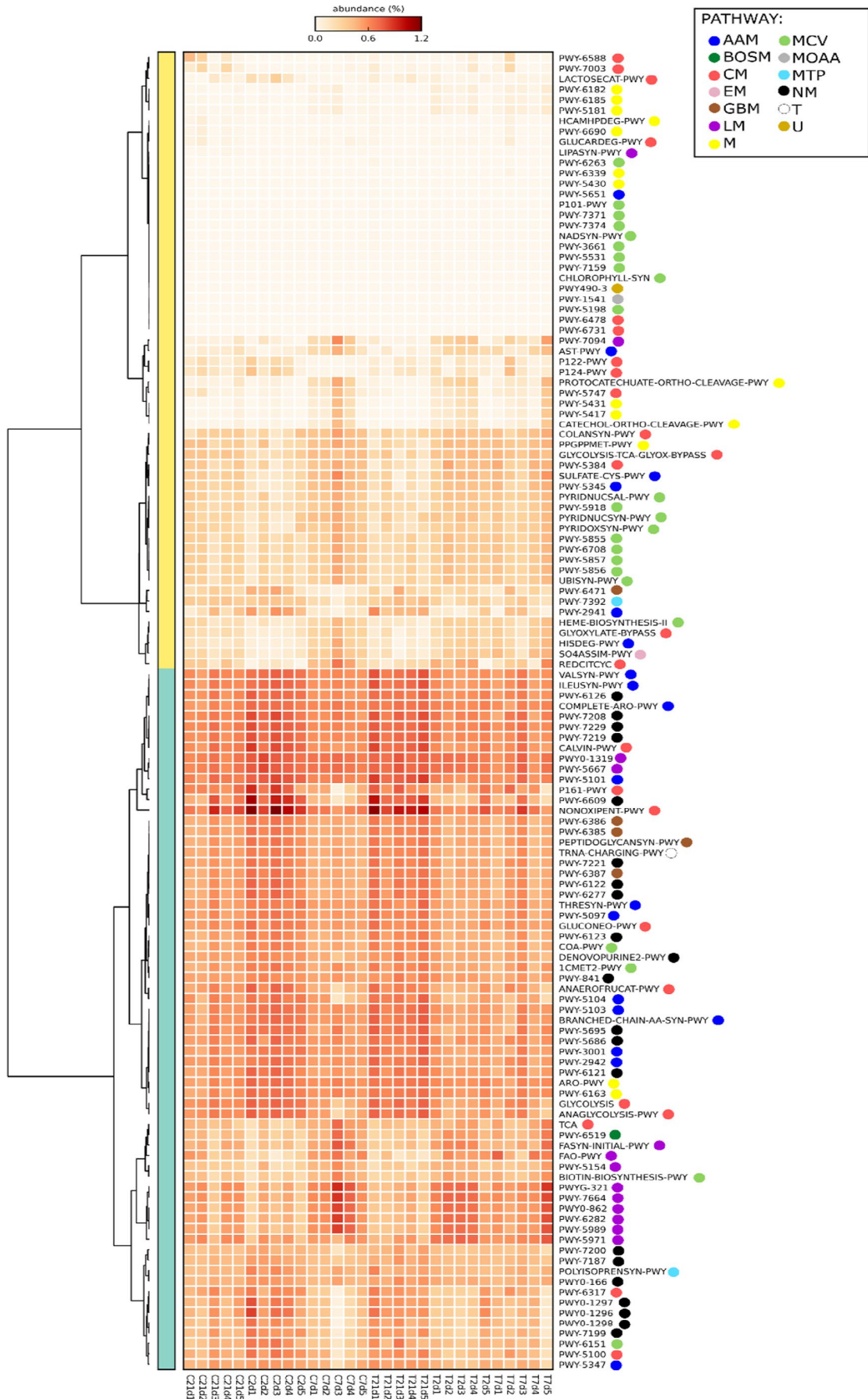


FIGURE 4 Composition of gut microbiota in males of *P. melas*. The relative abundance of major taxa at the family (a) and genus (b) level in control and pendimethalin-treated beetles at different time points. Taxa with sequence abundance <1% of total sequences were pooled together as 'Other' in all the taxonomic ranks and reported in Table S1 of Supplementary files

FIGURE 5 Heat map illustrating the functional profile predicted at level 3 KEGGs using PICRUSt analysis represents the overall functional contribution of gut bacterial communities present in *P. melas*. Acronyms are coloured according to the metabolic pathways. AAM, amino acid metabolism; BOSM, biosynthesis of other secondary metabolites; CM, carbohydrate metabolism; EM, energy metabolism; GBM, glycan biosynthesis and metabolism; LM, lipid metabolism; M, metabolism; MCV, metabolism of cofactors and vitamins; MOAA, metabolism of other amino acids; MTP, metabolism of terpenoids and polyketides; NM, nucleotide metabolism; T, translation; U, unclassified



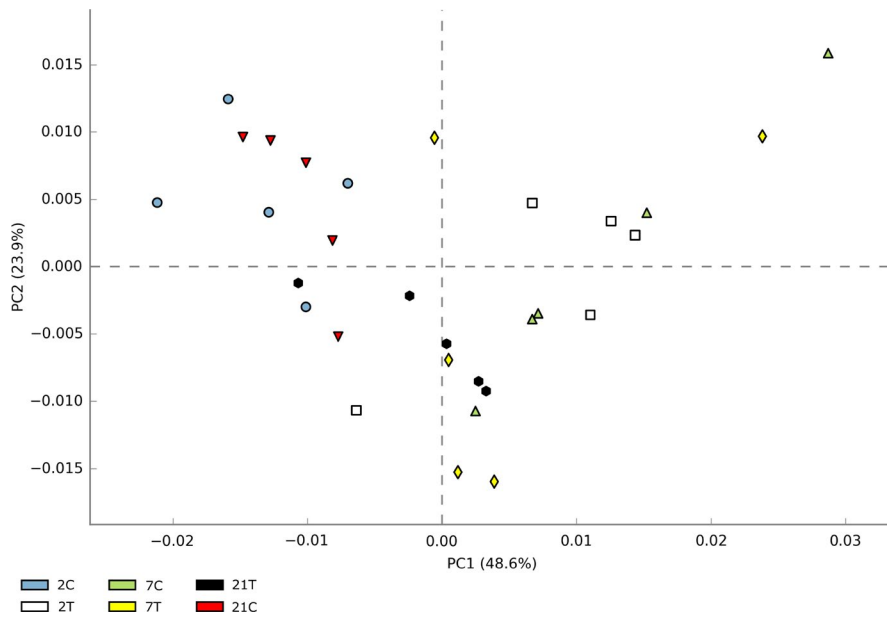


FIGURE 6 Projection of the bacterial function distances into the first and second principal components (PCA) in sampled beetles from control (C) and pendimethalin-treated (T) group at days 2 (2C, 2T), 7 (7C, 7T) and 21 (21C, 21T) after the start of treatment

1774) (Giglio et al. 2019; Vommaro et al. 2021). Microbiota bears a variable range of physicochemical parameters along the gut axis, including pH and redox conditions, oxygen availability and enzyme activities (Dillon & Dillon, 2004). Nevertheless, the herbicide exposure causing modifications in the host environment or acting on the structure and function of its organs could have indirect effects on the structure and function of the gut microbiota, affecting the colonization ability of mutualistic microbes. The hindgut, mainly the ileum compartment, is considered the primary colonized portion of the alimentary canal in insects (Douglas, 2015), supplying the most suitable conditions in terms of ion and metabolite concentrations, because of the excretory and osmoregulatory activity of Malpighian tubules. These organs are involved in the detoxification of xenobiotics, and their morphological and functional alterations are well known to be a good marker in ecotoxicological studies (Giglio & Brandmayr, 2017). Although we did not evaluate this in our study, we speculated that the exposure to PND interferes with the network of acid-base reactions, which together contribute to the organism's homeostasis (Harrison, 2001), modifying functional and morphological conditions of the gut and affecting the microbiota in *P. melas*. Indeed, using level 3 KEGG predictions, differences in the functional potentials of the bacterial communities were also observed mainly after 2d from the initial exposure.

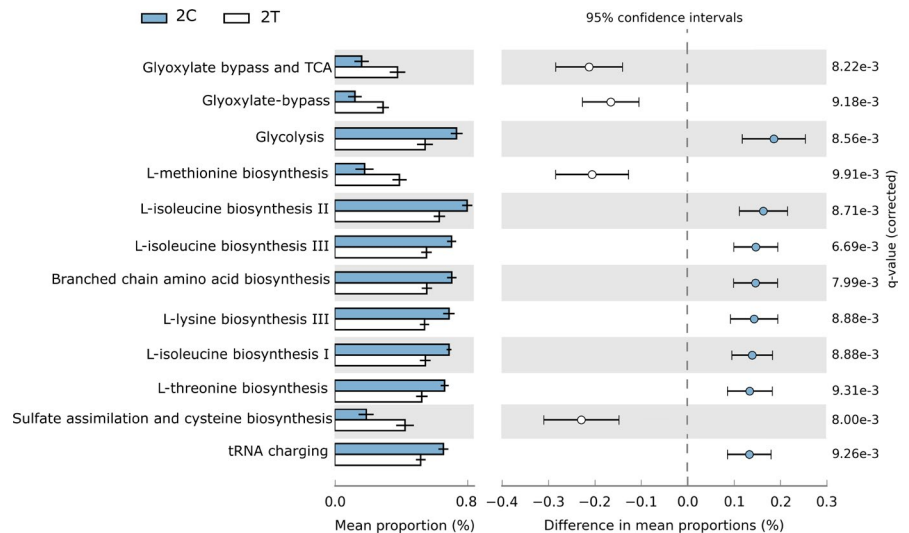
Gut bacteria such as *Enterobacter* spp., *Pseudomonas* spp., *Pantoea* spp. are also found to assist in the detoxification processes of potentially toxic compounds in insect exposed to pesticides (Douglas, 2015; Itoh et al. 2018; Kucuk, 2020). The nitroreduction has been indicated as the initial degradation and detoxification step for pendimethalin (Ni et al. 2016). Numerous pendimethalin-degrading microorganisms have been isolated in the environment (Elsayed & El-Nady, 2013; Strandberg & Scott-Fordsmann, 2004) including *Paracoccus* sp. that degrades approximately 100 and 200 mg/L pendimethalin after 2 and 5 days of incubation, respectively, producing an alkane metabolite (Ni et al. 2018). These observations

are consistent with the increase of *Paracoccus* relative abundance recorded in 21 days of exposure. Thus, the microbe-mediated detoxification may explain the tolerance to exposure recorded in *P. melas*.

Gut microbiota affects the robustness of host immunocompetence, by playing a role in antimicrobial peptide expression and phenoloxidase secretion, as shown in samples of *Rhynchophorus ferrugineus*, that devoid of their mutualistic microbial community showed a reduced ability to cope with infections (Muhammad et al. 2019). Moreover, a previous study on the ground beetle *Harpalus rufipes* has been revealed a reduction in circulating phenoloxidase levels after exposure to PND field rate (Giglio et al. 2019). In addition, gut microbiota takes part in endocrine system regulation, being involved in development and growth processes, as observed for honey bees and red palm weevil (Habineza et al. 2019; Zheng et al. 2017). Thus, further studies are needed to investigate the effects of this herbicide on gut microbiota related to immune responses and the regulation of hormones controlling moulting, pupation, metabolism and reproduction in insects.

The association and interaction between bacteria have a crucial role in host homeostasis due to the non-pathogenic insect-associated microorganisms involved in a range of functions such as nutritional processes (digestion, provisioning and assimilation), development and pathogen resistance (Douglas, 2015; Engel & Moran, 2013). Bacterial species may act independently to assisting functional traits of the host, as observed in the carabid beetles *Harpalus pensylvanicus* (Schmid et al. 2014). However, some functions are the product of interactions among bacterial species, and the prevalence of a bacterium can be correlated negatively or positively with the abundance of other bacteria. In *Drosophila melanogaster*, an increased abundance of *Acetobacter* has been recorded in the presence of some *Lactobacillus* species modulating microbiota-dependent traits such as larval development rates and the levels of glucose and triglycerides (Newell & Douglas, 2014). *Serratia* spp consuming oxygen maintains strict anaerobes that digest cellulose in the gut of termites (Adams & Boopathy, 2005), but it is negatively correlated with the

FIGURE 7 Inferred functions of bacterial communities associated with *P. melas*. All of the predicted significant KEGG (Kyoto Encyclopedia of Genes and Genomes database) metabolic pathways (Welch's *t* test with Benjamini–Hochberg correction) are shown at the third hierarchical level and grouped by major functional categories, for both control (2C) and pendimethalin-treated (2T) group after 2 days of exposure



abundance of other bacteria in the gut of crickets (Douglas, 2015). Moreover, modifications observed in the microbiota structure of *P. melas* exposed to PND likely cause a variation of the antagonistic interaction among different bacteria with different metabolic requirements, favouring the growth of facultative pathogens such as *Pantoea* and *Empedobacter* at 2d and 7d, respectively. Thus, we assume that the alterations in the microbiota community could lead to dysbiosis, compromising other microbiota-dependent life traits.

There is a growing need for studies that contribute to our understanding of the herbicide impact on the gut microbiome helpful to protect beneficial soil insects that have a crucial role in the ecosystem services. In this study, the changes in the microbiome structure may alter the community functions, and thus, fallouts can occur for beetles' physiology and behaviour. In generalist predators such as *P. melas*, bacteria enter the gut by horizontal transmission from the surrounding environment (Kolasa et al. 2019; Kudo et al. 2019). The reduction in soil microbiota is well known in pendimethalin field application, and the exposure of this carabid to PND by contact or ingesting contaminated food caused modifications of microbiota structure and related functions. The PND exposure may indirectly affect microbiota because of alterations of gut physical, chemical or structural conditions. Such modifications could bring out a niche competition among different bacterial species for nutrient sources, changing their colonization ability. This interference with consolidated symbiotic relationships may have effects on different life-history traits, compromising the ecological role of *P. melas* as pest control, such as feeding behaviour, reproduction as well as the capability to withstand the colonization of the gut by non-indigenous species, including pathogens and therefore prevent infections. Thus, our results contribute to evaluating the risk assessment of herbicides such as pendimethalin on soil invertebrates in agroecosystems.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

AG, VML and AP conceived research. AG and VML conducted field sampling and exposure assays. FG conducted sequencing experiments. AP conducted statistical analyses. AG and MLV wrote the manuscript. AG secured funding. All authors read and approved the manuscript.

DATA AVAILABILITY STATEMENT

The raw reads from 16S rRNA gene sequencing were deposited into the Zenodo public repository at <http://doi.org/10.5281/zenodo.4663948> (Pallavicini et al. 2021). The data that support the findings of this study are available in the supplementary material of this article (Table S1).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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