LORENA LISBETD BOTINA JOJOA

BEHAVIOR AND GUT MICROBIOME OF THE STINGLESS BEE *Partamona helleri* UNDER SUBLETHAL EXPOSURE TO A LEAF FERTILIZER AND A BIOINSECTICIDE

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título *Magister Scientiae*.

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APROVADA: 26 de julho de 2018.

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CONTENTS

ABSTRACT

JOJOA, Lorena Lisbetd, Botina, M.Sc., Universidade Federal de Viçosa, July, 2018. **Behavior and gut microbiome of the stingless bee *Partamona helleri* under sublethal exposure to a leaf fertilizer and a bioinsecticide.** Advisor: Gustavo Ferreira Martins. Co-advisor: Wagner Faria Barbosa.

The gut microbiome is related with nutritional health and immunocompetence of insects, besides is essential to neutralize impairs caused by pathogens or xenobiotics. Agrochemicals are regarded as one of the culprits often associated with the decline of bees, which occur by lethal and sublethal exposures during the foraging and, posteriorly with the food contamination within colony. In natural condition, is know that stingless bees have been submitted to chronic exposures by agrochemicals, which can compromise the gut microbiome and behavior in bees favoring for colony collapse, however, the effect of the leaf fertilizers and bioinsecticides remain neglected and are potentially important. Herein, we assess the sublethal effect of leaf fertilizer copper sulphate and bioinsecticide spinosad on forage bee behavior and gut microbiome composition of the stingless bee *Partamona helleri* (Friese), an important pollinator native in Neotropical region. Behavior bioassays and composition of gut microbiome were performed with forage bees orally exposed to LC_5 estimated from copper sulphate and spinosad. The characterizing of gut microbiota was through sequencing on illumina Miseq platform of the V4-V5 region of the bacterial 16S rRNA gene. The sublethal exposure to copper sulphate and spinosad did not affect in pattern on the overall activity, flight take-off and feed consumption. In contrast, copper sulphate decreases respiration rate and caused accumulation of copper in bodies of bees exposed. Neither copper sulphate nor spinosad altered the richness of the gut microbiome, but spinosad increased the differential abundance of the genus *Gilliamella*. In conclusion, agrochemicals considered safe for pollinators can exhibit high toxicity to stingless bees, revealing sublethal effects on physiology and changes in the differential abundance the gut microbiome of *P. helleri.* compromising the pollination services in Neotropical region that provide these pollinators, and therefore deserve further investigations.

RESUMO

JOJOA, Lorena Lisbetd, Botina, M.Sc., Universidade Federal de Viçosa, julho de 2018. **Comportamento e o microbioma intestinal da abelha sem ferrão *Partamona helleri* sob exposição subletal a um fertilizante foliar e um bioinseticida.** Orientador: Gustavo Ferreira Martins. Coorientador: Wagner Faria Barbosa.

O microbioma intestinal está relacionada a saúde nutricional e a imunocompetência dos insetos, além de ser essencial para neutralizar prejuízos causados por patógenos ou xenobioticos. Agroquímicos tem sido frequentemente associado ao declínio de abelhas, os quais ocorrem pela exposição letal e subletal durante o forrageamento e, posteriormente, com a contaminação do alimento dentro da colônia. Em condições naturais, sabe-se que abelhas sem-ferrão têm sido submetidas a exposições crônicas de agroquímicos, o que pode comprometer o microbioma intestinal e favorecer o colapso da colônia, no entanto, o efeito dos fertilizantes foliares é bioinseticidas nesse contexto é pouco explorado e são potencialmente importantes. Aqui, nós avaliamos o efeito subletal do fertilizante foliar sulfato de cobre e do bioinseticida espinosade na abelha sem-ferrão *Partamona helleri* (Friese), um importante polinizador nativo da região Neotropical. Os bioensaios comportamentais e a composição do microbioma intestinal foram realizados com abelhas forrageiras expostas oralmente à CL5 estimadas de sulfato de cobre e espinosade. A caracterização da microbiota foi através do sequenciamento na plataforma ilumina MiSeq das regiões V4-V5 do gene bacteriano 16S rRNA. A exposição subletal ao sulfato de cobre e ao espinosade não evidenciaram mudanças no padrão da atividade geral de grupo, decolagem para voo e consumo de alimento. Por outro lado, o sulfato de cobre diminuiu a taxa respiratória e causou um acúmulo de cobre no corpo das abelhas expostas. Nem o sulfato de cobre ou espinosade alteraram a riqueza do microbioma intestinal, mas o bioinseticida espinosade aumentou a abundância diferencial do gênero *Gilliamella*. Em conclusão, agroquímicos considerados seguros para os polinizadores podem exibir alta toxicidade para abelhas sem-ferrão, revelando mudanças sobre a fisiologia e induzindo mudanças na abundância relativa diferencial do microbioma intestinal de *P. helleri*. comprometendo os serviços de polinização nas regiões neotropicais desses polinizadores, portanto, merece futuras avaliações.

1. INTRODUCTION

Pollination services provided by native bees are essential for many crops, specially for plants cultivated in the tropics. Furthermore, native bees favor and maintain native plant biodiversity (Roubik, 2014; Valk and Koomen, 2012). Since stingless bees are predominant native pollinators in neotropical ecosystems are key pollinators in the region, together with the exotic honey bees (Barbosa et al., 2015b; Giannini et al., 2015; Valk and Koomen, 2012). However, there is increasing evidence that native bees, including stingless bees, are in decline in many regions of the globe (Potts et al., 2010), but it has received little attention in the risk assessment between pollinators (Barbosa et al., 2015b; Lima et al., 2016).

The decline of stingless bee colonies over the last decade has been attributed to several factors, among which are deforestation, habitat fragmentation and intensive agriculture production (Freitas et al., 2009; Roubik, 2014). In fact, accumulating evidences suggest that the large-scale application of agrochemicals has played a critical role in the increased rate of colony loss (Johnson, 2015; Lima et al., 2016; Valk and Koomen, 2012). Many lethal and sublethal effects of agrochemicals have already been reported in bees, although most studies are limited to exotic honey bees and convectional insecticide (Johnson, 2015; Potts et al., 2010; Tomé et al., 2017). Moreover, Stingless bees exhibit higher susceptibility in comparison with *A. mellifera*, representing more suitable models for risk assessments in neotropical ecosystems (Tomé et al., 2017). In this regard, understanding the possible negative impact of agrochemicals on stingless bees should not be neglected (Lima et al., 2016; Roubik, 2014).

Surprisingly, recent studies revealed that agrochemicals regarded as safe for bees such as fungicides, fertilizers, herbicides and biopesticides indicate that some of them exhibit high lethal toxicity to stingless bees (Barbosa et al., 2015b; Rodrigues et al., 2016; Tomé et al., 2017). In field conditions however, bees are exposed to lower doses of these agrochemicals than those reported in lethal assessments, withem the sublethal range which may affect the cognitive function, behavior, and physiology of stingless bees (Barbosa et al., 2015c; Bernardes et al., 2018; Rodrigues et al., 2016; Tomé et al., 2015a, 2017). Agrochemicals such as fungicides and fertilizers are sources of heavy

metals and their repeated application can cause the build up of metals such as copper (Cu) in crop plants (He et al., 2005; Johnson, 2015). Cu acts as an essential trace element in plants and is able to accumulate in different plant tissues (Hladun et al., 2015), including pollen and nectar, which are subsequently harvested by bees and leading to their bioaccumulation in these insects. Therefore, even at low concentrations, or sublethal concentrations, these compound may prove harmful for the bees behavior and health (Di et al., 2016; Nikolić et al., 2016).

High acute mortality by agrochemicals in bees depend on the route of exposure and mode of action (Johnson, 2015). Leaf fertilizers are a sources of heavy metal input into agricultural systems, including bioelements such as copper (Cu), iron (Fe), zinc (Zn), manganese (Mn), cobalt (Co) and selenium (Se) that can be detrimental for bees (He et al., 2005; Hladun et al., 2016). Their toxicity is revealed in several ways: they can inactivate many enzymes, replace essential metal ions in biomolecules which leads to inhibition or loss of function, and disrupt redox homeostasis in the cell impairing vital all activities (Tchounwou et al., 2012). Indeed, laboratory and field studies indicate that chronic exposure to metals such as Cu, even at levels below what is currently considered toxic, have a major impact on survival, physiology and behavior of bees (Di et al., 2016; Hladun et al., 2016; Nikolić et al., 2016; Rodrigues et al., 2016).

Spinosad is a bioinsecticide based on spinosyns, which are fermentation products of the actinomycete *Saccharopolyspora spinosa* (Mertz and Yao) (Actinomycetales: Pseudonocardiaceae). Spinosad is more effective when ingested, but it can also affect insects through contact and exhibit some systemic action (Biondi et al., 2012). The spinosyns cause hyperexcitation, and ultimately disruption of the insect central nervous system, acting 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). In bees these toxins can impair nicotinic synaptic transmission and functions related to the mushroom bodies, harming multisensory integration, learning, and memory formation (Menzel, 2012). Sublethal doses of spinosad compromises normal larval development and cause behavioral disturbances on *Melipona quadrifasciata* (Barbosa et al., 2015c; Tomé et al., 2015a).

Several studies revealed the close relationship between the gut microbiota and the health of bees, in addition to augmented resilience to environmental stressors, where colony success in influenced by their microbial community (Dillon and Dillon, 2004; Engel et al., 2012; Raymann and Moran, 2018). Bees exhibit a distinctive gut community which is highly conserved despite the environment, geography, and host subspecies (Cox-Foster et al., 2007; Kwong et al., 2017b; Martinson et al., 2011). Furthermore, the gut microbiome appears to span a much greater range of possible states, exhibiting highly variable in composition, richness, and evenness among honey, bumble, and stingless bees (Kwong et al., 2017b).

In bees, the gut microbiota plays an important role for the metabolism, hormonal signaling, behavior, gut physicochemical conditions, growth and development, protection against pathogens and immune response (Engel et al., 2012; Kešnerová et al., 2017; Kwong et al., 2017a; Raymann and Moran, 2018; Zheng et al., 2017). Gut bacterial communities of adult stingless bees are dominated by five genera. The bacteria in these communities, particularly members of the *Snodgrassella*, *Gilliamella*, *Lactobacillus*, *Firmicutes* and *Bifidobacterium* (Díaz et al., 2017; Kwong et al., 2017b) and their abundance can shift according to age, season and food sources (Anderson et al., 2016; Corby-Harris et al., 2014; Hroncova et al., 2015; Ludvigsen et al., 2015). Nonetheless, the core members of the gut microbiota are highly conserved (Kwong et al., 2017b), and important for both nutrition and defense against xenobiotics (Raymann and Moran, 2018). This is so because the gut microbiota modulates the nutritional status across pollen and nectar digestion, providing energy and enhancing immunocompetence against invading pathogens, subsequently, improving the colony ability to defend against environmental stress (Koch et al., 2012; Mockler et al., 2018; Zheng et al., 2016).

Several studies suggest that alteration on gut microbiota leads to increased susceptibility to pathogen infection and increases insect mortality of individuals in nest (Cariveau et al., 2014; Hamdi et al., 2011; Mockler et al., 2018; Raymann and Moran, 2018). A metagenomic analysis of honey bees identified an alteration in the composition of the gut microflora between hives exhibiting colony collapse disorder (CCD) and normal hives (Cox-Foster et al., 2007); which provides support to the

notion that symbiotic bacterial communities can provide functional benefits to bees and colony health.

In this context, the aim of the present study was to assess the sublethal effects of the leaf fertilizer copper sulphate and the bioinsecticide spinosad on behavior and gut microbiome composition of the stingless bee *Partamona helleri*. Moreover, we measured copper (Cu) and sulfur (S) concentrations in the individual bee bodies after exposure to copper sulphate.

2. MATERIALS AND METHODS

2.1. Insects and agrochemicals

Five colonies of the stingless bee *P. helleri* were collected from the rural area of Viçosa county (State of Minas Gerais, Brazil; 20∘ 45′ S and 42∘ 52′ W) and maintained in the campus of Universidade Federal de Viçosa (UFV). Foragers were collected at the hive entrance from each colony using glass jars. The foraging bees obtained in the jars were anesthetized with exposure to carbon dioxide for 5 s and 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% of relative humidity (RH) until starting the bioassays. The fasting period before pesticide exposure was necessary to acclimatize the bees to experimental conditions and force them to feed on the diet in oral exposure tests (Tomé et al., 2015b).

Two commercial formulations of agrochemicals commonly used in Brazilian tomato crops were used, as follows: the bioinseticida spinosad (Tracer; suspension concentrate; 480 g/liter, Dow AgroSciences, Santo Amaro, SP, Brazil) and the leaf fertilizer copper sulfate (Sulfato de Cobre Penta 24; a salt formulation containing 240 g/Kg Cu and 110 g/Kg S; Multitécnica Industrial, Sete Lagoas, MG, Brazil) (MAPA, 2017). Decreasing concentrations of the compounds were obtained based on the maximum recommended label rates of each agrochemical (spinosad: 204 µg a.i./mL; CuSo4: 5000 µg a.i./mL) and were prepared by diluting them in a 50% (w/w) sucrose solution using deionized and distilled water.

2.2. Concentration-mortality bioassays

Foraging bees were subjected to concentration-mortality bioassays with a series of concentrations below the maximum recommended field rates of each agrochemical. The compounds were diluted in sucrose solution (50%, v/v) and offered to bees during 24 h (chronically exposure) using drilled 2-mL microtubes (Eppendorf, São Paulo, SP, Brazil) inserted through a hole into the 500-mL polyethylene containers (Tomé et al., 2015b). Food consumption was recorded at the beginning and end of each bioassay, as described in the next section. 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) and ten concentrations of copper sulfate (100, 120, 140, 160, 200, 250, 333, 500, 1000 and 5000 µg a.i./mL). Each colony comprised a biological replicate for each concentration, i.e., five replicates were used per concentration. Exposure to uncontaminated sucrose solution (control treatment) was used to assess natural mortality and subsequently correct the mortality data. Mortality was recorded after 24 h of exposure and insects were counted as dead if they were unable to walk the length of their body. 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 microbiome of the bees.

2.3. Feeding behavior bioassay

The average food consumption per foraging bee was estimated by weighting the food microtubes at the beginning and end of the oral exposure on analytical balance (Rodrigues et al., 2016). Plastic containers with food microtubes and without bees were maintained at the same experimental conditions to estimate the weight loss of the diet by evaporation, which was subsequently used to correct the diet consumption.

2.4. Overall activity bioassay

The overall group activity of *P. helleri* foragers were performed 24 h after acute oral exposure to the estimated lethal concentrations (LC_5) for spinosad and copper sulfate, and the non-exposed bees, as previously described. Exposed foragers were anesthetized as previously described and five insects were subsequently placed into

glass Petri dishes arenas (9 cm diameter and 2 cm high). The petri dish bottoms were covered with a filter paper (porosity of 3 microns, 0.5% ash content, 9 cm diameter, 80 g/m2 density; Nalgon Equipamentos Científicos Ltda, Itupeva, SP, Brazil) and the inner walls were coated with Teflon TPFE® (DuPont, Wilmington, DE, USA). After five minutes of acclimation, the movement of the insect group 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) (Tome et al., 2015a; Tomé et al., 2015b). Each Petri dish containing five insects comprised the experimental unit and each treatment (i.e. LC_5 spinosad, LC_5 copper sulphate or control) was recorded for three experimental units per colony. The bioassays were carried out in a room with artificial fluorescent light at 25 ± 2ºC and 70 ± 5% relative humidity.

2.5. Flight take-off behavior bioassay

The bees previously subjected to the group activity bioassay were subsequently subjected to a flight take-off bioassay (1 h after the previous bioassay or 25 h after the exposure period). Briefly, a 105-cm tower was formed with three stacked wooden cages (35 × 35 × 35 cm each) opened in their interior to allow free insect flight through 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 explore the vertical bee flight towards the light source after the insect release from the center 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 in the previous bioassay of overall activity. Bees were released after 1 min after placing the petri dishes the center at bottom of the tower; this procedure allowed a brief acclimation of the bees to the new experimental conditions. The vertical flight take-off was recorded for 1 min and the time for each bee to reach the light source was recorded by using a stopwatch. Bees that did not reach the light source within 1 min were treated as censored data.

2.6. Respirometry bioassay

Respirometry bioassays were carried out with a new batch of 24 h-treated bees explored to the LC_5 of either agrochemical, or control. The treated bees were

individually placed in a 25 mL plastic chambers in a completely closed system (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. The air stream was directed to an infrared reader connected to the system allowing the determination of the CO_2 produced per each bee. The residual CO_2 from the purified air was also determined in three chambers without insects during the experiment to correct the final quantity of CO_2 produced (Tomé et al., 2015a). All treatments were performed at the same time for each colony and four bees were used from each of the five colonies (i.e., twenty bees per treatment)

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

The quantification of Cu and S in the bee bodies was performed by the method of nitro-perchloric digestion (Sarruge and Haag, 1974). Groups of fifty bees from each colony (i.e., 250 insects) from each treatment were kept in 100% alcohol in centrifuge tubes (15 mL) and stored at room temperature until further use. The bee samples were subsequently dried for 24 h at 120 °C, weighed and destructed by boiling the sample at 200 °C (Heater Plates, Nova Ética modelo 208/D, Brasil) in a mixture of 10 mL HNO_3 and HCl at a ratio of 4:1. The volume was completed to 25 mL with deionized water. Copper quantification was determined by atomic absorption spectroscopy (Varian modelo SpectrAA 220FS, Australia) and quantification of sulfate was determined by UV-visible spectrophotometry (420 nm; FEMTO 600S, Brasil).

2.8. DNA extraction and 16S rRNA gene sequencing

Total bacterial 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 again the LC_5 of each agrochemical as well as the control (i.e., untreated sucrose solution). Before DNA extraction, each bee was submitted to surface disinfestation individually surface-sterilized by rinsing them in an increasing ethanolic series (70, 80 and 90 % v/v of ethanol) for 30 s each, followed by multiple washes in sterile phosphate buffer saline (PBS) and subsequently in 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 manufacturer's instructions. DNA concentration was determined using Qubit 2.0 fluorometer and dsDNA BR Assay kit (Invitrogen, Carlsbad, CA, USA). The DNA samples were stored at −20 °C until further use.

The quality of the extracted bacterial DNA samples was checked by amplifying 16S gene using the 5F (TGGAGAGTTTGATCCTGGCTCA) and 1513R (TACIGITACCTTGTTACGACTT) primers (Weisburg et al., 1991). Briefly, bacterial DNA was amplified in a 25 μL reaction mix (GoTaq® DNA Polymerase, Promega Corporation, USA), 0.4 mM of 5F primer and 1513R primers each, 2 μl template DNA and 1.25 U of Taq DNA polymerase, 0.2 mM dNTP, 1X (1.5 mM $MgCl_2$) of 5X Green GoTaq® Reaction Buffer and 16.375 μL free water. The PCR was performed under following conditions: 5 min of initial denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 53 °C for 1 min, and extension at 72 °C for 1,5 min with a final extension at 72 °C for 5 min. Negative controls without DNA were included in all amplifications. The PCR products were evaluated by electrophoresis in a 1.2 % agarose gel with 1 X TAE buffer. PCR amplicons were stored at -20 °C. DNA samples kept in the freezer at -20 °C were stored inside ultrafreezer (Bio memory 515L, ETHIK) at -80 °C for 24h and then lyophilized in Lyophilizer L101 (Liotop) for 24h.

The lyophilized DNA was sent to be sequenced at the Argonne National Laboratory (Illinoi, 31USA). Bacterial and archaeal 16S rRNA genes (V4 – V5 region) were amplified using primers 515F (GTGCCAGCMGCCGCGGTAA)(Caporaso et al., 2011) and 926R (CCGYCAATTYMTTTRAGTTT) (Parada et al., 2016) for paired-end sequenced on the Illumina MiSeq platform at the High-throughput Genome Analysis Core (HGAC) (Walters et al., 2016).

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 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 using VSEARCH ver. 2.3.4 (Rognes et al., 2016) and QIIME ver. 1.9.1 default reference database (SILVA 123) (Caporaso et al., 2010), respectively. Sequences with more than 97% similarity were considered the some operational taxonomic units (OTU) and performed following the UPARSE method at 97% similarity cutoff (Edgar, 2013), as recommended by the Brazilian Microbiome Project (Pylro et al., 2014) using the BMP Operating System (BMPOS) (Pylro et al., 2016). 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 measured using the alpha diversity metric Chao1 (Chao, 1984). Relative abundances (betadiversity) of 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

The probit model was suitable to the data 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). Therefore, bee mortality increased with the increase of both spinosad and copper sulphate concentrations. The LC_5 and LC_{50} estimates were

respectively 0.81 and 2.89 µg a.i./mL for spinosad and 120 and 362.60 µg a.i./mL for copper sulfate (Fig. 1). Concentrations corresponding to the maximum label rate of both agrochemicals caused 100% mortality within 24 h of exposure.

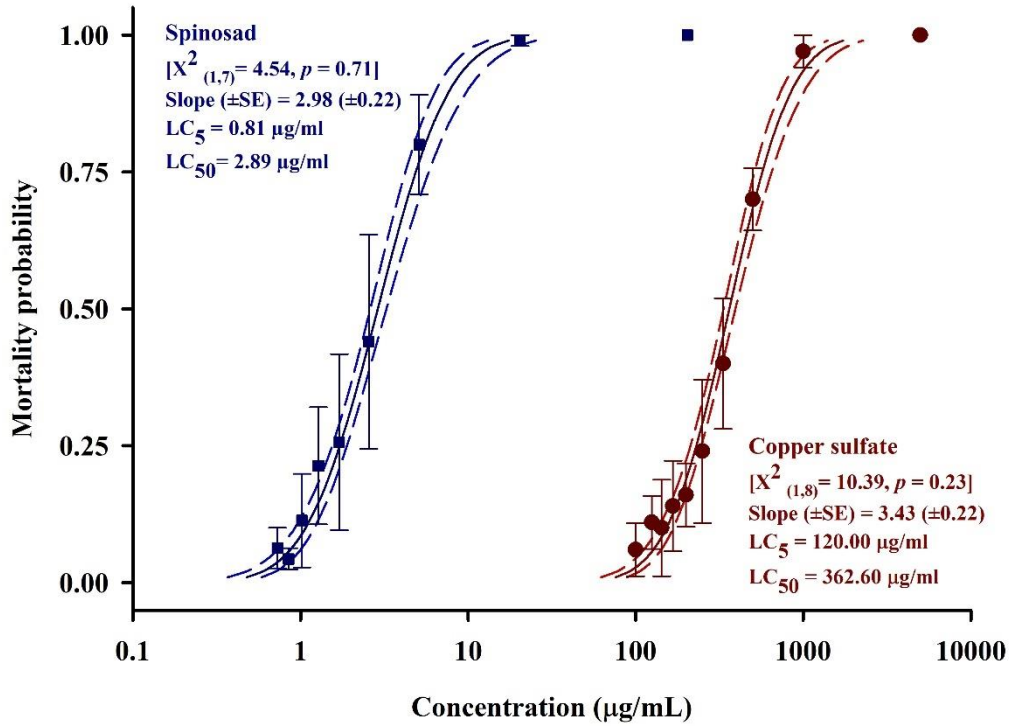

Figure 1. Concentration–mortality curves of foragers of the stingless bee *Partamona helleri* orally exposed to spinosad or copper sulfate. LC_5 and LC_{50} values are indicated and dotted lines represent the 95% fiducial limits of each curve. Vertical bars represent standard errors (SE).

3.2. Behavioral bioassays

The overall walking activity (Δ pixel × 100^{-2} s) of the foraging bees did not exhibit significant differences among spinosad (295.74 ± 9.86), copper sulfate (285.32 ± 14.75) or control (269.90 ± 24.07) ($F_{6,38} = 0.63$; $P = 0.54$). Similarly, the time (s) of the foragers to reach the light source was not significantly different among spinosad (20.01 ± 2.36), copper sulfate (16.36 ± 2.04) or control (18.45 ± 2.42) ($\chi^2 = 0.97$; df = 2; $P = 0.61$). Food consumption (mg) did not vary either spinosad (0.434 ± 0.10), copper sulfate (0.470 ±0.11) or control (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 foraging bees was significantly impaired by the agrochemicals ($F_{2,52}$ = 3.60; P = 0.03). Copper sulfate (130.074 ± 32.99) significantly reduced the respiration rate of the bees compared to the control diet (196.004 ± 33.25) (P = 0.02). In contrast, spinosad (166.629 ± 32.99) did not significantly affect this parameter (P = 0.45) (Fig. 2).

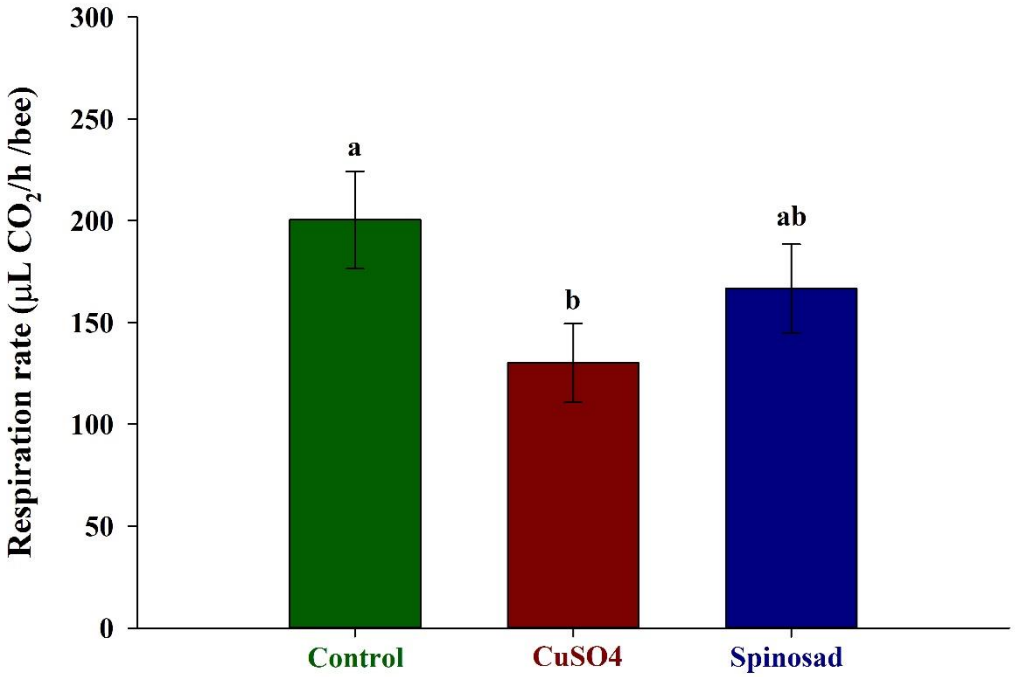

Figure 2. Respiration rates (± SE) of foraging workers of the stingless bee *Partamona helleri* orally exposed to spinosad or copper sulfate. Different letters indicate significant differences among treatments based on Tukey's HSD test (P = < 0.05).

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

Foragers accumulated significantly more copper (Cu) when they were exposed to $CuSO_4$-contaminated sucrose solution (22.15 µg/g) than the control treatment (9.40

µg/g) (U= 1; *P* = 0.01). Nonetheless, the sulfur (S) content had similar values between control (5.3 mg/g) and $CuSO_4$-treated bees (5.5 mg/g) (U= 7.5; *P* = 0.31).

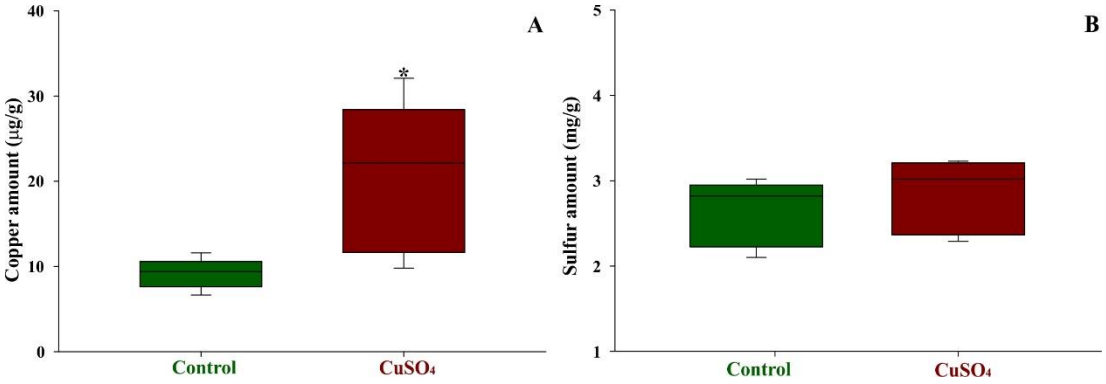

Figure 3. Copper and sulfate content (± SE) in forager's bodies of *Partamona helleri* orally exposed to copper sulfate: (A) Copper quantification, (B) Sulfur quantification. Asterisks indicate significant differences by the Mann-Whitney test ($P < 0.05$).

3.5. Bacterial 16S rRNA Gene Sequences analysis

The community composition of the gut microbiome was accessed using deep ~400 bp amplicon sequencing of the bacterial 16S rRNA V4 – V5 gene produced a total of 2933 reads of 15 samples, on averages of reads per treatment were 195.41 $CuSO_4$; 194.57 Spinosad and 196 control with coverage of 99%. The gut symbiont of *P. helleri* were identified in 33 genera in accordance with OTUs (Fig. 4). The main bacterial OTUs identified here are phylogenetically related to other ones found in the gut microbiome of corbiculate bees (Kwong et al., 2017b; Kwong and Moran, 2016). According to the taxonomic assignment, gut microbiome was dominated by seven genera based on 16S rRNA gene sequence similarities: *Apibacter*, *Commensalibacter*, *Gilliamella*, *Izhakiella*, *Lactobacillus*, *Pseudomonas* and *Snodgrosella*.

The bacterial diversity and composition were consistent regardless of the treatment (Fig 5). Spinosad- and copper sulfate treatments did not elicit any change in the microbiota diversity. Chao1 Alpha diversity (richness) measured for *P. helleri* did not differ between treatments and control (F= 0.60; *P* = 0.58) (Fig 5A). Beta diversity analysis was similar among control and exposed bees ($R^2 = 0.21$; *P* = < 0.1) (Fig. 5B). Furthermore, the gut symbionts diversity between treated and control bees displayed

similar clustering patterns based on principal coordinate analysis (PERMANOVA) (Fig. 5B). In contrast, differential abundance analysis indicated a significant increase of *Gilliamella* in spinosad-treated bees compared to control and copper sulfate treatments (FDR= 0.029) (Fig. 5C).

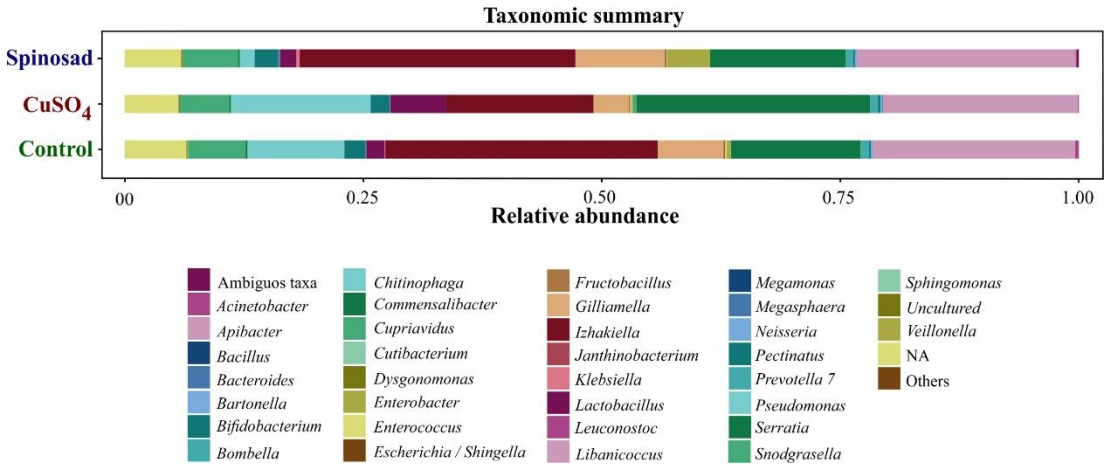

Figure 4. Relative abundance of bacterial taxa (genera) found in the gut of foraging workers of the stingless bee *Partamona helleri* orally exposed to spinosad or copper sulfate. Each column in the graph is the average (n = 75) of the percentage abundance of each taxa in each treatment.

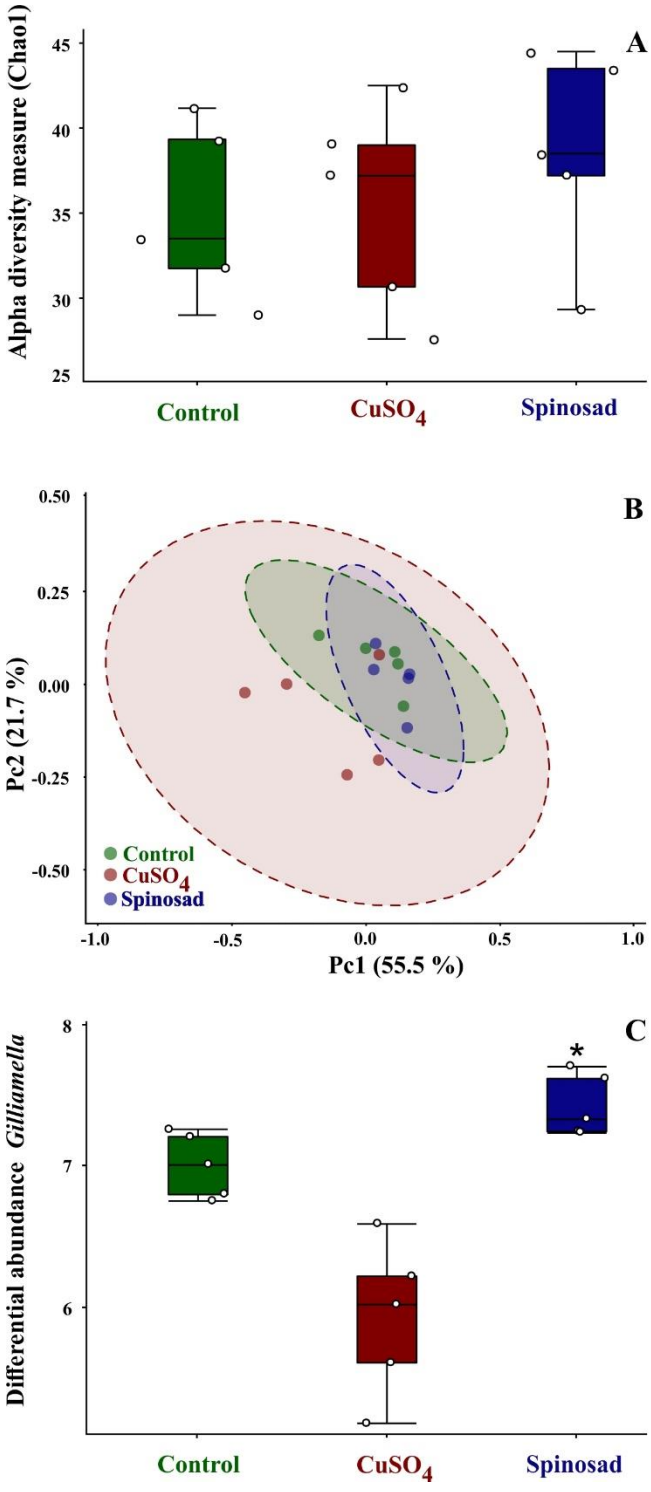

Figure 5. Alpha and beta diversity of bacteria of the gut of foraging workers of the stingless bee *Partamona helleri* orally exposed to spinosad or copper sulfate. (A) Comparison of alpha-diversity (measured by observed OTUs) of the gut microbial community among treatment and control. Relative abundance of bacterial genera (n=15). (B) Principal coordinate analysis using the average Bray-Curtis dissimilarity in gut communities among control bees and exposed bees. Circles with the same color indicate foragers that received the same treatment. Dotted circles delineate the extent of each treatment. (C) Comparison of *Gilliamella* differential abundance on bees

exposed to spinosad and copper sulfate using the bioconductor metagenomeSeq. 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.

4. DISCUSSION

The mortality of foraging bees of *P. helleri* increased with increasing concentrations of both bioinsecticide (spinosad) and leaf fertilizer (copper sulphate). In addition, LC_{50} values were much lower than recommended concentrations for their respective agrochemicals (spinosad: 70.6x; copper sulphate: 13.8x); hence spinosad was also more toxic than copper sulphate. These results reinforce that agrochemicals considered safe for pollinators can also exhibit high toxicity to stingless bees, especially regarding spinosad, which was already recognized as harmful to Meliponi bees (Barbosa et al., 2015b, 2015c; Rodrigues et al., 2016; Tomé et al., 2015b). Nonetheless, although mortality curves and LC_{50} values are frequently used to assess the susceptibility of living organisms to xenobiotics, such estimates are deficient because sublethal effect frequently occur; this is so because in practice the dose initially applied is subjected to environmental degradation and the exposure to sublethal concentrations are extended (Guedes et al., 2017). Therefore, we also assessed possible sublethal effects on important behavioral and physiological activities, as well as the gut bacterial composition. The latter is also very important to the use of nutrients and secondarily to the immunocompetence of bees (Cariveau et al., 2014; DeGrandi-Hoffman et al., 2010; Engel et al., 2012; Evans and Armstrong, 2006; Zheng et al., 2016, 2017)

Behavioral responses are integrated to environment-insect interactions. Thus, these are important traits that can exhibit high sensitivity to environmental perturbations (Guedes et al., 2016). Previous laboratory studies with spinosad reported negative impacts on the flight performance, respiration rate, locomotion and orientation of workers of either honeybees, bumblebees or stingless bees (Barbosa et al., 2015a, 2015c; Lopes et al., 2018; Tomé et al., 2015b). A recent report also pointed out altered respiration rate, flight and feeding activities on stingless bees of *Friesella schrottkyi* caused by copper sulfate-based leaf fertilizer (Rodrigues et al., 2016). Heavy

metals are known to have negative effects on different organisms, including vertebrates and invertebrates, which may reflect in reduced survival and altered ingestion, locomotion, reproduction, behavior and physiology (Di et al., 2016; Mogren and Trumble, 2010; Moroń et al., 2013). Nevertheless, behavioral impairments were not evident in our study for any of the treatments (either spinosad or copper sulphate), although copper sulfate-exposed bees showed reduced respiration rate. This physiological stress caused by the exposure to the leaf fertilizer indicated that the level of the impairment on this biological parameter was not able to reflect on the behavioral changes assessed. Therefore, further assessments on other biological parameters that are complementary or linked to labor activities of this stingless bee species are needed. The divergence in the response to sublethal concentrations between spinosad and copper sulfate are 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).

Many studies have considered the use of bees as bioindicator of environmental pollution, especially the one caused by heavy metals (Giglio et al., 2017; Nikolić et al., 2016). Bioaccumulation of metals may occur through feeding on contaminated food as heavy metal and other elements intake in bees takes place via ingestion of contaminated pollen and/or nectar is relatively common (Johnson, 2015). Our results indicated that significant quantities of Cu were bioaccumulated in foragers' bodies orally exposed to $CuSO_4$. Untreated forager bees also showed low concentration of Cu and S. All the concentration of Cu measured in our study fall within the range of values reported in literature for honey bees (11.65 – 24.5 µg/g Cu) (Di et al., 2016; Giglio et al., 2017; Roman, 2010). The levels of toxic metals for bees depend on the kind and form of chemical compounds in which the elements occur (Roman, 2010). The fluctuations in concentration indicated a significant variation in exposure of bees to these metals in the environment. Some stingless bees species, such as *P. helleri*, exhibit generalist habits and can adapt well to urban ecosystems (Brown and de Oliveira, 2014). Thus, *P. helleri* is potentially useful as bioindicators for detecting and monitoring environmental pollution because they are able to bioaccumulate heavy metals.

The OTUs identified in our study are phylogenetically and taxonomyc reported in diverse corbiculate species, including honey (*Apis*), bumble (*Bombus*) and stingless bees (Tribe Meliponini) and are distinct strains of five genera of the core gut bacteria, which includes: *Snodgrassella*, *Gilliamella*, two of *Lactobacillus*, and a *Bifidobacterium* genera; all ubiquitous and found mainly in adult workers worldwide (Díaz et al., 2017; Koch et al., 2013; Kwong and Moran, 2016; Leonhardt and Kaltenpoth, 2014). Multiple studies have correlated the lack of core gut bacteria, or abundance of non-core bacteria to high susceptibility of bees to pathogens, impaired hormonal signaling and shifted gut physicochemical conditions (Kešnerová et al., 2017; Zheng et al., 2017). Honeybees and bumblebees with high diversity of non-core bacterial species showed higher prevalence of the pathogens *Crithidia* and *Nosema* (Cariveau et al., 2014; Koch et al., 2012; Zheng et al., 2017). The bacterial gut community observed here was similar to those characterized in previous studies. Screens of the *P. helleri* genome-sequence database revealed sequences corresponding to several of these bacterial groups, indicating that they are probably part of the normal gut microbiota, thus supporting the development and maintenance of specialized microbiome in social bees.

Previous studies in honey bees documented that the gut microbiome was altered by xenobiotics affecting host health (Motta et al., 2018; Raymann and Moran, 2018). Therefore, our results suggested that copper sulfate and spinosad did not affect significantly composition of the gut bacterial. However, the differential abundances of *Gilliamella* increased with spinosad exposure. This result is consistent with previous assessments in relative abundance level of *Gilliamella* on honeybees exhibited increased significantly after antibiotics treatments (Raymann et al., 2017b). Nevertheless, bees exposed to fungicide did not exhibit shift in the relative abundance of the genera *Gilliamella* between pesticidal and control treatments (Kakumanu et al., 2016). Moreover, pathogen infection may reduce the abundance of this bacterial genus in *Bombus terrestris* (Mockler et al., 2018). In honey bees, *Gilliamella* 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). Strains of *Gilliamella* are dominant gut bacterium in bees (Kwong et al., 2017b). *Gilliamella* may also confer protective benefits and so changes in the relative abundance of these taxa could disrupt stingless bee metabolism, although the importance of its increase is still unknown.

Nonetheless, because the increase of the differential abundance of *Gilliamella* may represent an unbalance of the symbiotic equilibrium, we hypothesized that the metabolism of bees can be also affected.

The presence of *Gilliamella*, *Lactobacillus*, *Bacillus* and *Bifidobacterium* bacteria at *P. helleri* can contribute with benefits in this species, because these bacteria are known to be beneficial gut symbionts in honey bees, and they contribute to digestion of complex carbohydrates, enabling the enzymatically break down and fermentation of sugars found in pollen, honey, and nectar (Engel et al., 2012; Kwong et al., 2014; Lee et al., 2015; Zheng et al., 2016). Our results suggest that the presence of these gut symbionts across different treatments could provide the host with advantages in nutrition and health, providing some resistance to sublethal concentration of the tested agrochemicals. Interestingly, *Bacillus* strains may prevent the growth of two major honey bee pathogens such as *Paenibacillus larvae* and *Ascosphaera apis* (Koch and Schmid-Hempel, 2011; Sabaté et al., 2009). Likewise, acids produced by sugar-fermentation of symbionts that can be highly beneficial for host energy metabolism (Lee et al., 2015), also confers pH-mediated resistance to infection in bees, whereas depletion of core microbiota results in low-acid conditions that favor pathogen growth (Palmer-Young et al., 2018). In contrast, bees exposed to antibiotic and agrochemicals such as chlorothalonil (fungicide) exhibited changes on the diversity of the bacterial composition, increasing the mortality and susceptibility to *Nosema* infection (Kakumanu et al., 2016; Li et al., 2017; Raymann et al., 2017b, 2017a). This disruption of gut bacteria by antibiotic could cause the inhibition of honeybee immunity response by down-regulation of the expression of genes encoding antimicrobial peptides (Li et al., 2017). Therefore, certain pesticides and antibiotics have the potential to impact the bee's gut microbiome and its function, impairing host health and thus require further assessments.

Some of the rarer bacterial genera found in gut at *P. helleri* have been reported to be opportunistic organisms in honeybee guts including species belonging to the genera *Enterobacter*, *Klebsiella*, and *Serratia* (Engel et al., 2012). These genera were also found in our samples *Serratia* strains, for instance, can be pathogenic and cause sepsis and death (Burritt et al., 2016). Other groups of bacteria belonging to the genera *Pectinatus*, *Bartonella, Leuconostoc*, *Apibacter* were reported in previous studies of

Meliponini microbiota (Kwong et al., 2017b) and *Bombela*, *Commensalibacter*, *Fructobacillus* were reported in Bumblebee (Jessy et al., 2017). In addition, the genera *Bacteroides*, *Acinetobacter*, *Chitinophaga*, *Cupriavidus*, *cutibacterrium*, *Dysgonomas*, *Enterococcus*, *Escherichia/Shigella*, *Izhakiellam*, *Janthinobacterium*, *Libanicoccus*, *Megamonas*, *Megasphaera*, *Neisseria*, *Pseudomonas, Shingomonas* and *Veillonella* were also observed in our study, although the importance and the role that these bacteria play in bees are unknown.

Understanding the factors affecting the health of stingless bees is vital to agriculture, as well as to the sustainability of natural ecosystems. In summary, as expected, our results indicated that exposure to copper sulfate and spinosad at their respective label concentrations may cause high mortality on *P. helleri*. In addition, LC_5 exposure to copper sulphate and spinosad did not altered behavioral patterns. However, copper sulphate altered physiological patterns as respiration rate and caused bioaccumulate of copper in bodies of bees exposed. Copper sulfate and spinosad did not interfere in the diversity of the gut microbiome of *P. helleri* species, but spinosad showed potential for induce changes on differential abundance of bacterial genera. It is possible that these agrochemicals affect early colonization of the microbiota by interfering in the ability of bees to regulate bacterial populations, since the bacterial diversity increases as the bee ages through larval stages to newly emerged worker and old worker (Martinson et al., 2012). Furthermore, it is possible that other bee species with different genetic backgrounds or gut bacterial compositions across different developmental stages have different responses to agrochemicals. Thus, this knowledge gap deserves further attention in future inquiries

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