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MEASURING THE CHANGES IN *APIS MELLIFERA* GUT MICROBIOME COMPOSITION AFTER PESTICIDE EXPOSURE

Thesis of the International Master's in Applied Ecology supervised by Doctor Luís Cunha and Doctor Diogo Neves Proença and presented to the Department of Life Sciences of the Faculty of Sciences and Technologies of the University of Coimbra.

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Abstract

The western honey bee, *Apis mellifera*, harbors a large bacterial community in the gut, with roughly 1 billion bacterial cells in a mature worker. This gut microbiota composition is known to host a unique set of distinct bacterial components, which is similar across the globe and even between different honey bee species. The microbiome is dominated by eight bacterial species that promote weight gain and reduce pathogen susceptibility by breaking down toxic substances. Some of these bacteria, such as the members of *Lactobacillus* and *Bifidobacillus* genus, have been linked to play a crucial role in the immunity and defence against pathogens.

As beneficial bacteria, they were hypothesized to be crucial groups that are affected by pesticides. Furthermore, pesticides are known to affect pollinators' health near agricultural sites. In order to reduce the toxic load and impact, a new generation of biological pesticides, believed to be more eco-friendly, is currently being developed. The main objective of this study was to evaluate the effect of pesticide exposure on the gut microbiome of adult individuals of the native subspecies of honey bees *Apis mellifera* ssp. *iIberiensis* (Engel, 1999), testing a fungicide and an insecticide widely used in Portugal., It was hypothesized that sublethal concentrations of these pesticides would affect the diversity between taxa and the overall abundance of different bacterial groups, in particular the ones known to be beneficial to the bee's health. The experiment was conducted with a 7-day chronic feeding test to evaluate the effects of different sublethal concentrations of a pyrethroid insecticide named Cypermethrin (formulation: Sherpa® 100 EC)) and a well-studied fungicide, Difenoconazole (formulation: Cérimonia® 250 EC) and compared their effects on bee survival and gut-associated microbial composition. The research was done in the facilities of the University of Coimbra. Illumina DNA sequencing technique and subsequent metabarcoding were used to calculate and define the changes in major bacterial taxa. Data analysis and significance tests were performed using the *DADA2, phyloseq* and *vegan* packages in the RStudio software.

As expected, mortality was not affected by pesticide intake since sublethal concentrations were used. The discovered core microbiome of local honey bees was consistent with the existing literature and included 38 species. Overall richness and abundance of species increased significantly in bees treated with Concentration 3 (1/25 of LC50) of both Difenoconazole and Cypermethrin, showing the disruption of gut community structure. Overall, the core microbial community showed resilience while exposed to the lower doses in this study (Concentration 1 and 2) for both pesticides. Most of the significant differences were obtained while comparing bees in the control treatment with Concentration 3, which indicates that doses below the 1/25 of LC50 are

too low to cause a noticeable shift in the honey bee microbiome. In Cypermethrin treated bees, results showed that an increase in pesticide dose causes a sharp decline in *Raoultella* (formerly known as *Klebsiella*). In Cypermethrin treated bees, results showed that an increase in pesticide dose causes a sharp decline in *Raoultella* (formerly known as *Klebsiella*). Difenoconazole led to a shift in the microbial community, namely in such beneficial bacteria as *Shodgrassela* and *Bombilactobacillus* spp. Our results support the need for further investigating the influence of pesticides on the Honey bee microbiome and promoting sustainable beekeeping.

Keywords: gut microbiota, difenoconazole, Cerimonia, honeybee, Cypermethrin, Sherpa, chronic oral exposure, bacterial composition.

Resumo

A abelha melífera, *Apis mellifera*, contém uma larga comunidade de bactérias no seu intestino, compreendendo cerca de 1 bilião de células bacterianas numa abelha adulta. A composição deste microbioma intestinal é constituída por um conjunto de diferentes componentes bacterianos, sendo esta semelhante ao longo do globo e ainda entre diferentes espécies de abelhas melíferas. O microbioma é dominado por oito espécies de bactérias que promovem o ganho de peso e reduzem a suscetibilidade a agentes patógeneos ao degradar substâncias tóxicas. Algumas destas bactérias, como por exemplo, espécies pertencentes ao género *Lactobacillus* e *Bifidobacillus*, têm um papel crucial na imunidade e na defesa contra patógeneos.

Como bactérias benéficas, elas foram tomadas como sendo grupos cruciais que são afetados por pesticidas. Além disso, pesticidas podem afetar a saúde dos polinizadores perto de zonas agrícolas. Com o intuito de reduzir a carga de toxicidade e o seu impacto, está a ser desenvolvida uma nova geração de pesticidas biológicos que se acredita serem mais amigos do ambiente. Estes podem ser de origem animal ou vegetal, mas os seus efeitos em insetos ainda não foram profundamente testados. O principal objetivo deste estudo foi avaliar o efeito da exposição de pesticida no microbioma intestinal de indivíduos adultos da espécie nativa de abelhas melíferas, *Apis mellifera* ssp. *iIberiensis* (Engel, 1999), utilizando um fungicida e um bio-inseticida largamente usado em Portugal. Gerou-se a hipótese de que concentrações subletais destes pesticidas iriam afetar a diversidade entre taxa e a abundância geral de os diferentes grupos de bactérias, particularmente aqueles conhecidos como sendo benéficos para a saúde da abelha. A experiência foi realizada com um teste de alimentação crónica de 7 dias de forma a avaliar os efeitos de diferentes concentrações subletais de biocida chamado Cypermethrin (formulação: Sherpa® 100 EC) e um fungicida bem estudado, Difenoconazole (formulação: Cérimonia® 250 EC) e ainda comparar os seus efeitos na mortalidade da abelha, o *uptake* do pesticida e a composição do microbioma intestinal., Este estudo foi realizado nas instalações da Universidade de Coimbra. A técnica de sequenciação Illumina DNA e a subsequente *metabarcoding* foram usadas para calcular e definir as alterações no *taxa* bacteriano. A análise dedados e o teste de significância foi feita usando o *vegan, DADA2* e *phyloseq* packages no Rstudio software.

Como esperado, a mortalidade não foi afetada pela ingestão do pesticida, já que foram usadas concentrações subletais. O microbioma de abelhas melíferas descoberto, que inclui 38 espécies foi consistente com a literatura já existente. A riqueza específica e a abundância das espécies aumentaram significativamente em abelhas sujeitas à concentração 3 (1/25 of LC50) de ambos os pesticidas, Difenoconazole e Cypermethrin,

demostrando perturbações na estrutura da comunidade microbiótica intestinal. No geral, a comunidade microbiana principal mostrou resistência enquanto exposta às doses mais baixas neste estudo (Concentração 1 e 2) para ambos os pesticidas. A maioria das diferenças significativas foram obtidas ao comparar o tratamento de controlo das abelhas com a Concentração 3, o que indica que as doses abaixo de 1/25 de LC50 são demasiado baixas para causar uma mudança perceptível no microbioma das abelhas. Os resultados obtidos em abelhas tratadas com Cypermethrin evidenciam que um aumento na dose do pesticida causa um declínio acentuado no género Raoultella (antes conhecido como Klebsiella). Difenoconazole levou a uma alteração na comunidade microbiótica, principalmente em bactérias de carácter benéfico como Shodgrassela e Bombilactobacillus spp. Os nossos resultados sustêm a necessidade de estudar a influência de pesticidas no microbioma intestinal das abelhas melíferas e promover uma apicultura sustentável.

Palavras-chave: microbiota intestinal, difenoconazol, Cerimónia, abelha, cipermetrina, Sherpa, exposição oral crônica, composição bacteriana.

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List of Abbreviations/ Acronyms

LAB - lactic acid bacteria

DNA - deoxyribonucleic acid

PE - paired-end reads

LD50 - lethal dose, 50%

LC50 - lethal concentration, 50%

a.i. - active ingredient

mL - milliliters

nL - nanoliters

ng - nanograms

mg - milligrams

bp - base pairs

ANOVA - Analysis of Variance

DADA2 - Divisive Amplicon Denoising Algorithm 2

OECD - Organisation for Economic Co-operation and Development

SD - Standard Deviation

SE - Standard Error

mM - millimolar, a decimal unit of molar concentration

ASV - amplicon sequence variant

µg - micrograms

1. Introduction

1.1. The ecological importance of the Honey Bee

Western honey bee *Apis mellifera* (Linnaeus, 1758) is spread on all the continents except Antarctica (Mortensen et al., 2013). And its economic value played a big role in this distribution since it was often intentionally done by humans. The vital role of this insect in the modern world cannot be denied. Since ancient times, bees have been the source of honey, as well as several other products such as propolis, wax and pollen (Mortensen et al., 2013). Honey bees provide an essential ecosystem service - pollination - to crops and wild plants and consequently great economic benefits for humans. In the United States of America, the economic dependence of the agricultural sector on pollination services is estimated to be around 14.2−23.8 billion dollars (Chopra et al., 2015). This number includes the estimated profits from the production of such crops as various vegetables, fruits, cotton, flax, nuts, seeds and flowers. The benefits to society obtained from honey bees reach far beyond food production. It is important to take into consideration other economic sectors, where jobs and revenue are generated for beekeepers, as well as through transportation, equipment manufacturing and food processing (Southwick & Southwick, 1992).

Honey bees also play a critical part in maintaining the health of natural and agricultural ecosystems (James et al., 2018). According to James and colleagues, honey bees are the most commonly occurring visitors of wild flowers in pollination networks worldwide. They appear in networks in their native range as well as in areas in which they were brought by humans. This has an enormous value in maintaining the diversity of plant species, including medicinal and endangered plants (James et al., 2018).

In the recent decades, honey bee colonies became wider distributed due to their usage in agriculture and beekeeping activities, although high colony mortality during the winter season has been a growing threat all around North America and Europe since 2007 (Currie, Pernal, & Guzmán-Novoa, 2010; De la Rúa et al., 2009).

1.2. The global threats and main stressors to the Honey Bee

Considering the constant increase of the economic dependence on honey bees for pollination services, their decline poses a growing danger. Nowadays, pollination by honey bees is threatened by many factors that negatively affect bee population health and could lead to such phenomena as Colony Collapse Disorder across Europe and the USA, which is a sudden disappearance of most part of the worker bees in a colony without obvious reasons (Dainat et al., 2012). Plenty of reasons for this decrease have been appointed, but the ones that seem to be the main contributors are anthropogenic and include pesticide exposure, poor nutrition, monocultures in crop farming and increased loads of pests and diseases (Potts et al., 2010). The massive change in land use patterns is also among the factors that negatively affect honey bee populations. An increased amount of agricultural food production and monoculture crop plantations leads to natural habitat fragmentation and a decrease in floral food sources for all the pollinators (Kluser et al., 2011). In some parts of Europe, crops overtook the main food source position for honey bees as the native flower resources are lacking - for example, in the United Kingdom, rapeseed has become the main pollen source for bees (Kluser et al., 2011). These shifts cause malnutrition and diet disturbance, which lowers the overall health and sustainability of a colony (Brodschneider & Crailsheim, 2010).

Migratory beekeeping is gaining popularity over the years as a commonly practiced method of boosting honey bee colonies' productivity in producing honey and for pollination services. It is, however, a source of worry because it increases the spread of illnesses and may jeopardize the genetic identity of the colonies involved (Jara et al., 2021). By now, it is a well-known fact that bee parasites, namely the parasitic mite *Varroa destructor,* that have spread across the colonies are one of the main reasons for decreasing longevity (Sammataro et al., 2000). The danger of this mite stems not only from the feeding on the fat body of the bee, but also from it being a vector for various viruses and facilitating their circulation (Tentcheva et al., 2004). The rapid spread of diseases has been aided by the importation of queen bees from afflicted areas together with the transportation of infested bee colonies for pollination, and apiculture has been seriously impacted. Additionally, it has been shown that migratory beekeeping and queen trade influence population diversity and gene flow (Kukrer et al., 2021). In his study, Kukrer and colleagues state that human activity led to local losses of native honey bees due to genetic swamping and replacing them with non-native strains.

Lastly, it is important to mention new and arising threats such as *Vespa velutina* or the Asian hornet. Once introduced, it spread rapidly across Europe, thanks to the ability of a single nest to spread many mated queens over a big region, which is further aided by favorable climatic circumstances and the absence of natural enemies (Budge et al., 2017). Notably, the Western honey bee has no effective defense mechanisms against this predator since they were never exposed to this species before (Budge et al., 2017). Different governments are working on proper management activities to limit the spread of this problem and researching different techniques for more effective nest location search, but the Asian hornet expansion continues (Nuñez-Penichet et al., 2021).

1.3. The role of pesticides in Honey Bee health status

Another consequence of extensive agricultural land use is massive pesticide application for crops protection. Over the last decades, pesticides were accused more and more of being the main reason for the decline of bee colonies (Woodcock et al., 2016). The main ways through which these substances enter the organism of a bee are ingestion, contact exposure, and seldom respiratory intake (Chmiel et al., 2020). Even in sublethal doses, or those significantly lower than LD50, different pesticide families have been proven to negatively affect honey bee reproduction and performance (Chmiel et al., 2020). Depending on the type of pesticide and concentration, it can permanently weaken the immune system of honey bees or inflict massive damage to their neurological systems, leaving them vulnerable to other influences (Leska et al., 2021).

It is challenging to make generalized statements regarding pesticides since the term “pesticide” includes an array of different classes of chemical substances with different properties and modes of application. Insecticides, herbicides, fungicides and nematicides are used on the fields with crops and often, they are applied simultaneously. To sum up, managed beehives near agricultural sites can be exposed to a set of various pesticides at the same time through residues, and the precise effect of it could be revealed only by a detailed ecotoxicological study (Tsvetkov et al., 2017).

Besides decreasing the population numbers, they can also diminish immunity and cognition (Siviter et al., 2018). It poses even a greater risk since most of the pesticides have alarmingly long half-life periods that range from several days to months and tend to accumulate in food and honey, ground and water bodies (Mitchell et al., 2017). For example, permethrin’s half-life in the soil is about 40 days in average, from 11 to 112 (Toynton et al., 2009). And this tendency for toxic build-up does not seem to be slowing down. In fact, since the time of the first attempts to buffer the extensive chemical pollution

by pesticides, their impact only grew stronger (DiBartolomeis et al, 2019). According to DiBartolomeis research, the total toxicity and environmental presence of insecticides in the US increased significantly over the last 25 years. The results of this 2019 study revealed a 48 times higher level of acute Insecticide Toxicity Load from 1992 to 2014 for contact toxicity in the soil. This metric was created by the author of the study and it measures both the amount and danger of the insecticide in the soil since this method accounts for the total mass of insecticides used in the US, acute toxicity to insects using honey bee contact and oral LD 50 as reference values for arthropod toxicity, and the environmental persistence of the pesticides. Authors believe that it may contribute to declines not only in pollinator numbers but also insect eaters such as birds, impacting the whole food web.

1.4 Pesticides used in this study

Difenoconazole, a substance that was studied in this work which is an active ingredient of Cerimonia®, is a broad-spectrum fungicide that is used worldwide. In general, there is a broad family of difenoconazole-based fungicide products which are used on all continents. Difenoconazole is a classical fungicide, effective against a wide range of fungus, including those belonging to the Ascomycetes, Basidiomycetes, and Deuteromycetes families. It has multiple applications - seed treatment, foliar spray, and systemic fungicide. It is taken up by the diseased plant's surface and translocated to all regions of the plant. It has both curative and preventive properties. Winter wheat, oilseed rape, Brussels sprouts, cabbage, broccoli/calabrese, and cauliflower can all be treated with difenoconazole against such diseases as Light Leaf Spot, Brown Rust, Pod Spot, Ring Spot and Septoria tritici (EFSA 2011).

A second substance that is used in this study is Cypermethrin, an active component of a commercial product Sherpa®. Cypermethrin-based insecticide products have been in use since the late 1970s. By acting as a sodium channel modulator of the nerve membrane, Cypermethrin induces both stomach and contact action (Harp et al., 2006). Products based on Cypermethrin are used against external parasites on cattle but also as poisons against household insects. This insecticide is of broad spectrum and is considered to degrade quickly on plants and in soil. Cypermethrin belongs to the chemical family of pyrethroids, substances that were chemically synthesized to resemble pyrethrins. According to the EPA - United States Environmental protection agency, pyrethrins are obtained naturally from Chrysanthemum flowers. Pyrethroids are manufactured to resemble pyrethrins and therefore considered to be less toxic than

conventional pesticides. Though they are more toxic to humans and show potent negative properties on insects and fish (https://www.epa.gov).
Despite the importance of honey bees for both terrestrial ecosystems and agriculture, we still don't know how their colonies cope with anthropogenic stress, or to what extent this stress is to blame for recent spikes in recorded mortalities (Goulson et al., 2015). In the 2019 study by Rouze and colleagues, the treatment of winter honey bees with different insecticide classes and infection by *Nosema ceranae* led to variations in the honeybee core microbiota composition, with a general decrease in *Lactobacillus* and *Bifidobacterium* (Rouze et al., 2019). Moreover, each tested insecticide led to a reduction in these species, independently from the season-specific holobiont.

Permethrin, a widely used pest control pyrethroid from the same family as Cypermethrin, is influencing insect behavior making them have fewer interactions (Erin et al., 2015). Studies, performed on both wild bees and honey bees show that Cypermethrin alters the production of energy metabolites and causes oxidative stress, as well as inhibits survival and food ingestion (Glory et al., 2021). In rats, Cypermethrin was reported to decrease *Bacteroidetes phylum*, *Bifidobacterium* and *Lactobacillus* (*Firmicutes)* which are beneficial bacteria (Nasuti et al., 2016).
Difenoconazole is reported to lower microbial activity in soil microbial communities and decrease their biomass (Roman et al., 2021). The influence of difenoconazole in the concentration of 3,75 -10 mg/kg of soil (different soils were tested) on the bacteria community was the decrease of diversity. Another study, performed on zebrafish, showed that difenoconazole led to an increase in *Proteobacteria, Firmicutes* including *Lactobacillus* and *Bacteroides* (Jiang et al., 2020).
A similar reduction is expected in these clades after the exposure to both pesticides accordingly. One of the goals of this study is to research from which dosage the effect would be detectable.

1.5. The Honey Bee gut microbiota

According to Rouze and colleagues, the gut microbiota, which contributes to host homeostasis, may be altered by the cumulative effect of the previously mentioned stressors - pesticides together with the malnutrition effects and parasite loads (Rouze et al., 2019). Therefore, it is necessary to understand the role of the microbiome in the overall bees' health. What is the evidence that microbiome plays a key role in the bee health and is susceptible to these factors? Studies showed that endosymbiotic microorganisms are responsible for different functions including synthesis of vitamins,

food processing (Mukherjee 2009; Thompson et al., 2014), regulation of immune system response (Kwong and Moran 2016), and defense against pathogens (Lozupone and Knight 2005). Bee microbiome composition was a subject of interest for researchers for the last 50 years. Pioneer studies of *A. mellifera* microflora began in the 1970s (Gilliam 1974) and it remains the area of high interest today. Tests, microscopy and culture-based approaches were used to identify microbial symbionts of bees before the metagenomics era. At the beginning of a new millennia, the image of the bee gut bacterial composition was changed. When genomic technologies and DNA sequencing became available to laboratories it became clear that the results obtained by inoculating are different from the data obtained by DNA sequencing (Jeyaprakash et al., 2003). It can be explained by the difficulties of inoculating endosymbionts *in vitro* since it was often problematic to find the right medium for multiplying those bacteria.

The honey bee adult worker harbors a large bacterial community in the gut, with roughly 1 billion bacterial cells in a mature worker (Powell et al., 2014). Of these bacteria, approximately 95% are located in the hindgut. The typical worker bee gut community reaches its usual composition 3–5 days after hatching (Powell et al., 2014) and does not change noticeably when insects leave the hive and shift to foraging activities (Corby-Harris et al., 2014). Thus, the adult gut composition seems to be stable and consisting of specific community that is not found elsewhere. In the event that pupae are evacuated from the hive and hatch inside a clean Petri dish within the research facility, bees will stay almost completely germ-free all through grown-up life stage and will lack the normal intestine bacterial species (Powell et al., 2014). Therefore, it can be confirmed that colonization happens through contact of recently eclosed grown-ups with nurture bees and with the hive environment. Adults that emerge at lab conditions can be experimentally colonized with known bacterial strains to determine effects on hosts.

Samples from the hindgut include both Gram-negative and Gram-positive bacteria and are dominated by taxa belonging to the genera *Lactobacillus*, *Bifidobacterium*, *Bartonella*, *Snodgrassella*, *Gilliamella* and *Frischella* (Killer, 2011). Bacterial species clusters that belong to these genera dominate in the guts of *A. mellifera* workers, making up over 95% of the bacteria in most individuals (Sabree et al., 2012). The microbiome of bumblebees (*Bombus* species) contains bacteria that are closely related to some of the *Apis mellifera* associates (Koch et al., 2013).

Fungi, as a component of Honey bee gut holobiont, are significantly less researched. Despite the fact that searching for fungal taxa began in the 1970s, there is still a lot undiscovered. The most frequently found molds in the alimentary canal of worker honey bees belonged to the genera *Penicillium* and *Aspergillus*. Commonly identified species included *P. frequentans*, *P. cyclopium*, *A. flavus*, and *A. niger* (Gilliam and Prest 1972).

These taxa display seasonal abundance, being always present during winter, but in March through May, only 20% of tested bees contained these species. Fungi in the microbiome also differ depending on the type of honey bee (Cornman et al., 2012). The fungal gut communities of most worker bees were highly dominated by *Saccharomyces,* but foraging bees and queens were colonized by different taxa of fungal species and *Zygosaccharomyces*, respectively. Whereas sequencing the gut metagenome of the honey bee revealed the existence of *Saccharomycetaceae* such as *Saccharomyces* and *Zygosaccharomyces* (Cornman et al., 2012), sequencing the metatranscriptome revealed that the honey bee gut contains a small fraction of microbial eukaryotic transcripts (Lee 2015). Honeybees also harbor yeast symbionts that are poorly understood concerning host nutrition and immunity. While pathogenic fungi are well-studied, symbiotic or opportunistic yeasts are generally not a focal point of microbiota research. The honeybee's intestine is predicted to contain a very small number of yeasts and other fungi compared to bacteria, even suggested to constitute less than 2% of the microbiome (Ptaszyńska 2016). The relationship between bacterial and fungal microbiota remains and their mutual influence remains unexplored. Studies show that *Nosema ceranae* infections lead to an increase of intestinal *Candida* and *Saccharomyces* titers which likely is caused by having excess sugars from *Nosema*-induced malabsorption (Ptaszyńska 2016). Moreover, yeasts are considered general indicators of honeybee stress (Gilliam 1973).

Diseased bees often show dysbiosis, with increases or decreases in the dominant microbial taxa or occurrence of transient bacteria not normally associated with the host (Raymann and Moran 2008). A 2020 study, done by Miller and colleagues, shows evidence that *Bombella apis*, a honey bee-associated bacterium has the capacity to suppress the growth of 2 insect fungal pathogens *Beauveria bassiana* and *Aspergillus flavus,* proving to be effective both *in vivo* and *in vitro* (Miller et al., 2020). As this topic gains popularity, state-of-art techniques are applied to improve bee health by manipulating the microbiota inside. A report, recently published in the *Science* magazine shows how genetically engineered symbiotic bee gut bacteria, *Snodgrassella alvi*, can induce eukaryotic RNA interference (RNAi) immune responses to decrease Varroa mites' survival by 70% and reduce the number of deformed wing virus-infected bees by 36% (Leonard et al., 2020). These discoveries open the path for future innovations in bee microbiome technologies.

Finally, while describing the microbiome of *A. mellifera* it is impossible not to mention the pathogens. Pathogens of the honey bee are heterogeneous and represented by different domains and kingdoms. They include trypanosomatid *Lotmaria passim* (Runckel 2014), the microsporidial pathogen *Nosema ceranae* and fungal *Ascosphaera apis* with

Nosema apis (Cornman et al., 2009), and bacterial pathogens such as *Paenibacillus larvae* (known as European foulbrood disease) and *Melissococcus plutonius* (American foulbrood disease). In addition, the ectoparasitic mite, *Varroa destructor*, contributes to decreased colony health by feeding on developing bees (brood) and facilitating virus transmission of such viruses as DWV - deformed wing virus (Ryabov et al., 2014), KBV - Kasmir bee virus (Chen et al., 2004), and iAPV- Israeli acute paralysis virus (Di Prisco et al., 2011).

1.6. Objectives

This work aims to compare and evaluate the effect of two widely used pesticides – Difenoconazole and Cypermethrin on honey bees. Analysis was focused on evaluating what effect will different doses have on parameters such as survival, microbial diversity, relative abundance and overall microbiome composition in the gut of *Apis mellifera* foragers. Specific objectives were defined, namely:

- To evaluate the effect of three different pesticide concentrations on Honey bee mortality and pesticide intake;
- To understand how the gut microbiome richness and abundance changes under exposure to sub-lethal field doses;
- To show which clades are the most susceptible and how the microbial community is disrupted.

2. Methods

The experiments were performed in the laboratories of the Centre for Functional Ecology - Science for People & the Planet (CFE), and in the group of Environmental Microbiology hosted by the Department of Life Sciences of the Faculty of Sciences and Technology of the University of Coimbra. The pesticide exposure methodology for the experiment was developed based on a protocol adapted from OECD - Organisation for Economic Co-operation and Development Guidelines for the Testing of Chemicals, namely the Chronic Oral Toxicity Test (OECD, 2017).

2.1. Honey bee sampling

Adult worker winter bees were collected in February 2021 by removing 1 Langstroth type beehive from a colony in the mountainous region of Lousã in the center of Portugal (apiary coordinates: 40° 10' 44.3346" & -8° 17' 2.0328") and installing it on the

premises of Soil Ecology and Ecotoxicology Laboratory, in Coimbra. The chosen beehive had no history of diseases, had known maintenance and physiological status and was not under any stress (no pesticides are used in this location and there is no deforestation in the foraging area). A total of 600 bees were collected from broodless frames into the plastic cages and transferred into the temperature chambers inside the laboratory facilities. Prior to the beginning of exposure, animals were acclimated to the laboratory conditions by being kept for 24 hours in the temperature-controlled chamber and being fed on the sugar syrup.

2.2. Exposure to Cypermethrin and Difenoconazole

An adaptation of the OECD protocol (OECD, 2017) was used with the following modifications: adult bees were selected instead of newly emerged, and 7-day duration instead of 10 days. A lower exposure time would avoid reaching mortality levels that would hinder the needed sample numbers for downstream sample processing.

A threshold of 33.3% mortality from the initial number of bees in a cage was used to define the last day of exposure in order to warrant an animal number that would suffice the needed replicates. To ensure the validity of the test, no chemical or *varroa* treatments were applied at least 3 months prior to the beginning of the experiment. 300 bees for each pesticide (600 in total) were collected from broodless frames from the same beehive for consistency in microbiome samples. Each cage/replicate contained 12 bees. There were 6 replicates per treatment, 4 treatments for each pesticide (3 concentrations and control) with a total of 24 cages for each pesticide.

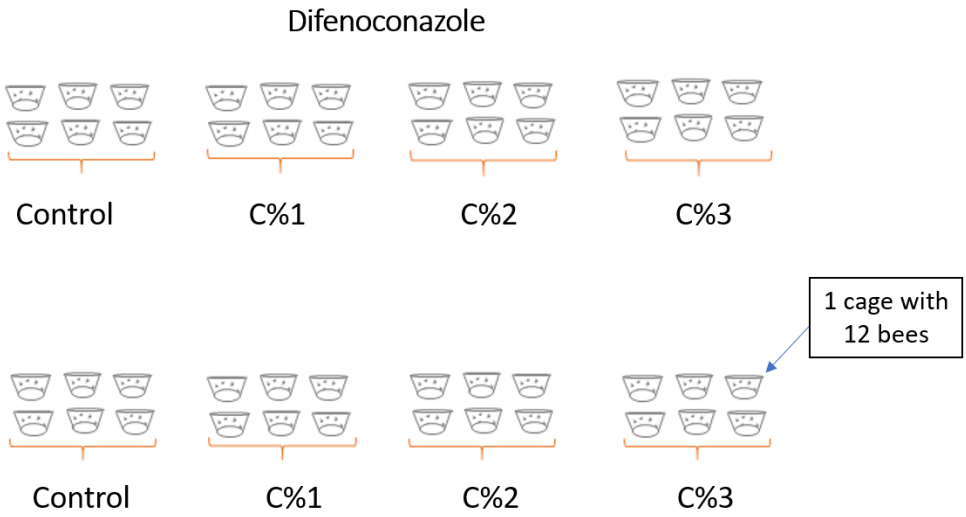

Figure 1. Schematic representation of the experiment layout. Each group of 6 cages represents a treatment. C%1,2 and 3 stand for Concentration 1,2 and 3 correspondingly.

Prior to the beginning of the experiment, one cage with 14 bees was collected and frozen in -20°C to serve as pre-control and display the initial state of Honey bee gut microbiota, unaffected by other factors such as laboratory conditions and stress caused by handling.

2.2.1. Pesticide selection

Pesticide selection was based mainly on ecotoxicology data for honey bees. The insecticide was chosen after a selection from a list of pesticides used in Portugal with moderate to low LD50 values. A preference was given to the pesticides with available data from performed acute and chronic ecotoxicity tests as per OECD standards while also being soluble in water, and not causing avoidance feeding behavior, which would result in lower pesticide consumption. These parameters are critical for mixing the substance with sugar syrup and further feeding. As a result, two selected pesticides were Cerimónia® Fungicide and Sherpa® Insecticide.

Sherpa® is a pesticide from a pyrethroid ester group of insecticides. The active ingredient of this insecticide is cypermethrin with the IUPAC name [Cyano-(3-phenoxyphenyl)methyl]3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1- carboxylate and CAS-Number 52315-07-8. Other common names for cypermethrin are NRDC 149 and WL43467. It has a chemical formula $C_{22}H_{19}Cl_2NO_3$ and a molecular weight of 416.30 g/mol. According to the report on Cypermethrin from Bayer, the melting point is 80.5

degrees C and density is 1.25 g/cm (Bayer company, 2014). It is not soluble in water but dissolves in organic solvents. The concentration of cypermethrin in Sherpa® is 100.0 g/L and the product is in the form of an emulsifiable concentrate. The substance is classified as a hazardous chemical for humans (hazard statements H302, H304, H319, H336, H373).

Cerimónia® is a fungicide from a triazole family of fungicides. It has an active ingredient called difenoconazole. The IUPAC name of Difenoconazole is 1-[[2-[2-Chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl]methyl]-1H-1,2,4-triazole and its CAS-Number is 119446-68-3. It has a chemical formula of $C_{19}H_{17}Cl_2N_3O_3$ and a molecular weight of 406.26 g/mol. Other formulation names for the substance are CGA 169374 or Dragon. The melting point is 70.6 °C. It is dissolved by most organic solvents. The concentration of difenoconazole in Cerimónia® is 250.0 g/L. The product is in the form of an emulsifiable concentrate. The mechanism of action of Cerimónia® is inhibition of demethylation in sterol biosynthesis and the recommended dose is up to 0.5L/ha depending on the type of plant. Difenoconazole is classified as a hazardous chemical for humans (hazard statements H302, H304, H315, H319, H373, H410). Information is taken from the manufacturer's website (https://www.ascenza.pt/products/cerimonia).

2.2.2. Pesticide dosage

For this study, sublethal concentrations were calculated according to already available data from studies on Cypermethrin and Difenoconazole. Previously used concentrations of Difenoconazole on native bees and honey bees range from 0.1 to 10 µg/L (Hanine et al., 2020, Leite et al., 2021) and from 3 ng/mL sucrose solution to several milliliters per L for Cypermethrin (Fent et al, 2020). In these studies, the effects on insect behavior or survival were observed even at sublethal dosages. The three concentrations for this study were derived as 1/100, 1/50 and 1/25 of LC50 and calculated in µg of active ingredient per 1 ml of food solution to resemble environmentally real exposure. According to OECD 245 guidelines, the main parameters that need to be determined when treating data from a chronic feeding experiment are toxic descriptors LC50 and LDD50 (OECD, 2017). They are used for risk assessment and hazard classification in fields like pharmacology and ecotoxicology.

According to the Encyclopedia of Toxicology, LC50 stands for lethal concentration affecting 50% of the population and it is expressed in terms of mass per volume. The units used are micrograms or nanograms of active ingredient per 1 milliliter of food solution (Gas, 2014). LDD50 is a lethal dietary dose affecting 50% of the population and it is expressed in micrograms or nanograms of active ingredient per one bee per day,

when it is calculated based on the average daily intake of the a.i.. Lethal dietary dose 50 can also be derived from the accumulated intake of the a.i. over the duration of the experiment. In that case, it is expressed in micrograms or nanograms of a.i. consumed by one insect.

Table 1: Dosage of active ingredients for Sherpa® and Cerimonia® and corresponding concentrations, used in this study for chronic exposure. C1, C2 and C3 corresponds from now on to Concentration 1 (the lowest), Concentration 2 and Concentration 3 (the highest), a.i. stands for active ingredient (Difenoconazole or Cypermethrin).

Pesticide name	Cerimonia	Sherpa
Active ingredient	**Difenoconazole**	**Cypermethrin**
Effective concentration, g of a.i./L	250	100
LC50, µg a.i./ml of food	810,81	0,95
LDD50, µg a.i./bee/day	30	0.028
C1 (1/100), a.i. µg/ml of food	8.108	0.0095
C2 (1/50), a.i. µg/ml of food	16.22	0.019
C3 (1/25), a.i. µg/ml of food	32.43	0.038

2.2.3 Pesticide feeding

Bees were fed *ad libitum* with 50 % water/sucrose solution in the control group, and same sucrose solution with pesticide in test cages. Conditions were kept at 25 ± 0.833 ºC and 65 ± 6.429 % relative humidity (RH). Throughout the duration of the whole test period, mortality and abnormal behaviors were recorded daily. The feeding was performed during 7 full days and food was provided around the same one hour period every day to achieve equal 24 hour feeding time periods. The feeding solution was given to bees in a horizontally installed Eppendorf tube, which was inserted into each cage (see Figure 2). Every day the feeders were weighted to check the consumption by calculating the difference in the weight before and after a day of exposure. The average evaporation

was also calculated each day through evaporation control cages and excluded from the intake calculations.

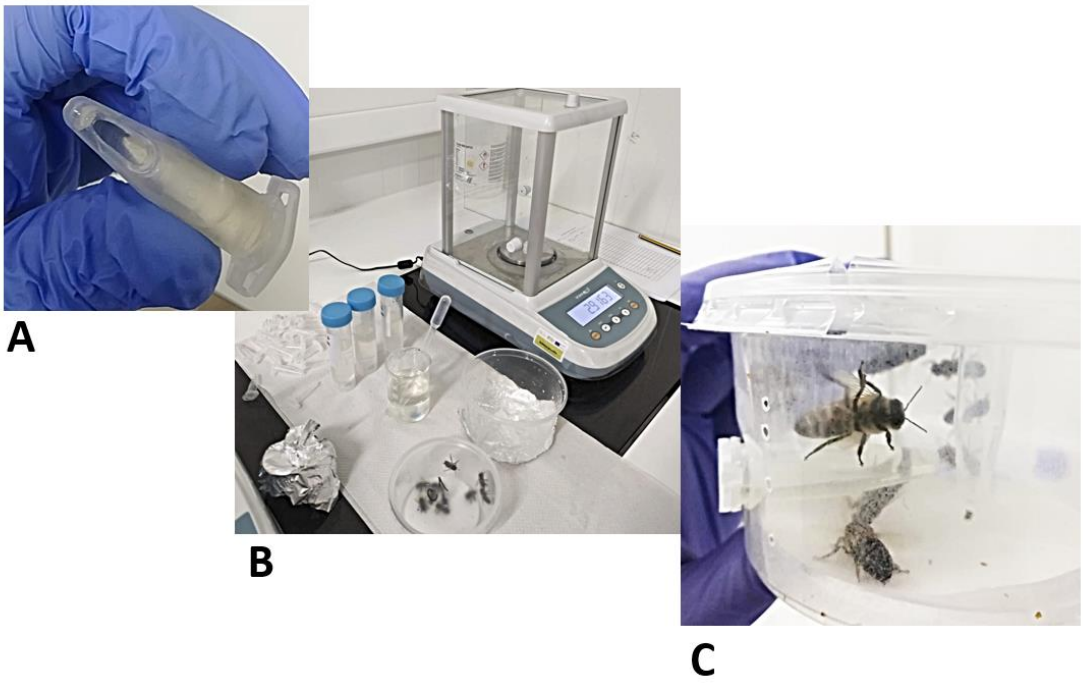

Figure 1: Experimental setup and feeding tubes description. (A) Modified Eppendorf tube with incision on top that was used for feeding. (B) Scale that was used for daily food solution weighting. (C) Feeder positioned inside the cage. Openings on the walls to allow airflow.

In the end, intake of feeding solution by each single bee during one day (approx. 24 hours) was calculated. For the detailed description please see the table above.

At the end of the experiment, daily food consumption and mortality of each cage were analyzed, with an average value of food consumption, intake of active ingredient, survival and accumulated consumption. The standard deviation for both food consumption and average pesticide active substance intake were calculated and are presented in the annex 1.

Each pesticide's survival and intake data were analyzed separately. Food solution was weighed before and after 24 hours of consumption. The data is presented for three different concentrations of both pesticides. Control treatment corresponds to zero pesticide intake. Cages with exceptional abnormal feeding behavior, and/or excess of mortality due to environmental contamination due to handling were removed for posterior analysis.

2.3 Isolation of DNA from the honey bee gut and sequencing

After the seventh exposure day, bees were frozen and stored at - 80 °C for further processing and DNA extraction. Bees were individually dissected in sterile conditions and the hindgut was isolated. The instruments and the surface on which the dissection was done were sterilized with 96 % ethanol and flamed and the dissection process was performed under aseptic conditions in the presence of a Bunsen burner. A pool of approximately 10 extracted bee guts from each cage (ranging from 8 to 11 based on the number of remaining bees in the cage) was placed in a 2 ml Eppendorf extraction tube with glass beads for further homogenization.

A blank control (extraction kit and environmental control) was added to the samples to ensure the absence of contamination. DNA was extracted using E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, USA) according to the manufacturer's protocol. Briefly, extraction was performed using the manufacturer's instructions, but modified to better meet the goals of the study. The glass beads and SLX buffer were used to lyse the samples in a QIAGEN TissueLyser LT instead of vortexing. To increase the yield, samples were incubated at -20°C for 1 hour prior to elution. Only 60 microliters of the Elution buffer, provided in the extraction kit were added as opposed to 100 microliters in the protocol due to the smaller quantity of sample material., After that, no changes were applied and the rest of the extraction was done matching the protocol.

In total, 50 samples (48 from the feeding experiment, 1 precontrol and 1 blank) were obtained with approximately 30 µl volume. DNA quality and quantity was evaluated using a NanoDrop™ 1000 Spectrophotometer and kept dissolved in 10 mM Tris-HCl pH 8.5 at -20 °C until sent to the sequence provider. The genomic DNA concentration ranged from 108 to 341 ng/µl, except for the blank where it equaled 0.

To find out the composition of the microbial community, isolated DNA material was sent to the Novogene facility (Cambridge, UK) for metabarcoding sequence analysis. The sequencing strategy used 200 bp paired ends (PE200) size in 16S V4 rRNA gene region and sequenced on Novaseq 6000 from Illumina. Amplicon sequencing on Illumina PE200 involved PCR amplification & library construction (PCR free library), of a 292 bp gene fragment of the 16S ribosomal gene with primers 515F (5'-GTGCCAGCMGCCGCGGTAA) and 806R (5'-GGACTACHVHHHTWTCTAAT) (Caporaso et al., 2012). Additionally, a blank control was added for library preparation

and sequencing to exclude contamination. PCR products were mixed at equal density ratios. The mixed PCR products were purified with Qiagen Gel Extraction Kit and the libraries generated with NEBNext® UltraTM DNA Library Prep Kit for Illumina and quantified via Qubit.

2.4 Data analysis

2.4.1. Survival and pesticide intake

The total number of alive animals in each cage was measured daily for the full lenght of the experiment (7 days). Three mock cages without bees were placed nearby on the same shelf of the temperature chamber to measure the daily average evaporation of food solution from the feeders. Pesticide intake was calculated using the following formula: $PI = \frac{m_i - m_{24h} - R}{N_{bee}}$ (1),

where:

m_i: Weight of a full feeder tube before it is placed in the cage

m_{24h}: Weight of the tube after 24 hours in the cage

R: Average evaporation

N_{bee}: Current number of alive bees in the cage

Data analysis for this study was performed in R (version 4.1.1) using the graphical interface RStudio (build 351). First, a Kruskal-Wallis test was used to evaluate the survival data. Chi-square and significance values were calculated for both the intake and survival per cage in control and three concentrations of each pesticide. The Kruskal–Wallis test is a nonparametric test that determines if all populations are identical or if at least one of them has a tendency to produce observations that differ from those of other populations (Encyclopedia of Statistics, 2008). It was used as an alternative for a one-way ANOVA since the survival data did not follow the standard normal distribution and the number of independent groups was higher than three (Control and three concentrations were analyzed separately for Difenoconazole and Cypermethrin).

2.4.2. Microbiome analysis

Sequence processing was done using the DADA2 workflow (Callahan et al., 2016), by implementing the default parameters for filtering, denoising, merging of paired reads, chimera identification and removal, and to obtain amplicon sequence variants (ASVs). Forward and reverse primer sequences as well as fragment ends with low quality scores were trimmed, yielding final lengths of 253 bp and 195 bp for forward and reverse reads, respectively. 17156 input sequences (ASVs) were obtained. Number of raw reads per sample ranged approximately from 400 in Control samples to 146000 and higher in pesticide treatments. Three samples were removed from Difenoconazole data before the analysis - 1.6 from Concentration 1, 2.4 from concentration 2 and Control 1.3. From Cypermethrin, samples 1.1 in C1 and 3.1 in C3 were removed as well. Chimera proportion was ~1% and taxonomy was assigned using IDTAXA (Murali & Bhargava, 2018) implemented in the R package DECIPHER (Wright, 2016) with a classifier trained on the SILVA v138 database (December 2019 release). The relative abundances of observed bacterial taxa at the genera level were compared between the different treatment groups using bar plot charts made with the package phyloseq (McMurdie et al., 2016). Alpha diversity (observed ASVs) of the bacterial microbiome was compared between the treatments using a non-parametric Kruskal–Wallis test, with $p < 0.05$ considered as statistically significant. Pairwise comparisons between the three treatments were made using the Wilcoxon test, with $p < 0.05$ considered as statistically significant. Non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity along with a graph-based permutation test (nperm = 999) using phyloseqGraphTest (v0.1.0) was performed to evaluate differences in bacterial community composition between the treatments and to construct a Minimum Spanning Tree.

2.5. Ethics Statement

The sampling and euthanasia were done according to international regulations. The sampling was adapted to be the least invasive for the rest of the beehive by taking the selected frame in a container in order not to disturb the rest of the colony and interact with one frame only. The dispatching method was putting the whole cage at - 15°C to ensure the suffering was minimal.

3. Results

3.1. Survival and pesticide intake

First, the information on pre-calculated and actual average Cypermethrin and Difenoconazole intake was analyzed. The results can be seen in the two tables below.

Table 2: Comparison of Difenoconazole doses provided in the feeding and average amount of pesticide ingested by each bee per day. a.i. stands for “active ingredient” and SD is the “standard deviation”.

Treatment	Average intake of a.i./bee/day	Given doses in µg a.i./bee/day	SD intake of pesticide (µg a.i./bee/day)
Control	0	0	0
C1	0.31	0.30	0.099
C2	0.57	0.60	0.142
C3	1.05	1.20	0.229

For Difenoconazole, bees treated with Concentration 1 and Concentration 2 showed consistent intake of pesticide. In the cages that contained food solution with Concentration 1, the difference between nominal dose and intake dose was 0.01 µg or 0.000001 gram. For Concentration 2, bees received on average 0,57 micrograms, which differs from the precalculated dose by 0.03 micrograms or 0.000001 g. In Concentration

3, intake varies the most from the precalculated dose with 0.15 micrograms less than the dose.

Table 3: Comparison of Cypermethrin doses provided in the feeding and average amount of pesticide ingested by each bee per day. a.i. stands for “active ingredient” and SD is the “standard deviation”.

Treatment	Average intake of a.i./bee/day	Doses in µg a.i./bee/day	SD intake of pesticide (µg a.i./bee/day)
Control	0	0	0
C1	0.00037	0.00035	0.0001
C2	0.00074	0.00070	0.0003
C3	0.00138	0.00141	0.0006

In cages that were fed on Cypermethrin, pesticide intake was consistent for all three pesticide concentrations. Bees that ingested Concentration 1 solution consumed on average 0.00002 micrograms more than the dose. For Concentration 2, on average each bee received an additional 0.00004 micrograms of Cypermethrin. In Concentration 3, 0.00138 micrograms were consumed as opposed to 0.00141 predicted dose.

As it can be seen from Table 3, the nominal chosen doses by treatment were close to average ingested ones, which supports the effectiveness of a chosen feeding method. Doses were calculated by multiplying the concentration by the weight of the food solution,

consumed on average every day by one bee known from the previous experiments. Therefore, the difference between assigned and actually ingested amounts of both Cypermethrin and Difenoconazole was minimal. For a more detailed description, SD of intake of the active ingredient and food consumption for both Difenoconazole and Cypermethrin please see *Table A-I-1* and *Table A-I-2* in the annex 1.

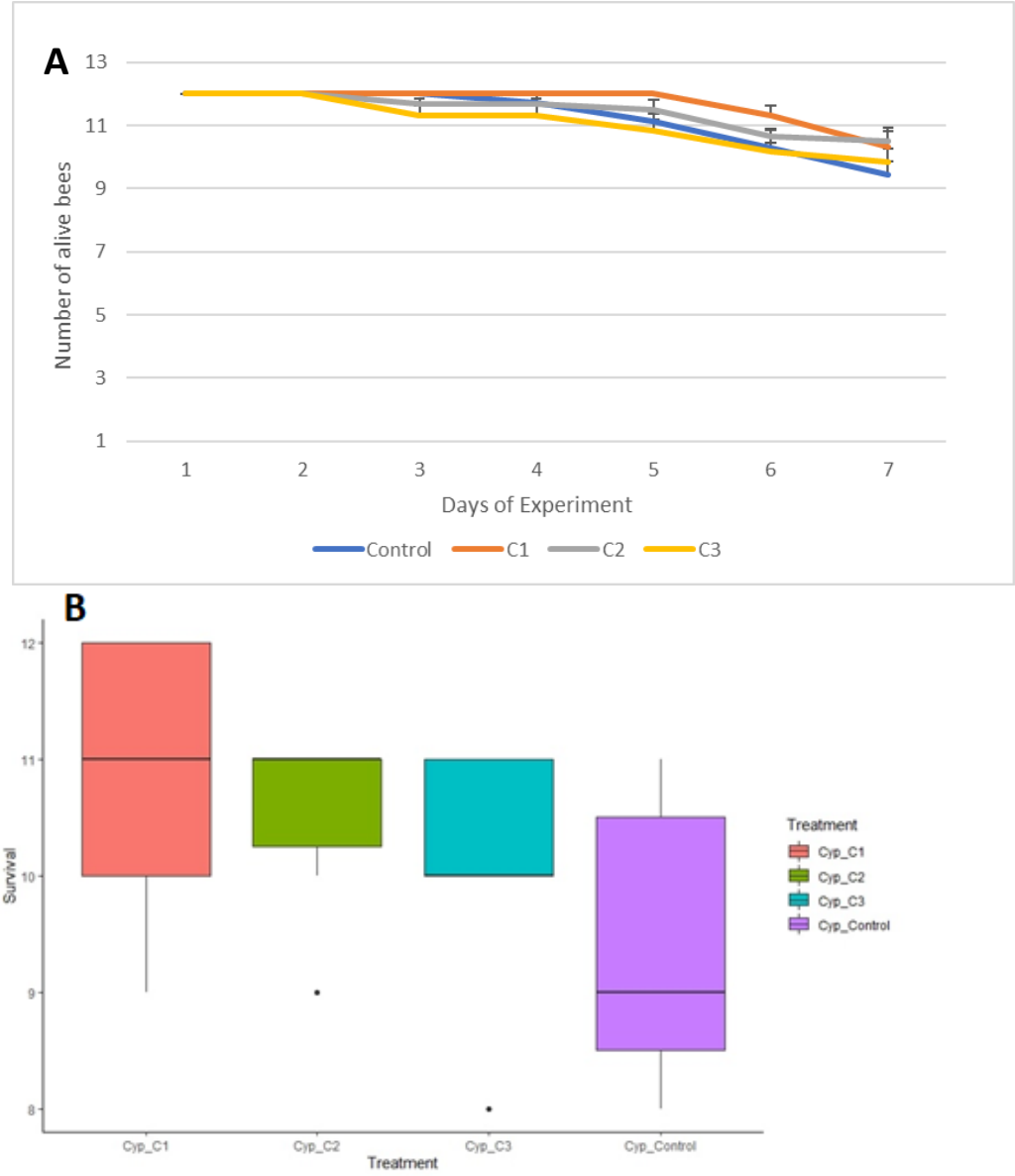

Figure 2: Survival data expressed by the number of alive bees per treatment for Cypermethrin chronic exposure feeding using three sublethal concentrations. (A) Remaining alive bees at each time point (by day, 7 days total). (B) Boxplot of alive bee numbers per treatment at the end of the exposure. Dots represent outliers, the median is represented as a horizontal line on the boxplot, and whiskers extend to the range of 1,5 interquartile. C1, C2, C3 correspond respectively to the sublethal concentrations

solution (Table 1), Cyp_control marks no pesticide intake (No Cypermethrin added to the feeding solution).

Moderate mortality was observed in all treatments and both controls. When compared to control, Concentration 1, Concentration 2 and Concentration 3 showed higher survival rates. On Day 5, a decline in the alive bee numbers was observed for all concentrations and control. The highest number of alive bees at the end of the experiment was in cages, treated with Concentration 1 and 2.

Analysis was performed in the RStudio programming environment to compare the effect of treatment on survival., Since our data were not normally distributed, a non-parametric Kruskal-Wallis test was chosen (McKight & Najab 2010). It is a subtype of a Mann-Whitney U Test, which does not assume that the data is distributed in any specific kind of way and is used when the number of groups is higher than or equal to 3. Each pesticide's survival and intake data were analyzed separately. Our assumed null hypothesis for both pesticides is that the mean ranks on survival numbers at the end of the experiment are equal across 4 treatments (Control, C1, C2, C3).

Kruskal-Wallis test revealed that there was no statistically significant difference in mean survival between the control treatment and pesticide concentrations (*n* of samples = 21 for Difenoconazole and 22 for Cypermethrin). The Levene test showed high confidence in results and that the variance was consistently homogeneous for both pesticides. There was no significant difference (Kruskal Wallis Chi-squared = 3.1456, $p = 0.1096$)

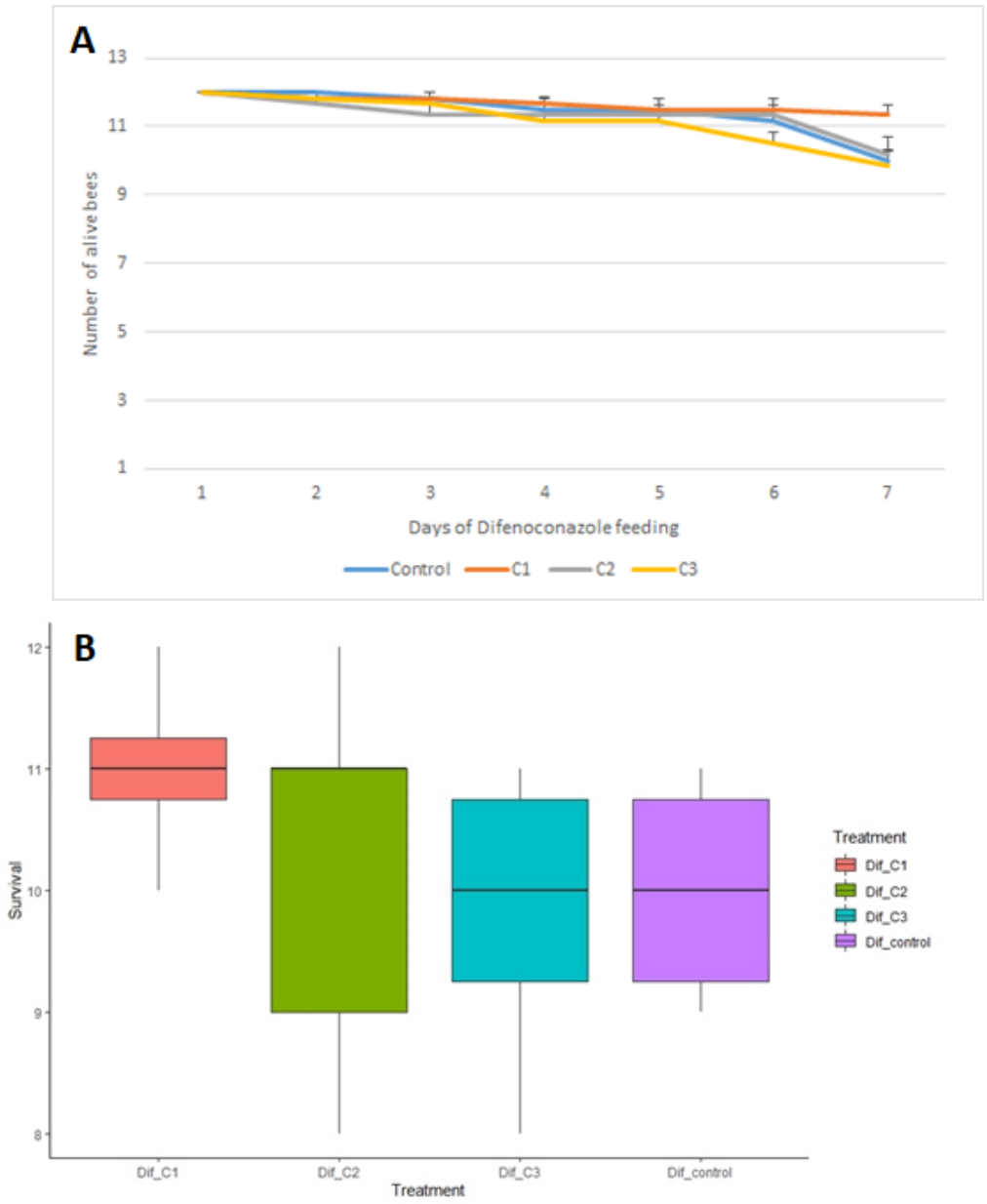

Figure 3: Survival data expressed by the number of alive bees per treatment for Difenoconazole chronic exposure feeding using three sublethal concentrations. (**A**) Remaining alive bees at each time point (by day, 7 days total). (**B**) Alive bee numbers by the end of exposure. In the box plot, the median is represented as a horizontal line on the boxplot, whiskers extend to the range of 1,5 interquartile. C1, C2, C3 correspond respectively to the sublethal concentration solutions (Table 1), Dif_control corresponds to the untreated bees, consuming pure sugar solution (0 ml of Difenoconazole).

Similarly to the results obtained during the Cypermethrin feeding, no significant difference was observed in mortality between Difenoconazole treatments (chi-squared = 4.1382, p = 0.2469). Concentration 1 showed the highest survival when compared to

control and showed the highest average number of alive bees per cage. A sharp decline in survival was observed in the cages of Concentration 2 and 3 and Control on day 6.

3.2. Metabarcoding and DNA sequencing

In total, 228 sequence variants were inferred from 18,827 input unique sequences of the 16S rRNA gene. Quality filtering reduced the total number of samples to 49 by removing those with no replication. Taxonomy was assigned, diversity and abundance of microbial communities were estimated. The overall results were consistent with the rarefied data curves. The rarefaction depth chosen is the 90% of the minimum sample depth in the dataset (425 reads),

3.2.1. The effect of Difenoconazole on Honey bee gut microbiome

Compared to the control group, Difenoconazole treatments had a significant impact on the total number of bacterial species of tested honey bees. Concentration 3 showed an increased richness (664 ASVs). The Kruskal-Wallis test was used to evaluate the differences in observed number of ASVs based on the concentrations of pesticide consumed and a significant difference was detected (Chi-squared = 10.367, df = 4, p = 0.03468) between Control and C3. Therefore, different Difenoconazole doses resulted in major changes in gut microbiota richness.

First, alpha diversity was measured. Chao1 index values were equal to the number of observed ASVs for all the samples, indicating the absence of uniquely present species (singletons). Treatment had a significant effect on diversity (number of observed ASvs) (p = 0.006072), but Kruskal-Wallis test proved the difference between control and Concentration 1 to be not significant (p = 0.14), as well as between Concentration 2 and Control (p = 0,25). Effect of Concentration 3 on Alpha diversity is shown on the figure below.

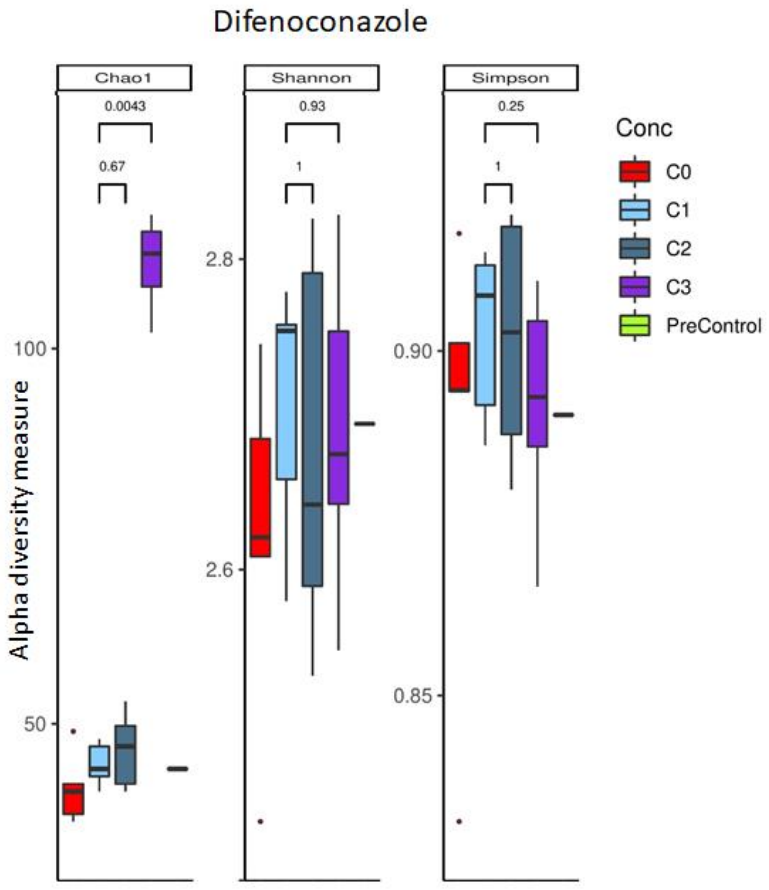

Figure 4: Alpha diversity estimation by Chao, Shannon and Simpson indices for treated and unexposed bees. C0, C1, C2, C3, indicate respectively Difenoconazole Control treatment, Concentration 1, 2, 3 and PreControl describes bees that were sampled before the beginning of the experiment. Interquartile range is represented by boxes, with a median identified as a line. Whiskers extend to 1.5 of the interquartile values and dots represent outliers.

Alpha diversity, measured by Chao1 index varies significantly (chi-squared = 14.419, df = 4, p = 0.006072) across Difenoconazole treatments. There were no significant differences in alpha diversity when measured with Shannon index across samples when measured with ANOVA (F value = 0.367, Pr(>F) = 0.828), which might occur due to a high evenness of ASVs in all samples. Furthermore, Simpson index did not significantly vary between treatments as well (p = 0.8744), which indirectly may reveal that dominance by few ASVs is not present.

Next, abundance and presence of different taxa were analyzed. The main gut microbial taxa, described earlier, were found to be fully present in our samples (genera *Gilliamella, Shodgrasella, Lactobacillus, Bombella*). Interestingly, our data shows low

levels or near absence of species in the order *Bifidobacteriales* in some samples (Figures 5 and 6), which are also considered to be core species.

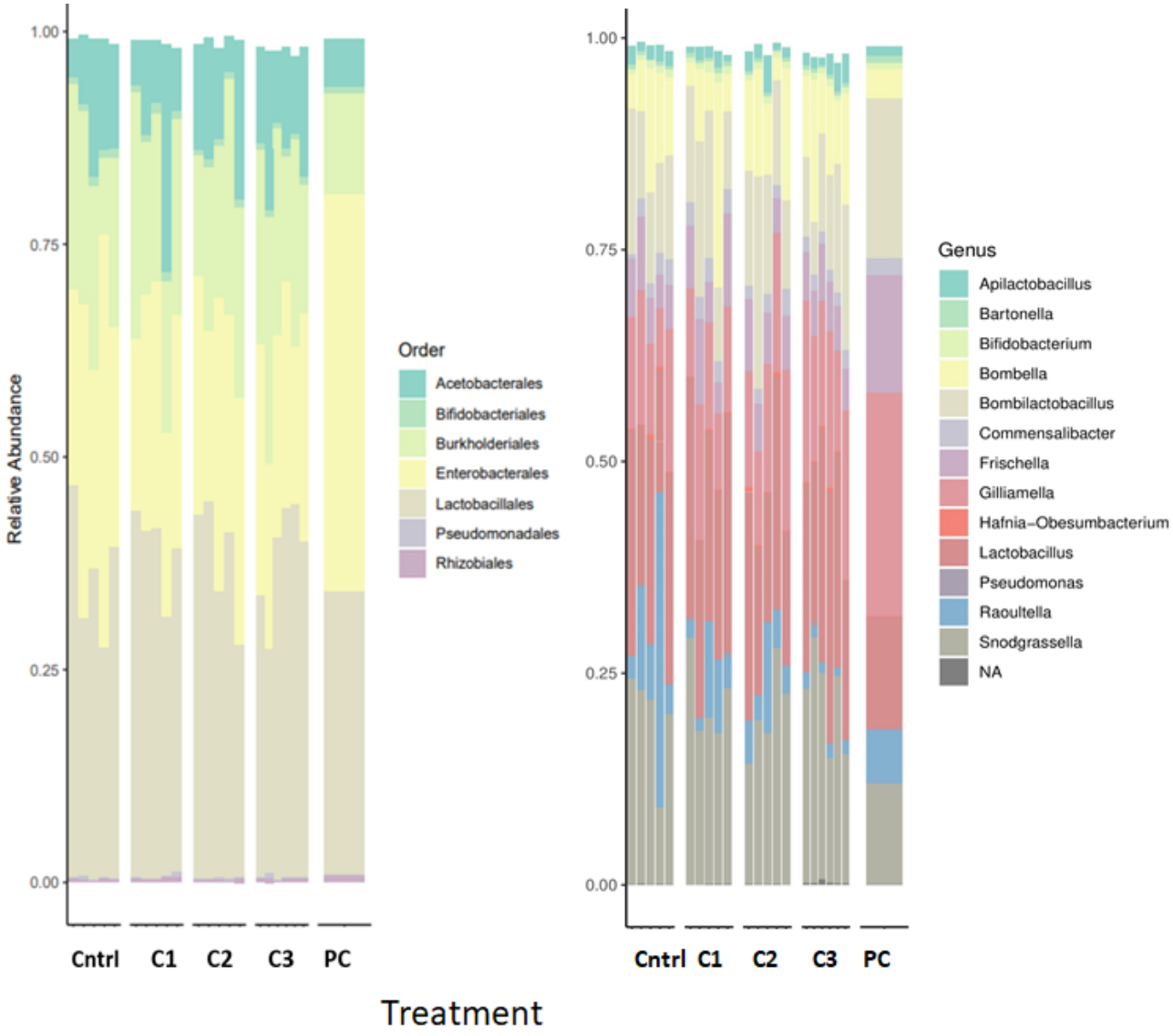

Figure 5: Relative abundance of Honey bee microbiome phyla in Difenoconazole treatments, sorted by Order and Genus. Cntrl indicates control and the absence of exposure, while C1, C2 and C3 represent Concentrations 1,2 and 3, and PC represents bees sampled before the beginning of exposure (pre-control). Each single line in a bar chart signifies one cage.

The gut microbial composition was affected by Difenoconazole treatments observed at order and genus levels. The abundance of order *Enterobacterales*, represented by *Raoutella* spp. (formerly *Klebsiella*) decreases in response to higher doses of pesticide, until it almost completely disappears in Concentration 3, as it is present in higher amounts in pre-control and control and decreases with the pesticide introduction. While

Lactobacillus and *Burkholderiales* abundance is constant throughout treatments, fluctuations appear in such order as *Acetobacterales*. They show higher abundance at the Concentration 3 than in the control group. The overall number of non-identified ASVs increases in concentration 3, showing higher total abundance of microbial components in response to Difenoconazole.

Lastly, Beta diversity was analyzed using PCA – principal component analysis. 5 dimensions were plotted: ASV1 – *Snodgrassella alvi;* ASV6 – *Raoultella* spp.; ASV10 – *Bombilactobacillus* spp.; ASV2 – *Gilliamella* spp.; and ASV3 - *Lactobacillus melliventris.* All of these bacteria were previously reported and are considered as core bee gut microbiome participants, with the exception of *Raoutella* (recently *Klebsiella*), which is found in low amounts and is an environmental bacterium, found in plants and soil. Results show strong negative correlation between *Snodgrassella* and *Gilliamella, a*nd positive correlation between the pairs of ASV1 and ASV3, as well as ASV10 and ASV2. No clear clustering was observed for the Control group (red circles) or the different concentration treatments. Dimension 1 (Principal component 1) explained 28% of the microbiome composition variance.
In PCA1 vs PC3 analysis, ASV1 (S. alvi) continued to play a big role as well as ASV6 (Raoutella spp.). The main axis was created by ASV4 - Bombella spp., and nost of the concetration 3 samples clusteres on the other side between it and ASV 15, which belongs to Lactobacillaceae family, indicating the trend for absence of Bombella in higher concentrations. Control samples were dispersed evenly indicating no clear clustering as it can be seen on the graphs below.

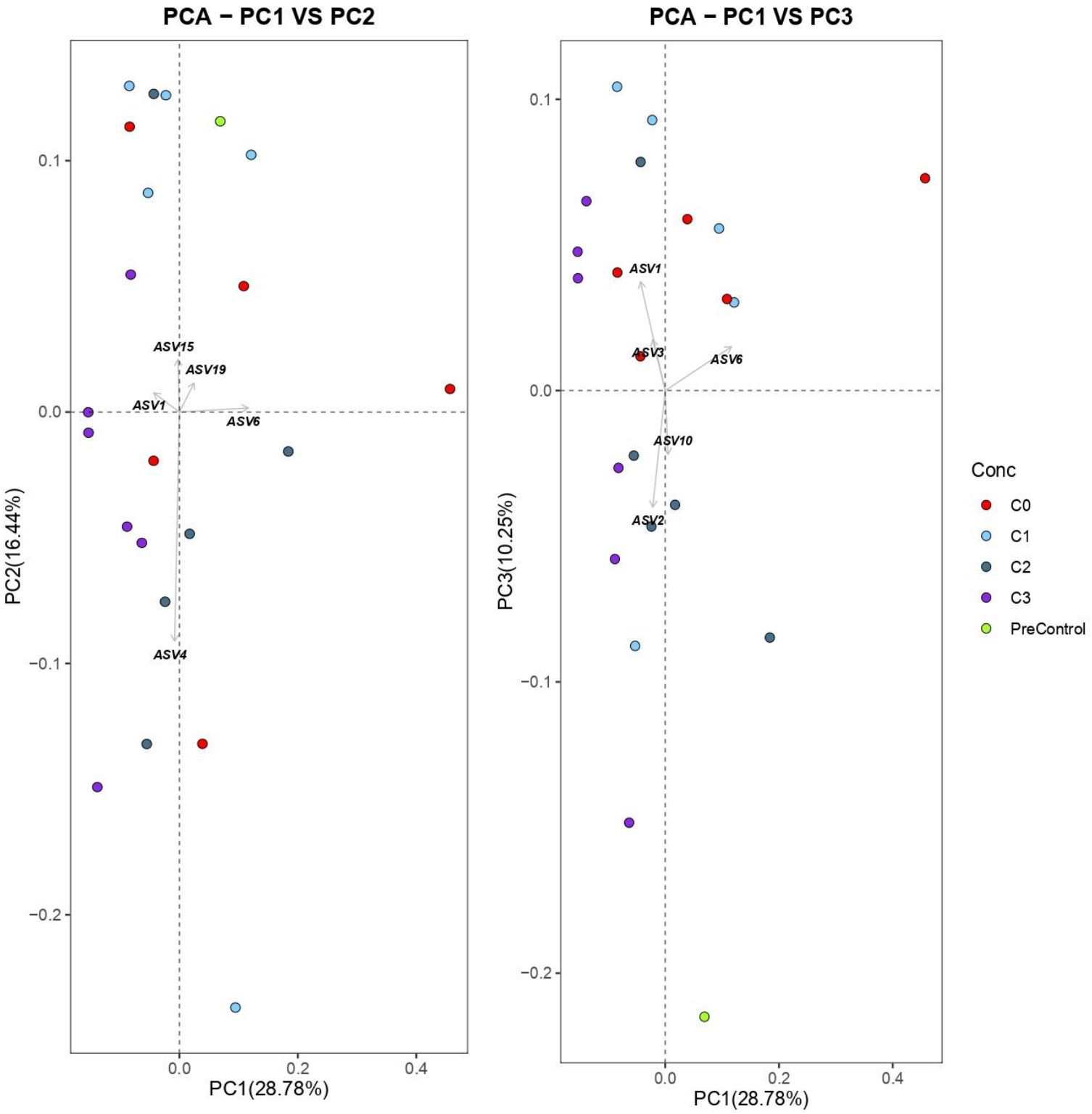

Figure 6: Principal component analysis for Difenoconazole treatments. Each circle represents a bacterial community of one cage.

PERMANOVA analysis was performed using Bray - Curtis distances. Results showed that 37% of differences can be explained by concentration. Pairwise comparisons

revealed that while there is no significant difference between both Control and PreControl and Concentration 1, as well between concentration 2 and controls. However, permutational analysis showed significant differences between C3 and C1 ($p = 0.027$), C3 and Control ($p = 0.01$), and C3 and C2 ($p = 0.032$). The lowest *p* value resulted from comparing Concentration 3 to Control, essentially proving that the treatment with higher concentration of Difenoconazole leads to a change in microbiome community structure.

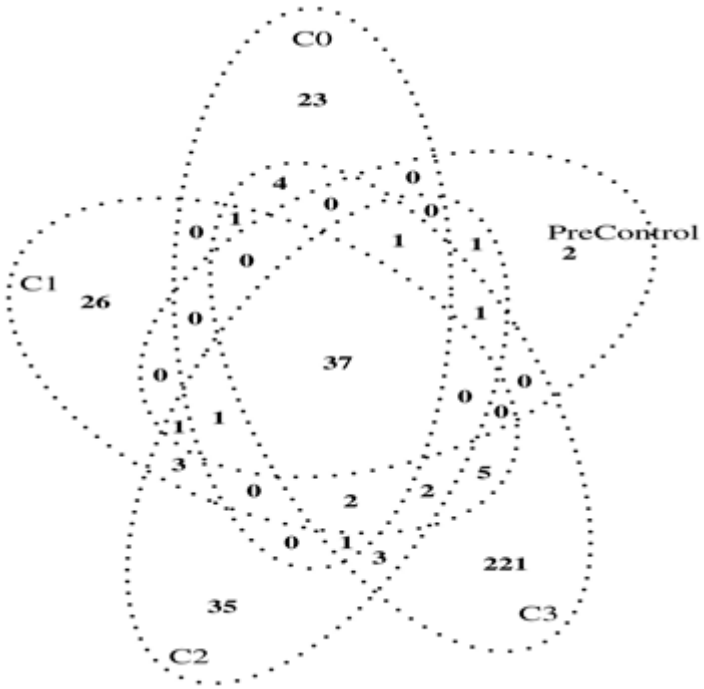

Figure 7: Venn diagram showing the number of ASVs that are shared and unique to each treatment in Difenoconazole chronic feeding.

The highest number of unique ASVs is presented in Concentration 3 (221 ASVs). The core microbiome is composed of 37 sequenced variants. These 37 species belong to the genera *Shodgrassella*, *Lactobacillus*, *Gilliamella*, *Commensalibacter*, *Frischella*, *Bombella*, *Bifidobacterium*, and *Bartonella* which is in line with the results of relative abundance analysis. The sequences, unique to Concentration 3, are representative of all taxa and include bacteria that are beneficial to honey bees such as *Bifidobacterium* as well as opportunistic pathogens like *Streptococcus* spp. They also include an array of opportunistic environmental bacteria, belonging to the *Microbacteriaceae* and *Legionellaceae.* Few species of genus *Flavobacterium* were discovered, and only in Concentration 3 exposure. *Flavobacterium* is an environmental bacterium that is found in soil and fresh waters. Some of its species are opportunistic pathogens of fish (Bernardet, 1996).

3.2.2. The effect of Cypermethrin on Honey bee gut microbiome

When compared to control cages with no exposure to active substance, treatment with C3 resulted in showed a significantly higher richness as measured by Chao1 index as per the Kruskal-Wallis test (Chi-squared = 6.5942, df = 3, p = 0.08602). Shapiro- Wilk test revealed a non-normal distribution of data, and Levene test showed homogeneity of variance for both pesticides.

The effect of Cypermethrin on both richness and diversity was similar to one obtained from Difenoconazole, as can be seen in the figure below (Figure 8).

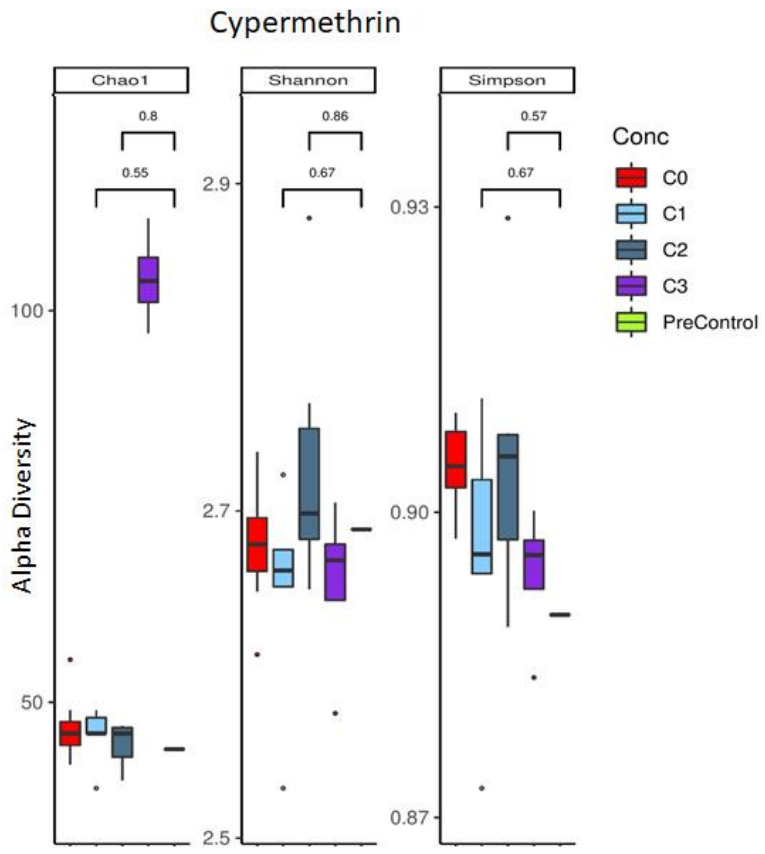

Figure 8: Alpha diversity estimation by Chao, Shannon and Simpson indices for treated and unexposed to Cypermethrin honey bees. C0, C1, C2, C3, indicate respectively Cypermethrin Control treatment, Concentration 1, 2, 3 and PreControl describes bees that were sampled before the beginning of the experiment. Interquartile range is represented by boxes, outliers by single dots and median is identified as a line. Whiskers extend to 1.5 of the interquartile values. Significance bars are 0.05.

Kruskal-Wallis test showed significant differences for the amount of ASVs, obtained in different treatments (chi-squared = 10.367, p = 0.03468). Chao1 index equals the amount of observed ASVs since no singletons were observed, similarly to Difenoconazole.

Abundance by Chao1 index was affected significantly only in Concentration 3 when compared to Control (p = 0.034) but not in other comparisons. Both dominance, indicated by Simpson index, and alpha diversity estimate by Shannon, do not show any dependence on Cypermethrin doses (p values are 0.247 and 0.33 correspondingly).

In general, the influence of Cypermethrin on richness and alpha diversity is similar to Difenoconazole effects. Figure 9 shows how relative abundance is affected by Cypermethrin.

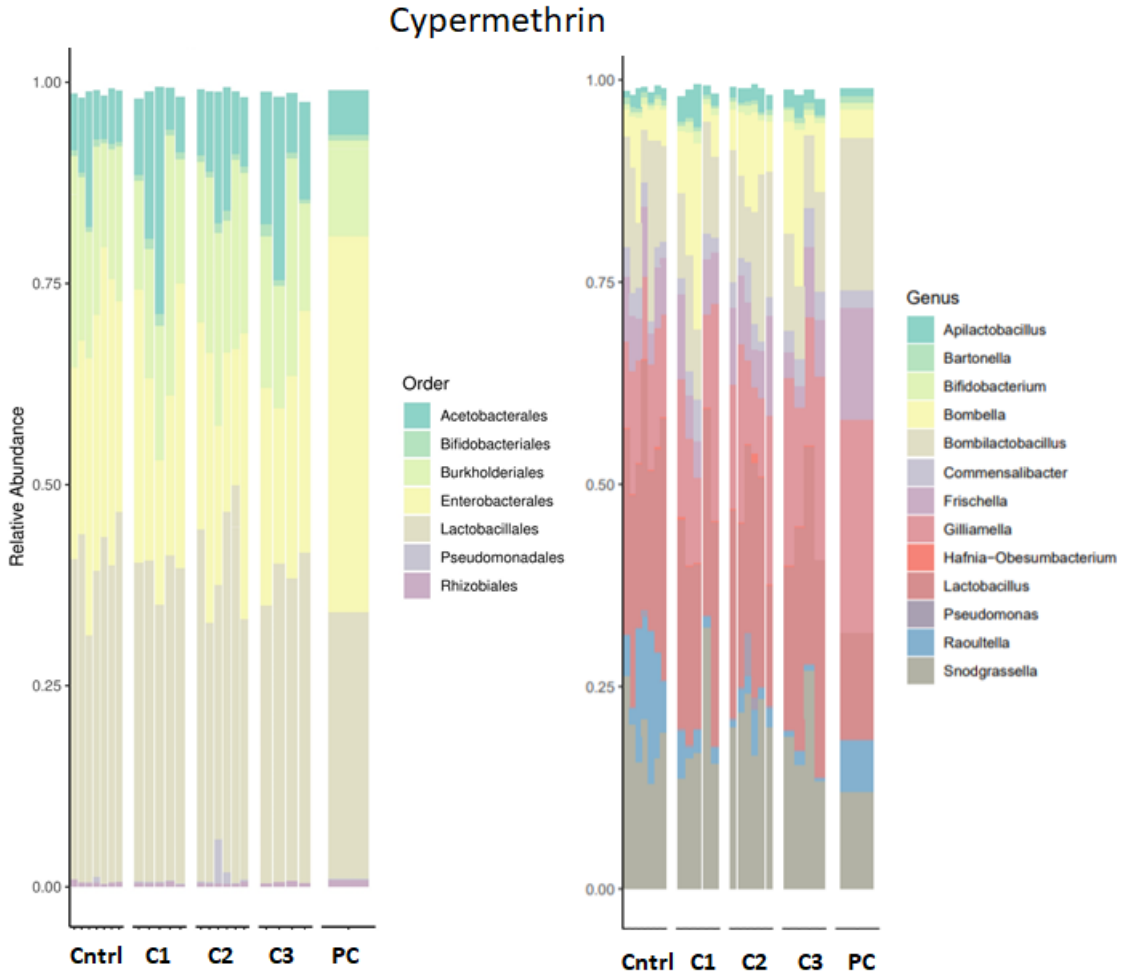

Figure 9: Relative abundance of Honey bee microbiome phyla in Cypermethrin treatments, sorted by order and genus. Cntrl indicates control and the absence of exposure, while C1, C2 and C3 represent Concentrations 1,2 and 3, and PC

represents bees sampled before the beginning of exposure (precontrol). Each single line in a bar chart signifies one cage.

The pattern changes for Cypermethrin exposure. An increase in order *Pseudomonales* was observed in Concentration 2, and a mild one in Concentration 3 when compared to Control as observed in relative abundance graphs. As before, all the main expected core species are observed. There is an inverse relationship between the Cypermethrin dosage and *Raoultella* abundance, as well as for Difenoconazole. *Frishella* abundance levels also are shown to decline in response to the higher pesticide dosage. The distribution of main orders is more stable and is not prominently affected except for orders *Acetobacterales* and *Enterobacterales*. These two orders are the most susceptible to both kinds of pesticides. The abundance of the main genera does not change except for the increase in *Bombella* in treated bees compared to controls. Contrasting with Difenoconazole, the overall remaining non-core species do not decrease in abundance,

Next, Principal component analysis of Bray-Curtis proportions was plotted. As it can be seen in Figure 10, Principal Component 1 explains 24% of the variance.

Four main dimensions were plotted: ASV2 - *Gilliamella* spp; ASV4 - *Bombella intestini;* ASV6 – *Raoultella* spp.; ASV11– *Snodgrassella alvi* and ASV13 - *Lactobacillus* spp. A strong negative correlation can be observed between *Bombella intestini* and *Raoultella* spp., as well as between *Snodgrassella alvi* and *Lactobacillus* spp.

and *Gilliamella.* Which corresponds to the previously reported results of Difenoconazole. At the same time, the highest concentration of Cypermethrin (C3) is explained and separated from other treatments by *Bombella intestini,* which is supported by the relative abundance data (Figure 9).

Lactobacillus and *Raoultella* carry high importance in explaining the differences for Control bacterial communities. Even though clear clustering is not present, the control cages samples are mostly grouped on the other side of ASV2 – ASV11 axis. *Snodgrassella alvi*, *Gilliamella* spp and *Bombella intestini* seem to be retained in exposed bees.

PCA1 vs PCA2 and PCA1 vs PCA3 show similar distribution and pattern pattern with 13,7% explained by PC3 and 19,3% explained by PC2. The biggest axis In PC2 vs PC1 analysis was plotted by ASV4 and a clear clustering of Control samples can be observed on the opposite side of it, indicating once again the influence of pesticide on Bombella presence.

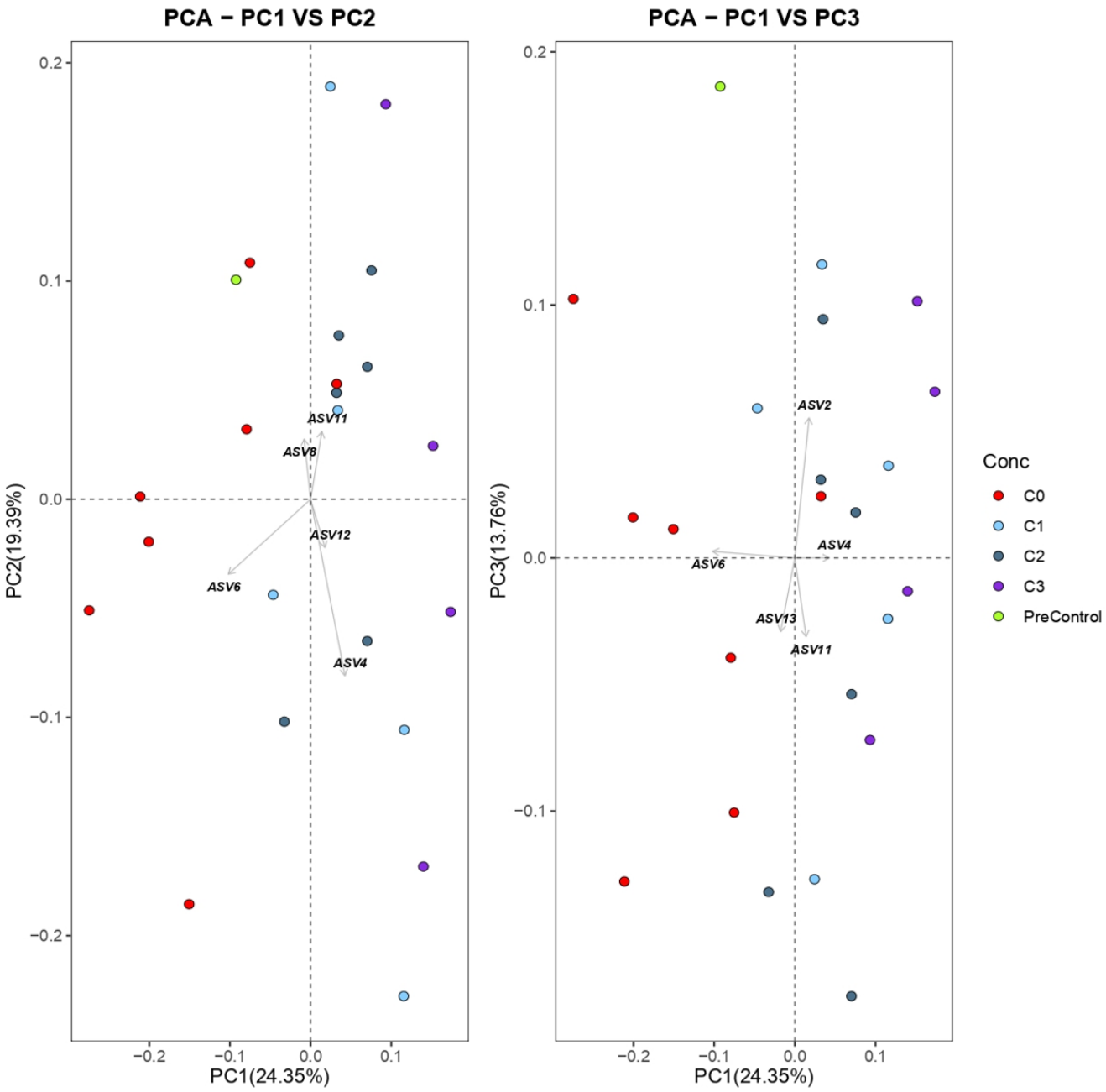

Figure 10: Principal component analysis for Cypermethrin treatments. Axes are represented by components 1 and 3. Each circle represents a bacterial community of one cage.

Bray-Curtis distances were used in the PERMANOVA analysis. The results were highly significant (p = 0.0001) and revealed that ingested concentration can account for 38% of the variances. While there is no significant difference between Control and PreControl and Concentration1, pairwise analyses revealed that there is a substantial difference between Concentration 2 and controls (0.036), as well as C3 versus

Control. Comparing Concentration 3 to Control yielded the highest $p = 0.01$.

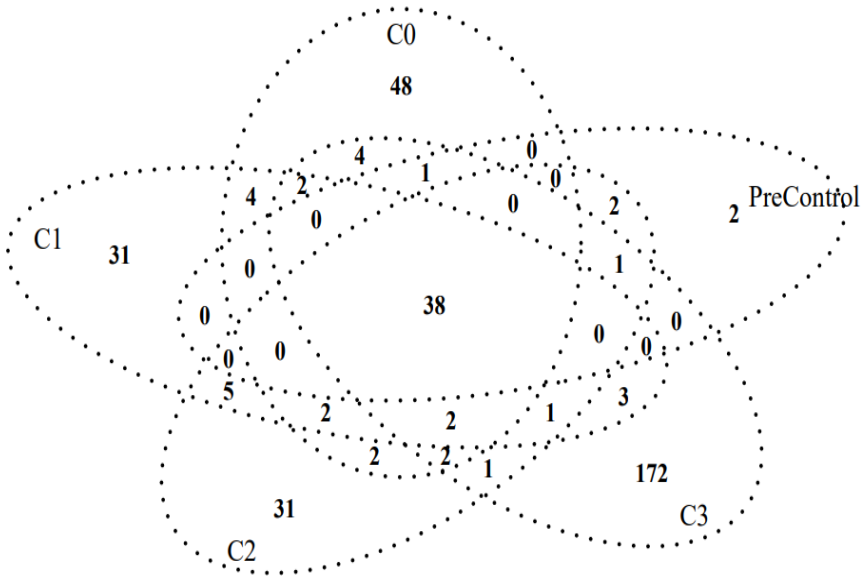

Figure 11: Venn diagram showing the number of ASVs that are shared and unique to each treatment in Cypermethrin chronic feeding. Each ellipse represents a kind of treatment.

The Venn diagram results support our findings and show once again the 38 core ASVs, shared between all treatments – Cypermethrin concentrations, Controls fed on sugar and bees, sampled before the start of the exposure (PreControl). These 38 species do not differ from those, reported from Difenoconazole or other studies, and belong to the genera *Shodgrassella, Lactobacillus, Gilliamella, Commensalibacter, Frischella, Bombella, Bifidobacterium, Raoultella* and *Bartonella.*

The highest number of unique ASVs is once again observed in Concentration 3, with 172 unique species. The difference, however, lays in the dynamic between Control and lower concentrations. Control treatment harbors 48 reported unique microorganisms, and Concentration 1 and 2 of Cypermethrin seem to negatively affect the richness of species, decreasing them compared to Control with 31 non-shared ASVs both. Bacteria, discovered as unique for Concentration 3 treatment only are mostly environmental and non-pathogenic, belonging to orders *Rhizobiales, Burkholderiales, Sphingomonadales, Myxococca* and other taxa, mostly found freely in the environment in soil and water.

4. Discussion and Conclusions

This work aimed to better understand the effect of sublethal doses of pesticides on honey bees microbiome composition. Two widely used pesticides - Difenoconazole and Cypermethrin were selected. This study expands the knowledge on how different members of the honey bee gut microbiome respond to exposure of sublethal pesticide doses. Results showed that while Difenoconazole and Cypermethrin do not influence survival, they disrupt the microbial community structure and increase the diversity of bacterial species in the hindgut.

4.1 Difenoconazole and Cypermethrin in sublethal concentrations do not impact animal survival

No significant differences in survival were observed for both Cypermethrin and Difenoconazole exposures. All doses proved not to have a statistically relevant effect on mortality when compared to their respective control treatments. This outcome was expected since the concentrations used here were lower than lethal ones (one-hundredth of LD50 is anticipated not to cause mortality) and is consistent with other reported results on honey bee mortality under chronic exposure to sublethal doses of pesticides (Cuesta-Maté et al., 2021). In a study by Cuesta-Mate et al. (2021), acetamiprid, thiacloprid, and oxalic acid were shown to both reduce and increase the survival of honey bees in sublethal doses depending on the location of the colony, respectively proving that mortality depended on the colony and not on the exposure type. Cypermethrin, however, has been shown to be highly toxic to honey bees with oral LD50 values ranging from 0.03 to 0.12 g/bee (WHO report, 1982). The action of the chemical is rapid (within 2 days).

In their study, Fent et al. (2019) exposed adult forager bees to a dosage of 0.3 ng/bee of Cypermethrin for 48 hours and no mortality occurred. Though, there is evidence that at the sublethal level of 0.3 ng/bee Cypermethrin induced differential expression of genes in the brain tissue. This further shows that even at sublethal level, Cypermethrin does induce changes in honey bee health status.

In the case of Difenaconazole, it has been shown that adult honey bees, exposed to contaminated food during a 4 day chronic exposure had a 76% survival rate, which is consistent with our results as well (on average, we had 61 alived bees at day 7 of 72 initial, which corresponds to 84%). While exposed to treated surfaces with Difenoconazole showed no mortality at all (survival was 100%) (Leite et al., 2021).

4.2 The diversity of the Honey bee gut microbiome increases in response to pesticide exposure

The diversity of the bacterial community changes dynamically across life stages and varies in bee workers of different ages (Zhi-Xiang et al., 2019). It is reported to be more susceptible to colonization by new species and exposure to toxins might induce this susceptibility.

According to the existing literature, Honey bee, wild bee and Bumble bee gut microbiomes share several similarities and are mainly composed by members of phyla such as *Firmicutes* and *Proteobacteria* (Engel et al., 2016; Moran et al., 2015). Our findings show the same main phyla, specifically belonging to the genera *Shodgrasella, Lactobacillus, Gilliamella, Bifidobacterium, Frishella and Bombella* (Engel et al., 2019). Their role in Honey bee is not completely established, as new evidence emerges every year and this field of research is growing. Bombella has been reported to suppress the growth of two opportunistic pathogens (Miller et al. 2020). *Lactobacillus spp* also plays an immune role, attenuating antibiotic - induced microbiota disruption (Daisley et al., 2020). Motta et al. (2018) showed that while glyphosate in sublethal concentrations perturbs the honey bee microbiota, the core microbiome taxa do not change and groups only change in terms of their relative abundance. These findings are consistent with our results, where we observed a similar effect of Difenoconazole and Cypermethrin. The treatments had a considerable impact on richness in terms of observed ASV numbers but not on dominance. None of the core taxa were fully erased, instead, their relative abundance was altered and new ASVs were added in exposed animals, showing in some cases different numbers of exclusive ASVs.

Overall, the core microbial community showed resilience while exposed to the lower doses in this study (Concentration 1 and 2) for both pesticides. Most of the significant differences were obtained while comparing bees in the control treatment with Concentration 3. This provides an answer for one of the questions posed by this study and indicates that doses below the 1/25 of LC50 are too low to cause a noticeable shift in the honey bee microbiome. Moreover, Cypermethrin decreased the relative abundance genera such as *Raoultella* and *Frishella,* and caused an increase in *Bombella* spp. Permethrin, a chemical that is very similar in structure to Cypermethrin, has shown antibacterial effects towards *Lactobacillus* in rats (Nasuti et al., 2016). Our results also showed a slight reduction of *Lactobacillus* abundance treated with Cypermethrin. The widespread use of pyrethroids like cypermethrin in the ecosystem may have affected the bacterial composition of insects, favoring pesticide metabolizing microbiota and hence fostering insecticide resistance, as has already been documented (Muturi et al., 2021).

Contrary to previous findings, Difenoconazole did not cause a significant increase in *Lactobacillus* spp., although it did cause higher abundance of non-assigned taxa. Similarly to Cypermethrin, there was a reverse correlation between the concentration of Difenoconazole and the presence of *Raoutella* in the samples. Both Difenoconazole and Cypermethrin showed reduction of Raoultella. Similarly, carbaryl pesticide also decreases Enterobacteriales order in exposed honey bee microbiota from initial 26.7% to 13,4% (Nogrado et al., 2019).

These findings imply that low doses of pesticides may not cause noticeable changes in behavior or mortality, but would still cause changes on the microbiome level and, by disrupting community in the bee hindgut, may open the "space" (available niches) for opportunistic pathogens. Consequently, this increases the pathogenic load in the environment and facilitates the spread of diseases between insect populations. It may also pose an additional risk for humans that are handling the insects, as *Legionella* and *Streptococcus* genera were identified in honey bees treated with Concentration 3 of Difenoconazole.In a previous study, the number of opportunistic pathogens has been reported to increase in response to Trichlorfon pesticide (Giambò et al., 2021)

4.3 Limitations and suggestions for further investigations

Considering our experimental design, several limitations restrict the generalization of our findings. The lack of possibility to perform the study on newly emerged bees was one of these factors. Due to the seasonal and timeframe limitations, the study was performed on adult winter bees. Increased age leads to increased mortality in samples, which makes it harder to have a chronic feeding test. Also, it is known that gut microbiomes differ during the foraging season in summer from the one in winter (Kesnerova et al., 2020). Moreover, there are indications that non-core members colonize older bees' guts more frequently, suggesting that their microbiome composition is more variable (Ellegaard & Engel, 2019). These findings suggest that age should be incorporated as one of the main factors in experimental design. Therefore, the effects of pesticides on summer bees might slightly vary from obtained ones and should be targeted in further studies. Secondly, recovery from the pesticide influence was not checked. Over the duration of chronic feeding, the microbiome of honey bees could have adapted and while being more affected during the first days could restore its structure by the end. Additional research should be conducted to check the microbial community response and composition changes in time. Additionally, a higher amount of pre-control samples and a more detailed analysis of bacterial communities before treatments would assist in understanding the role of acclimation and restraining to lab conditions, which may also be playing a factor. Lastly, even though most of ASVs were identified on the family level, a lot of species were not classified on the genus level. An improvement of

reference databases would help by identifying more taxa that in this case are kept unknown and may also be playing important roles in bee health status.

4.4 Conclusions

Treatment with both Cypermethrin and Difenoconazole led to changes in bacterial community structure and richness, but not in dominance or alpha diversity. Overall, slight changes were observed in microbiome community structure while core members of the holobiont showed resilience and were always present in exposure to both pesticides in all the studied concentrations.

Observed ASV richness significantly increased but only in response to the highest doses of both pesticides, proving that the effect of a pesticide (Difenoconazole or Cypermethrin) is altered at least for 1/25 from LC50.

This might lead to pesticide residues, that are present in the environment, to affect non-targeted pollinators.

Unique ASVs were represented by an array of different orders, belonging to beneficial bacteria previously found in bees (*Lactobacilliales*) as well as environmental opportunistic microbes (*Burkholderiales*).

While usually harboring a persistent and established microbiome, honey bees affected by pesticides could serve as reservoirs for opportunistic bacteria. These results contribute towards a better understanding of the microbial community of honeybees. A more thorough examination of the long-term consequences of pesticides on honeybee health is required.

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1. Annex

Annex 1.

Table A-I-1 **:** List of data of food consumption, pesticide intake and total bee survival at the end of the exposure for Difenoconazole.

Treatment	**Control**	**C1**	**C2**	**C3**
Number of cages	6	6	6	6
Nominal dose (µg a.i./bee)	0	0.3	0.6	1.2
Concentration (µg a.i./mL food)	0	8.108	16.22	32.43
Concentration (µg a.i./g food)	0	6.796	13.596	27.184
Surviving bees	60	68	61	59
Dead bees	12	4	11	13
Average food consumption (g/bee/day)	0.050	0.045	0.042	0.039

SD food consumption (g/bee/day)	0.013	0.015	0.010	0.008
Average intake of pesticide (µg a.i./bee/day)	0	0.308	0.573	1.050
SD intake of pesticide (µg a.i./bee/day)	0	0.099	0.142	0.229
Accumulated intake of pesticide (µg a.i./bee)	0	12.941	24.091	44.124

Annex 2.

***Table A-I-2*:** List of data of food consumption, pesticide intake and total bee survival at the end of the exposure for Cypermethrin.

Treatment	**Control**	**C1**	**C2**	**C3**
Number of cages	7	6	6	6
Nominal dose (µg a.i./bee)	0	0.0004	0.0007	0.0014
Concentration (µg a.i./mL food)	0	0.0095	0.0190	0.0014
Concentration (µg a.i./g food)	0	0.0080	0.0159	0.0380
Surviving bees	66	62	63	59
Dead bees	18	10	9	13
Average food consumption (g/bee/day)	0.0463	0.0459	0.0478	0.0435
SD food consumption (g/bee/day)	0.0137	0.0155	0.0192	0.0175
Average intake of pesticide (µg a.i./bee/day)	0	0.0004	0.0008	0.0014
SD intake of pesticide (µg a.i./bee/day)	0	0.0001	0.0003	0.0006
Accumulated intake of pesticide (µg a.i./bee)	0	0.0154	0.0312	0.0581