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Behavior and gut bacteria of *Partamona helleri* under sublethal exposure to a bioinsecticide and a leaf fertilizer

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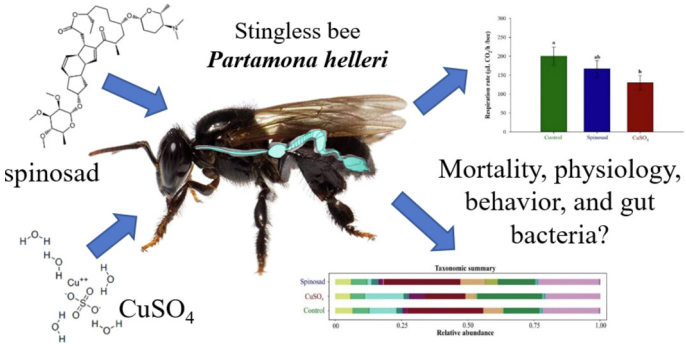
HIGHLIGHTS

- *Partamona helleri* survival was affected by exposures to spinosad and copper sulfate.
- The overall behavior was not affected by the exposures.
- The respiration rate of bees changed after exposure to copper sulfate.
- The richness of the gut bacteria was not affected by the exposures.
- Abundance of bacteria of the genus *Gilliamella* increased after spinosad exposure.

GRAPHICAL ABSTRACT



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ABSTRACT

The exposure of bees to agrochemicals during foraging and feeding has been associated with their population decline. Sublethal exposure to agrochemicals can affect behavior and the microbiota. Gut microbiota is associated with insect nutritional health, immunocompetence, and is essential for neutralizing the damage caused by pathogens and xenobiotics. Research on the effect of the bio-insecticides and fertilizers on the microbiota of bees remains neglected. In this study, we assessed the sublethal effect of both bioinsecticide spinosad and the fertilizer copper sulfate ($CuSO_4$) on the behavior and gut microbiota in forager adults of the stingless bee *Partamona helleri* (Friese), which is an important pollinator in the Neotropical region. Behavioral assays and gut microbiota profiles were assessed on bees orally exposed to estimated LC_5 values for spinosad and $CuSO_4$. The microbiota were characterized through 16S rRNA gene target sequencing. Acute and oral sublethal exposure to spinosad and $CuSO_4$ did not affect the overall activity, flight take-off, and food consumption. However, $CuSO_4$ decreased bee respiration rate and copper accumulated in exposed bees. Exposure to spinosad increased the proportional abundance of the genus *Gilliamella*, but $CuSO_4$ did not alter the composition of the gut microbiota.

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In conclusion, sublethal exposure to $CuSO_4$ induces changes in respiration, and spinosad changes the abundance of gut microorganisms of *P. helleri*.

1. Introduction

Pollination services provided by native bees are essential for many crops, especially those cultivated in the tropics. Stingless bees are the predominant pollinators in neotropical ecosystems and together with honey bees, comprise the key pollinators in the region (Barbosa et al., 2015a; Giannini et al., 2015; Lima et al., 2016; Valk and Koomen, 2012). The decline of stingless bee colonies over the last decade has been attributed to several factors, such as deforestation, habitat fragmentation, and intensive agriculture (Freitas et al., 2009; Roubik, 2014). Recent studies have shown that the large-scale applications of agrochemicals play a critical role in the increased rate of bee colonies loss (Johnson, 2015; Lima et al., 2016; Valk and Koomen, 2012). The impacts of agrochemicals on stingless bees have been neglected (Lima et al., 2016) and most studies focus on honey bees (Johnson, 2015; Tomé et al., 2017, 2019). However, stingless bees exhibit higher susceptibility in comparison with *Apis mellifera*, representing more suitable models for toxicity assessments in neotropical ecosystems (Barbosa et al., 2015b; Lima et al., 2016; Tomé et al., 2017).

Agrochemicals which are regarded as “safe” for bees, such as bioinsecticides, fungicides, fertilizers, and herbicides can exhibit a high lethal toxicity and deleterious effects on the cognitive function, behavior, and physiology in stingless bees, including *Melipona quadrifasciata* (Barbosa et al., 2015a, b; Seide et al., 2018; Tomé et al., 2015b), *Friesella schrottkyi* (Rodrigues et al., 2016), *Partamona helleri* (Bernardes et al., 2018; Tomé et al., 2017; Araujo et al., 2019a, 2019b), *Scaptotrigona xanthotrica* (Tomé et al., 2015a), and to honey bees (Lopes et al., 2018; Tomé et al., 2017).

Spinosad is a bioinsecticide widely used for crop protection and was primarily considered low risk to non-target arthropods, such as bees (Thompson et al., 2000). However, studies have shown that spinosad exhibits negative effects on bee health (Barbosa et al., 2015b; Biondi et al., 2012; Mayes et al., 2003; Tomé et al., 2015b). This bioinsecticide is derived from spinosyns, which are the fermented metabolites of *Saccharopolyspora spinosa* (Mertz and Yao) (Actinomycetales: Pseudonocardiaceae) (Sparks et al., 2001). The spinosyns cause hyperexcitation, and the disruption of the insect central nervous system, they act primarily as an agonist of nicotinic acetylcholine receptors (nAChRs) and secondarily as an agonist of γ-aminobutyric acid (GABA) receptors (Salgado, 1998; Sparks et al., 2001).

Fungicides and fertilizers contain heavy metals, and their repeated application can cause the accumulation of metals such as copper (Cu) in plants (Fageria et al., 2002). These pollutants have been detected in pollen and nectar, which are harvested by bees and bioaccumulated in the insects (Hladun et al., 2015; Johnson, 2015). Leaf fertilizers also input heavy metals into the agricultural ecosystem, such as Cu, iron (Fe), zinc (Zn), manganese (Mn), cobalt (Co), and selenium (Se), all of which can be detrimental for bee health (He et al., 2005; Hladun et al., 2016). These toxic heavy metals can inactivate many enzymes by replacing essential metal ions in biomolecules, resulting in their inhibition or function loss, the disruption of redox homeostasis, and therefore the impairment of essential activities (Tchounwou et al., 2012).

Several studies have confirmed a close relationship between gut microbiota and insect health, including in bees (Corby-Harris et al., 2014; Hroncova et al., 2015; Ludvigsen et al., 2015). Resilience to environmental stressors, and thus, colony success, is also influenced by the gut microbial community (Dillon and Dillon, 2004; Engel et al., 2012; Raymann and Moran, 2018). The core members of the gut microbiota in adult stingless bees are highly conserved (Kwong et al., 2017). The gut microbial populations are vital for the pollen and nectar digestion which are essential for providing energy and enhancing immunocompetence against pathogens, thus improving the colony's ability to withstand xenobiotics and environmental stresses (Koch et al., 2012; Mockler et al., 2018; Raymann and Moran, 2018; Zheng et al., 2016). The gut bacteria of adult eusocial bees are dominated by five lineages: *Snodgrassella*, *Gilliamella*, *Lactobacillus* Firm-4, *Lactobacillus* Firm-5, and *Bifidobacterium* (Díaz et al., 2017; Kwong et al., 2017), and their abundance varies according to age, season and food sources (Anderson et al., 2016; Corby-Harris et al., 2014; Hroncova et al., 2015; Ludvigsen et al., 2015).

Studies suggest that changes in the gut microbiota profile lead to an increased susceptibility to pathogen infection and increased mortality of individual bees, thus affecting the ability of bee colonies to withstand environmental stressors (Cariveau et al., 2014; Hamdi et al., 2011; Mockler et al., 2018; Raymann and Moran, 2018). Metagenomic analysis of honey bees identified a difference in the composition of the gut microbiota between hives exhibiting colony collapse disorder (CCD) and normal healthy hives (Cox-Foster et al., 2007), corroborating the idea that symbiotic bacteria can provide functional benefits to bees and colony health. In this context, the present study aimed to assess the sublethal effects of the bioinsecticide spinosad and the leaf fertilizer copper sulfate ($CuSO_4$) on behavior and composition of gut microbiota in *P. helleri*, a native pollinator in the Neotropical region, which is widely distributed in the Atlantic forest and open areas of southeastern Brazil. Moreover, this species is also found in agricultural landscapes and urban areas (Camargo and Pedro, 2003). Change in the gut microbiota was used as an indicator of sublethal stress to the two compounds evaluated.

2. Materials and methods

2.1. Insects and agrochemicals

Five colonies of the stingless bee *P. helleri* were collected from the rural area of the municipality of Viçosa (Minas Gerais State, Brazil; 20° 45′ S and 42° 52′ W) and maintained at the campus at the Universidade Federal de Viçosa (UFV). Foragers leaving the hive entrance were collected using jars. The foraging bees were anesthetized in the jars by exposing individuals to carbon dioxide for 5 s; they were then transferred to a transparent plastic container (500 mL). Bees were maintained without food for 1 h under complete darkness at 28 ± 1 °C and 75 ± 5% relative humidity (RH) until the bioassays began. The fasting period before pesticide exposure was necessary to acclimatize the bees to the experimental conditions and to encourage them to feed on the diet provided in the oral exposure tests (Tomé et al., 2015b).

Two commercial formulations of agrochemicals commonly used in Brazilian tomato crops (MAPA, 2017) were used as follows: the bioinsecticide spinosad (Tracer; suspension concentrate; 480 g L^{-1},

Dow AgroSciences, Santo Amaro, SP, Brazil) and the leaf fertilizer $CuSO_4$ (Sulfato de Cobre Penta 24; a salt formulation containing 240 g kg^{-1} Cu and 110 g kg^{-1} S; Multitécnica Industrial, Sete Lagoas, MG, Brazil). Decreasing concentrations of the compounds were obtained based on the maximum recommended label rates of each agrochemical (spinosad: 204 μg a.i. mL^{-1}; $CuSO_4$: 5000 μg a.i. mL^{-1}; 100 L ha^{-1}) and were prepared by diluting them in a 50% (w/w) sucrose solution using deionized water. Spinosad was used at its maximum field recommended label rate for the control of whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) (MAPA, 2017).

2.2. Concentration-mortality bioassays

Foraging bees were subjected to a concentration-mortality bioassay, corresponding to a series of concentrations below the maximum recommended field rates of each agrochemical. The compounds were diluted in sucrose solution (50% w/w) and offered to bees over a 24 h period (acute exposure) using drilled 2-mL microtubes inserted through a hole into the 500-mL polyethylene containers (Tomé et al., 2015b). Twenty bees per replicate were placed in each container at nine concentrations of spinosad (0.72, 0.85, 1.02, 1.27, 1.7, 2.5, 5.1, 20.4, and 204 μg a.i. mL^{-1}) and ten concentrations of $CuSO_4$ (100, 120, 140, 160, 200, 250, 333, 500, 1000, and 5000 μg a.i. mL^{-1}). Each colony comprised a biological replicate for each concentration, i.e., five replicates were used per concentration. Exposure to an uncontaminated sucrose solution (control treatment) was used to assess natural mortality for the correction of the mortality data. Mortality was recorded after 24 h of exposure, and insects were counted as dead if they were unable to walk. Lethal concentrations to kill 5% of the bees (LC_5) were estimated via probit curves for each agrochemical and subsequently used to assess sublethal effects on behavioral parameters and gut microbiota of the bees.

2.3. Feeding behavior

The feeding behavior was assessed in bees exposed to the estimated sublethal concentrations (LC_5) of each agrochemical, as well as a sucrose solution (control). The average food consumption per foraging bee was estimated by weighing the food microtubes at the beginning and the end of the oral exposure on an analytical precision scale (Rodrigues et al., 2016). Plastic containers with food microtubes but without bees were maintained at the same experimental conditions to estimate the weight loss of the diet by evaporation, which was used to correct the rate of diet consumption.

2.4. Overall activity bioassay

The overall group activity of *P. helleri* foragers was analyzed after 24 h of oral exposure to the estimated sublethal concentrations (LC_5) of spinosad and $CuSO_4$. Individuals feeding on a sucrose solution were used as a control, as described above. Exposed foragers were anesthetized, and five individuals were placed into Petri dishes (9 cm diameter and 2 cm high). The Petri dish bottoms were covered with a filter paper (porosity of 3 μm, 0.5% ash content, 9 cm diameter, 80 gm^{-2} density; Nalgon Equipamentos Científicos Ltda, Itupeva, SP, Brazil) and the inner walls were coated with Teflon TPFE® (DuPont, Wilmington, DE, USA). After 5 min of acclimation, the movement of the individuals within the arena was recorded for 10 min and digitally transferred to a computer using the ViewPoint automated tracking system equipped with a charge-coupled device (CCD) camera (ViewPoint LifeSciences, Montreal, QC, Canada) (Tomé et al., 2015a, 2015b). Each Petri dish of five insects was an experimental unit, and each treatment (i.e., LC_5 spinosad, LC_5 $CuSO_4$, and control) was recorded for three experimental units per colony. The bioassays were carried out in a room with artificial fluorescent light at 25 ± 2 °C and 70 ± 5% relative humidity.

2.5. Flight take-off assay

The same bees subjected to the group activity bioassay were subsequently used in a flight take-off bioassay (1 h after the previous bioassay or 25 h after the exposure period). A 105 cm tower was formed with three stacked wooden cages (35 × 35 × 35 cm each) which allowed insects to fly between them. A fluorescent lamp was placed 5 cm above the tower in a dark room to attract the flying insects. The flight take-off assay explored the vertical bee flight towards the light source after release from the center of the bottom of the tower (Tomé et al., 2015b). Fifteen bees were used per colony per treatment (i.e., 75 bees per treatment) and the bees were released in groups of five as used in the bioassay on overall activity. After the placement of the Petri dishes at the base of the tower, the bees were allowed a 1 min of acclimation period to the experimental conditions before release. The vertical flight take-off was recorded for 1 min, and the time for each bee to reach the light source was recorded using a stopwatch. Bees that did not reach the light source within 1 min were not included in the analysis.

2.6. Respirometry bioassay

Respirometry bioassay was carried out with a new batch of 24 h-treated bees and a control group (item 2.4). The treated bees were placed individually in a 25 mL plastic chamber connected to a TR3C respirometer equipped with a CO_2 analyzer (Sable Systems International, Las Vegas, NV, USA). The carbon dioxide production (μL CO_2 h^{-1} bee^{-1}) was determined after a 3-h period by injecting CO_2-free air into the chamber for 2 min at a flow rate of 600 mL min^{-1}. The air stream was directed to an infrared reader connected to the system allowing the determination of the CO_2 produced per bee. The residual CO_2 from the purified air was determined in three empty chambers without insects during the experiment to correct the final CO_2 produced (Tomé et al., 2015a). All analyses were performed at the same time for each colony, and four bees were used from each of the five colonies [i.e., 20 bees per colony (five independent replicates/treatment) or 60 individuals in total].

2.7. Copper (Cu) and sulfate (S) concentrations in bee bodies

The quantification of Cu and S in the bees was performed by the method of nitro-perchloric digestion (Sarruge and Haag, 1974). Groups of fifty dead bees from each colony (i.e., 250 bees) per treatment were maintained in 100% ethanol in centrifuge tubes (15 mL). The bee samples (including the ethanol) were dried for 24 h at 120 °C, weighed and then boiled at 200 °C (Heater Plates, Nova Ética 208/D, São Paulo, Brazil) in a mixture of 10 mL HNO_3 and $HClO_4$ at a ratio of 4:1. The fluid volume was increased to 25 mL with deionized water. Copper quantification was determined by atomic absorption spectroscopy (Varian SpectrAA 220FS, Mulgrave, Australia) and sulfate was determined by UV–visible spectrophotometry (420 nm; FEMTO 600S, São Paulo, Brazil).

2.8. DNA extraction and 16S rRNA gene sequencing

Total genomic DNA was extracted from pooled midguts and hindguts of fifteen bees per colony per treatment, with each colony corresponding to an individual biological replicate. The concentrations used were the LC_5 of each agrochemical as well as the control (i.e., untreated sucrose solution). Before DNA extraction, each bee was individually submitted to surface-disinfestation by

rinsing in an increasing ethanol ascending series (70, 80, and 90% v/v of ethanol) for 30 s each, followed by multiple washes in sterile phosphate buffer saline (PBS; 0.1 M; pH 7.6) followed by sterile water. Bacterial DNA was extracted from each sample (i.e., 15 pooled hindguts and midguts) using the PowerMax Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The DNA concentration was determined using Qubit 2.0 fluorometer and dsDNA BR Assay kit (Invitrogen, Carlsbad, CA, USA). The DNA samples were stored at −80 °C for 24 h and then lyophilized in Lyophilizer L101 (Liotop) for 24 h.

Bacterial and archaeal 16S rRNA genes (V4 –V5 region) were amplified using primers 515F (GTGCCAGCMGCCGCGGTAA) and 926R (CCGYCAATTYMTTTRAGTTT) (Walters et al., 2016) and paired-end sequenced on the Illumina MiSeq platform, at the High-throughput Genome Analysis Core (HGAC), Argonne National Laboratory (USA).

All raw sequences obtained by amplicon sequencing were submitted to the National Center for Biotechnology Information (NCBI) under the accession BioProject ID PRJNA529891 and Submission ID SUB5345934. BioSample accessions were SAMN11288628-42.

2.9. Statistical analyses

Concentration-mortality data were subjected to probit analyses (PROC PROBIT; SAS Institute, 2008). The overall group activity and respiration rate were subjected to analyses of variance followed by Tukey's HSD test when appropriate; colonies were inserted as a random effect (PROC MIXED; SAS Institute, 2008). The time data of flight take-off were subjected χ^2 log-rank test ($P < 0.05$) using Kaplan–Meier estimators (PROC LIFETEST in SAS). Mann–Whitney U test was used to analyze the heavy metal concentrations ($P < 0.05$) (PROC NPAR1WAY). The assumptions of normality and homoscedasticity were tested before each analysis (PROC UNIVARIATE; SAS Institute, 2008).

The 16S rRNA gene data pre-processing and diversity estimates were performed as recommended by the Brazilian Microbiome Project (Pylro et al., 2014) using the BMP Operating System (BMPOS) (Pylro et al., 2016). Briefly, an Operational Taxonomic Unit (OTU) table was built following the UPARSE pipeline (Edgar, 2013), using VSEARCH ver. 2.3.4 (Rognes et al., 2016). The paired reads were merged with FLASH (Magoc and Salzberg, 2011), quality-filtered using a maximum expected error value of 0.5 (on average, 1 nucleotide in every 2 sequences is incorrect) and then truncated at 250 bp. Singletons were removed after dereplication of filtered reads, and these sequences were clustered into OTUs (97% similarity cutoff).

After clustering, the sequences were aligned against the SILVA (Quast et al., 2013) reference database (version 132). The 16S rRNA datasets were rarefied to the same number of sequences per database (Lemos et al., 2011) and used to construct dissimilarity matrixes generated by Binary and Bray–Curtis distances using the "phyloseq" package in R. The "Adonis" function was used to calculate the permutational multivariate analysis of variance (PERMANOVA) and verify the strength and statistical significance of groups among treatments, used for Principal Coordinate Analysis with the vegan package (Oksanen et al., 2015). The microbial diversity changes were evaluated using the alpha diversity metric Chao1 (Chao, 1984). Relative abundances (beta diversity) of the bacterial genus from bees exposed to different agrochemicals were compared using analysis of variance (ANOVA). The dataset was summarized at the genus level. The hypothesis testing method used to compare taxonomic differences between treatments was made using the Bioconductor metagenomeSeq package for R (Paulson et al., 2013).

3. Results

3.1. Concentration-mortality curves

A probit model was used for the results from the concentration-mortality bioassays of both agrochemicals based on the low χ^2 and high p-values obtained in the goodness-of-fit tests (Fig. 1). Bee mortality increased with the concentration of both spinosad and $CuSO_4$ concentrations. The LC_5 and LC_{50} estimates were 0.81 and 2.89 μg a.i. mL^{-1} for spinosad and 120 and 362.60 μg a.i. mL^{-1} for $CuSO_4$, respectively (Fig. 1). Concentrations corresponding to the maximum label rate of both agrochemicals caused 100% mortality within 24 h of exposure.

3.2. Behavioral bioassays

The overall walking activity (Δ pixel × 100^{-2} s) of the foraging bees did not exhibit significant differences between the spinosad treatment (295.74 ± 9.86), $CuSO_4$ treatment (285.32 ± 14.75) or control bees (269.90 ± 24.07) ($F_{6,38} = 0.63$; $P = 0.54$). The time (s) taken for the foragers to reach the light source did not significantly differ between spinosad treatment bees (20.01 ± 2.36), $CuSO_4$ treatment (16.36 ± 2.04) or control bees (18.45 ± 2.42) ($\chi^2 = 0.97$; df = 2; $P = 0.61$). Food consumption (mg) did not vary between the spinosad treatment (0.434 ± 0.10), $CuSO_4$ treatment (0.470 ± 0.11) or control bees (0.584 ± 0.30) ($F_{2,12} = 0.78$; $P = 0.48$).

3.3. Respiration rate

The respiration rate (μL CO_2 h^{-1} bee^{-1}) of the bees did not exhibit significant differences between spinosad treated bees (166.629 ± 32.99) and control bees (196.004 ± 33.25) ($P = 0.45$). In contrast, $CuSO_4$ (130.074 ± 32.99) reduced the respiration rate of the bees compared to the control ($P = 0.02$) ($F_{2,52} = 3.60$; $P = 0.03$) (Fig. 2).

3.4. Copper (Cu) and sulfate (S) concentrations in bee bodies

Bees accumulated significantly more copper (Cu) when they were exposed to $CuSO_4$ contaminated sucrose solution (22.15 μg g^{-1}) than the control treatment (9.40 μg g^{-1}) (U = 1; $P = 0.01$). However, the sulfate (S) content had similar values

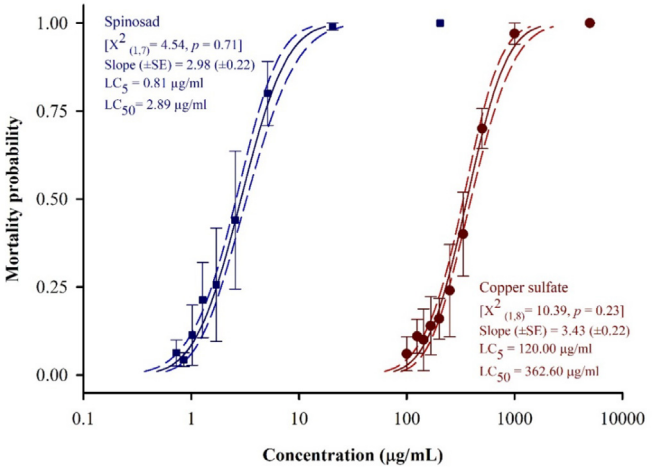

Fig. 1. Concentration–mortality curves of foragers of *Partamona helleri* after the oral exposition (during 24 h) to spinosad or copper sulfate ($CuSO_4$). LC_5 and LC_{50} values are indicated, and dotted lines represent the 95% fiducial limits of each curve. Vertical bars represent standard errors (SE).

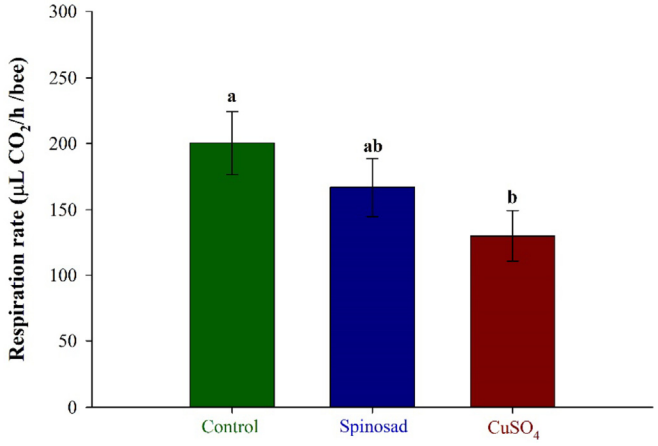

Fig. 2. Respiration rates (±SE) of foragers of *Partamona helleri* after the oral exposure (during 24 h) to spinosad or copper sulfate ($CuSO_4$). Different letters indicate significant differences among treatments based on Tukey's HSD test ($P < 0.05$).

between control (5.3 mg g^{-1}) and $CuSO_4$ treatment (5.5 mg g^{-1}) (U = 7.5; $P = 0.31$) (Fig. 3).

3.5. Bacterial 16S rRNA gene sequences analysis

A total of 231,643 high-quality sequences longer than 250 bp were obtained in this study. The average Good's coverage of 99% was calculated (Table 1), indicating the dataset was representative of the microbial communities analyzed. The average number of unique sequences read between treatments was 195.53 (±4.11). The dominant gut bacteria of *P. helleri* were assigned to 33 genera (Fig. 4). The main bacterial OTUs classified here are similar to those previously reported in the gut microbiota of corbiculate bees (Kwong et al., 2017; Kwong and Moran, 2016). The most abundant genera were *Apibacter*, *Commensalibacter*, *Gilliamella*, *Izhakiella*, *Lactobacillus*, *Pseudomonas*, and *Snodgrossella*.

The bacterial diversity and composition were similar regardless of the treatment (Fig. 5). Both spinosad and $CuSO_4$ treatments did not elicit any change in diversity. The Chao1 estimator of alpha diversity (richness) measured for *P. helleri* did not differ between the control and treatment groups (F = 0.60; $P = 0.58$) (Fig. 5A). The beta diversity analysis was similar among control and exposed bees ($R^2 = 0.21$; $P = < 0.1$) (Fig. 5B). Also, the gut bacteria diversity in control and treated bees displayed similar clustering patterns based on the Permutational Multivariate Analysis of Variance (PERMANOVA) (Fig. 5B). However, the differential abundance analysis indicated a significant increase of the genus *Gilliamella* in bees

Table 1
Number of quality-filtered reads per sample and average α-diversity metrics for the gut microbiota of foragers of *Partamona helleri* after the oral exposition (during 24 h) to spinosad or copper sulfate ($CuSO_4$).

Treatment	Colony	OTUs[a]	Chao1[a,b]	Good's coverage[c]	Reads[d]
Control	1	209	239.03	99%	16,188
Control	2	193	212.59	99%	16,654
Control	3	262	280.50	99%	25,407
Control	4	165	216.27	99%	14,537
Control	5	159	173.09	99%	8818
Spinosad	1	161	187.04	99%	13,332
Spinosad	2	222	240.50	99%	22,948
Spinosad	3	205	218.41	99%	16,352
Spinosad	4	185	202.88	99%	17,816
Spinosad	5	218	252.87	99%	20,472
$CuSO_4$	1	357	368.88	99%	23,587
$CuSO_4$	2	150	179.29	99%	9679
$CuSO_4$	3	179	250.72	99%	10,233
$CuSO_4$	4	101	147.75	99%	3213
$CuSO_4$	5	167	204.84	99%	12,407

[a] 97% identity was used to define OTUs (Operational Taxonomic Unit).
[b] Non-parametric estimator used to predict species richness (Total numbers of OTUs).
[c] Estimate of the proportion of the total diversity sampled.
[d] After quality filter.

treated with spinosad compared to the control and the $CuSO_4$ treatment (FDR = 0.029) (Fig. 5C).

4. Discussion

The mortality of *Partamona helleri* foragers increased with increasing concentrations of both the bioinsecticide (spinosad) and the leaf fertilizer ($CuSO_4$). Spinosad was more toxic than $CuSO_4$ to *P. helleri*. Also, LC_{50} values were much lower than the field recommended concentrations for both agrochemicals (spinosad: 70.6x; $CuSO_4$: 13.8x). These results reinforce the view that agrochemicals considered safe for pollinators can also exhibit high toxicity to stingless bees, especially regarding spinosad, which has already been recognized as harmful to Meliponini bees (Barbosa et al., 2015b; Bernardes et al., 2018; Rodrigues et al., 2016; Seide et al., 2018; Tomé et al., 2015b). Mortality curves and LC_{50} values are frequently used to assess the susceptibility of living organisms to xenobiotics; such estimates may not be realistic because sublethal effects often occur owing to the applied dose being subject to environmental degradation. Here, we explored a low concentration (LC_5) of the agrochemicals as considering the degradation of the compounds and due to the higher probability to obtain surviving individuals subjected for sublethal assessments (Guedes et al., 2017). Therefore, we assessed potential sublethal effects on behavioral and physiological activities, as well as on the bacterial

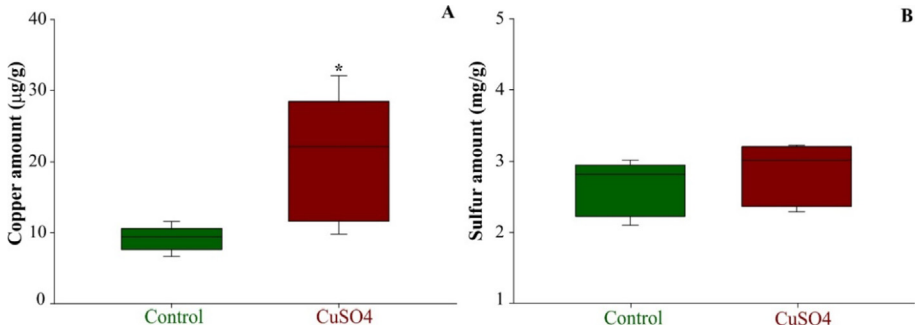

Fig. 3. Copper and sulfate content (±SE) in forager's bodies of *Partamona helleri* after the oral exposure (during 24 h) to spinosad or copper sulfate ($CuSO_4$): (A) Copper quantification, (B) Sulfur quantification. Asterisk indicates significant differences by the Mann-Whitney test ($P < 0.05$).

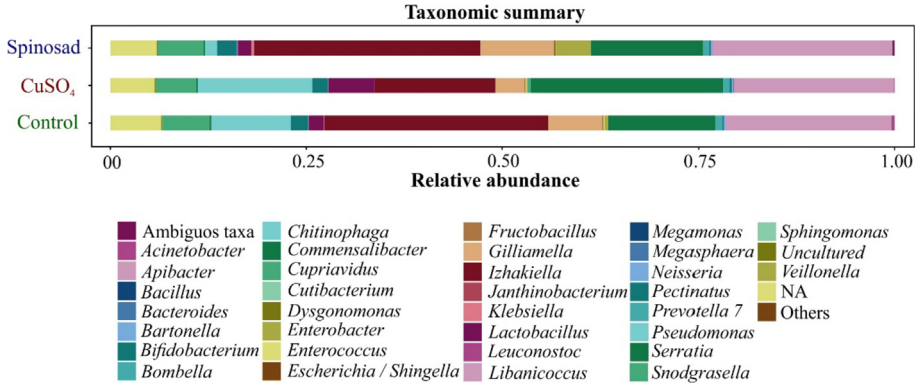

Fig. 4. Relative abundance of bacterial taxa (genus) found in the gut of foragers of *Partamona helleri* after the oral exposition (during 24 h) to spinosad or copper sulfate ($CuSO_4$). Each column in the graph represents the average (n = 75) of the percentage abundance of each taxon in each treatment.

composition of the gut. Gut bacteria are crucial for the release of nutrients and the immunocompetence of insects (Cariveau et al., 2014; DeGrandi-Hoffman et al., 2010; Engel et al., 2012; Evans and Armstrong, 2006; Zheng et al., 2016, 2017).

Bioinsecticides and heavy metals negatively affect different organisms, including bees, reducing survival (Di et al., 2016; Moroń et al., 2013; Tomé et al., 2015a), altering ingestion (Bernardes et al., 2017), behavior (Barbosa et al., 2015b; Mogren and Trumble, 2010), and physiology (Bernardes et al., 2018; Lopes et al., 2018; Rodrigues et al., 2016). Despite this, behavioral impairments were not evident in our study for any of the treatments (either spinosad or $CuSO_4$); however, bees exposed to copper sulfate showed a reduced respiration rate. The reduction in respiration rate observed was not sufficient to affect the behavioral parameters assessed for *P. helleri* in this study. Therefore, further assessments on other biological parameters that are complementary or linked to labor activities of this stingless bee species are necessary. The divergence in response to sublethal concentrations between spinosad and $CuSO_4$ is likely due to the different modes of action and concentrations of each agrochemical. Likewise, the sublethal effects detected may vary among stingless bee species (Lima et al., 2016). These results suggest that even low concentrations can lead to potential toxicity on bees and, therefore, these agrochemicals should be applied in an appropriate way to minimize their impact on native bees.

The bioaccumulation of heavy metals occurs through feeding on contaminated food. The typical method for the intake of heavy metals (or other elements) is via the ingestion of contaminated pollen and nectar (Johnson, 2015). Our results indicated that significant quantities of Cu were accumulated in foragers orally exposed to $CuSO_4$. In contrast, untreated forager bees showed low concentrations of Cu. All concentrations of Cu measured in our study are consistent with the range of values previously reported for honey bees (11.65–24.5 $\mu g\, g^{-1}$ Cu) (Di et al., 2016; Giglio et al., 2017; Roman, 2010), indicating a wide variation in the exposure of bees to these metals in the environment.

Metataxonomic analysis of the gut microbiota showed that *P. helleri* host several bacterial groups, which are reported in a diverse range of corbiculate species, including honey bees, bumblebees, and stingless bees (Kwong and Moran, 2016). These groups are dominated by a complex of distinct bacteria belonging to five bacterial lineages, which are considered as the core gut bacteria in stingless bees such as *Snodgrassella*, *Gilliamella*, *Lactobacillus* (two strains), and *Bifidobacterium*, all ubiquitously and consistently found in the digestive tract of foraging bees worldwide (Díaz et al., 2017; Koch et al., 2013; Kwong et al., 2017; Kwong and Moran, 2016; Leonhardt and Kaltenpoth, 2014). The findings of this study support the hypothesis that these bacterial groups are part of the usual gut microbiota, thus supporting the development and conservation of specific microbial community in social bees.

Non-core bacterial genera identified in our study were reported in previous studies in bees. The genera *Pectinatus*, *Bartonella, Leuconostoc*, *Apibacter* were reported in Meliponini microbiota (Kwong et al., 2017), while *Bombela*, *Commensalibacter*, *Fructobacillus* genera were reported in Bumblebees (Jessy et al., 2017) and *Enterobacter*, *Klebsiella*, and *Serratia* genera were found in Honey bees (Saraithong et al., 2017). However, the role played by these bacteria on their hosts is not fully understood. Also, the genera *Bacteroides*, *Acinetobacter*, *Chitinophaga*, *Cupriavidus*, *Cutibacterium*, *Dysgonomonas*, *Enterococcus*, *Escherichia/Shigella*, *Izhakiella Janthinobacterium*, *Libanicoccus*, *Megamonas*, *Megasphaera*, *Neisseria*, *Pseudomonas, Sphingomonas*, and *Veillonella* were also observed in our study. Likewise, the importance and role played by these bacteria is unknown and deserves further attention.

Studies on honey bees exposed to agrochemicals have shown changes in the gut microbial communities (Dai et al., 2018; DeGrandi-Hoffman et al., 2017; Kakumanu et al., 2016). However, our results did not find evidence that exposure to spinosad and $CuSO_4$ affect the overall composition of the gut bacteria, except for *Gilliamella*. Such divergence is likely due to the different genetic backgrounds found between bee species, which may play an essential role in the response of bees when exposed to sublethal levels of agrochemicals.

We found that the differential abundances of *Gilliamella* increased in response to spinosad exposure. This finding is consistent with previous studies which suggest that this bacterial genus is susceptible to pesticides (DeGrandi-Hoffman et al., 2017) and antibiotics (Raymann et al., 2018). The changes in the abundance of *Gilliamella* might be related to its function within the hindgut. *Gilliamella* is a dominant gut bacterium in bees (Kwong et al., 2017), where it produces a biofilm on the ileum wall and may provide a barrier to attachment or entry of gut pathogens (Kwong and Moran, 2016; Martinson et al., 2012). Considering that *Gilliamella* may confer protective benefits, changes in the relative abundance of this taxon could disturb stingless bees, although the potential of *Gilliamella* in protecting individuals against xenobiotic is still unknown.

Our approach to assessing the toxicity of agrochemicals by next-generation sequencing opens new avenues to fill gaps regarding the interaction between agrochemicals and the bee microbiota. Understanding the factors affecting the health and survival of stingless bees is vital to agriculture, as well as to the sustainability of natural ecosystems. In summary, our results indicated that exposure to spinosad and $CuSO_4$ at their recommended field application

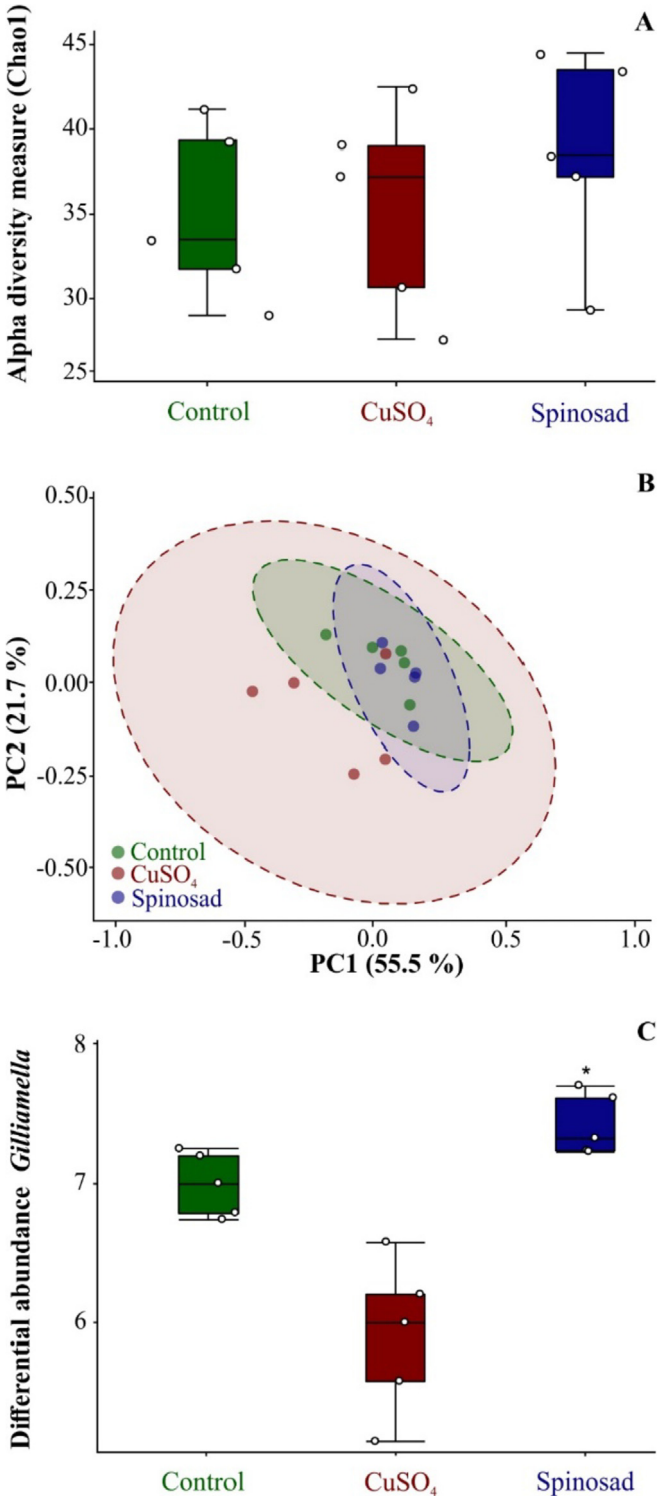

Fig. 5. Alpha and beta diversity of bacteria of the gut of foragers of *Partamona helleri* after the oral exposition (during 24 h) to spinosad or copper sulfate ($CuSO_4$). (A) Comparison of alpha-diversity (measured by Chao1) of the gut microbial community between exposed bees and control. (B) Comparison of beta-diversity by principal coordinate analysis using the average Bray-Curtis dissimilarity in gut communities between control and exposed bees. Spots with the same color indicate foragers that received the same treatment (n = 15). Dotted circles delineate the extent of each treatment. (C) Comparison of *Gilliamella* differential abundance on bees exposed to spinosad and $CuSO_4$ using the Bioconductor metagenomeSeq package. Box-and-whiskers plots show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles and asterisk indicate significant differences of *Gilliamella* differential abundance between control and exposed bees.

concentrations might cause high mortality on *P. helleri*. LC_5 exposure to spinosad and $CuSO_4$ did not alter behavioral patterns. However, $CuSO_4$ changed the respiration rate, and Cu accumulated in orally exposed bees. The sublethal concentrations of spinosad and $CuSO_4$ were not able to induce significant changes in gut bacterial communities of *P. helleri*. Studies investigating the effect of

chronic exposure on larvae are necessary to understand the impacts of both agrochemicals and gut microbiota on the health of stingless bees during the development process.

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