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KILMER OLIVEIRA SOARES

EFEITO DE AGROQUÍMICOS SOBRE A MICROBIOTA INTESTINAL DE ABELHAS

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EFEITO DE AGROQUÍMICOS SOBRE A MICROBIOTA INTESTINAL DE ABELHAS

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PARECER DE DEFESA DO TRABALHO DE TESE

TÍTULO: "EFFECT OF AGROCHEMICALS ON THE INTESTINAL MICROBIOTA OF HONEY BEES"

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Dedico

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[1]"From my rotting body, flowers shall grow
and I am in them, and that is eternity."

[1] "Do meu corpo apodrecido, flores crescerão, e eu estarei nelas e isso é a eternidade".
- Edvard Munch

RESUMO

Apesar das abelhas serem responsáveis por garantir a estabilidade ecológica de inúmeros ambientes, são suscetíveis a várias atividades antrópicas que podem resultar na extinção das espécies devido à degradação ambiental, como por exemplo, o uso de agroquímicos. As abelhas africanizadas (*Apis mellifera scutellata* x spp.) desempenham um papel significativo na comercialização de produtos derivados da apicultura no Brasil. No entanto, muito pouco se conhece sobre essas abelhas, uma vez que se originaram recentemente no Brasil (1956), através de cruzamento acidental envolvendo algumas abelhas europeias (*Apis mellifera* spp.) e africanas (*Apis mellifera scutellata*). Considerando o papel fundamental da microbiota intestinal das abelhas para a saúde e, consequentemente, polinização dos diversos biomas, essa tese objetiva investigar a composição da microbiota intestinal de abelhas africanizadas relativamente a abelhas europeias e africanas, além de avaliar, experimentalmente, alterações da microbiota associados à exposição a agroquímicos. O capítulo 1 (The honey bee gut microbiome: a review from the perspective of bees threatened by agrochemicals) trata de uma revisão sobre o microbioma intestinal de abelhas e suas alterações associadas ao uso de agroquímicos. No Capítulo 2 (Africanized honey bees (*Apis mellifera scutellata* x spp.) gut microbiota: is it similar to African or European bees?), apresentamos uma avaliação comparativa *in sílico*, utilizando dados de sequenciamento do gene RNAr 16S de 126 amostras intestinais de abelhas européias (n=34), africanas (n=82) e africanizadas (n=10). Os resultados demonstraram que a microbiota intestinal de abelhas africanizadas tem maior similaridade àquela das abelhas europeias, embora a abundância relativa do gênero *Snodgrassella* foi maior em abelhas africanizadas e africanas em comparação com abelhas européias. O gênero *Gilliamella* foi mais abundante em abelhas africanizadas em comparação com abelhas africanas. O capítulo 3 (Tetracycline exposure alters key gut microbiota in africanized honey bees (*Apis mellifera scutellata* x spp.) objetivou avaliar, experimentalmente, o efeito da exposição ao antibiótico tetraciclina sobre a microbiota intestinal de abelhas africanizadas. Amostras (DNA total do intestino) de abelhas do grupo controle (CON: alimentadas diariamente com xarope (10 g) composto por uma solução 1:1 de açúcar demerara e água, além de uma dieta protéica sólida (10 g) composta por 60% de extrato de soja e 40% de xarope de açúcar) e do grupo exposto (TET: alimentadas de forma idêntica ao CON, mas com a adição de cloridrato de tetraciclina na dose de 450 μg/g ao xarope de açúcar) foram obtidas de cada grupo antes (dia 0) e após a exposição à tetraciclina (dias 3, 6 e 9). Após sequenciamento da região V3-V4 do gene 16S rRNA em Illumina MiSeq, e processamento através do QIIME2 e DADA2, os resultados demonstraram diferenças significativas na composição (Jaccard) e na diversidade (Shannon) da microbiota intestinal, sendo tais diferenças cada vez mais acentuadas conforme maior o tempo de exposição. TET reduziu a abundância relativa de *Bombella* e *Fructobacillus*, juntamente com a diminuição da microbiota essencial, como *Snodgrassella*, *Gilliamella*, *Rhizobiaceae* e *Apibacter*. Como esses microrganismos são críticos para o metabolismo de nutrientes e defesa de patógenos, é possível que a diminuição deles possa afetar negativamente a saúde das abelhas. O Capítulo 3 (The effects of zinc and laboratory emerge date on honey bee gut microbiota) refere-se à um estudo experimental sobre o efeito do zinco sobre a microbiota de abelhas européias. O zinco é frequentemente encontrado no escoamento agrícola, uma vez que é usado em fertilizantes, inseticidas e aditivos alimentares. Estudos sobre os supostos efeitos do zinco na saúde das abelhas são, no entanto, escassos. Neste estudo, as abelhas foram expostas a sete concentrações de zinco (0, 50, 100, 250, 500 ou 1000 mg/L). A sobrevivência foi apenas minimamente impactada (>89% de sobrevivência) em concentrações de zinco até 100 mg/L. Para avaliar os efeitos do zinco na microbiota intestinal, as abelhas foram expostas a concentrações de 5 ou 100 mg/L de zinco. Amostras de intestino foram coletadas antes (dia 0) e após exposição (dias 3, 6 e 9). O conteúdo abdominal foi submetido à extração de DNA e

sequenciamento da região V3-V4 do gene 16S rRNA (V3-V4) em um Illumina MiSeq. As sequências foram filtradas e processadas através de QIIME2 e DADA2. Apesar da exposição ao zinco ter efeitos mínimos nas concentrações de DNA bacteriano e contagens absolutas microrganismos, foi observada diferença significativa nas concentrações entre os diferentes dias de emergência (momento que a abelha emerge do favo de cria). Nessa situação, observou-se diminuição das concentrações bacterianas. Diferenças significativas na diversidade e abundância taxonômicas foram observadas em abelhas expostas à maior concentração de zinco (100 mg/L), no qual observou-se redução de vários táxons benéficos (*Lactobacillu*s, Rhizobiaceae, *Gilliamella*) e aumento de *Paenibacillus*, um táxon potencialmente patogênico. Os resultados indicam que a exposição ao zinco, mesmo em níveis relativamente baixos, pode comprometer a saúde das abelhas, mesmo que a sobrevivência não seja drasticamente afetada.

Palavras-chave: flora bacteriana de abelhas; subespécies de abelhas; tetraciclina; zinco.

ABSTRACT

Effect of agrochemicals on the intestinal microbiota of bees

Although bees are responsible for ensuring the ecological stability of numerous environments, they are susceptible to various human activities that can result in the extinction of species due to environmental degradation, such as the use of agrochemicals. Africanized bees (*Apis mellifera scutellata* x spp.) play a significant role in the commercialization of beekeeping products in Brazil. However, very little is known about these bees, since they originated recently in Brazil (1956), through accidental crossing involving some European (*Apis mellifera* spp.) and African (*Apis mellifera scutellata*) bees. Considering the fundamental role of the intestinal microbiota of bees for health and, consequently, pollination of the various biomes, this thesis aims to investigate the composition of the intestinal microbiota of Africanized bees in relation to European and African bees, in addition to evaluating, experimentally, changes in the microbiota associated with exposure to agrochemicals. Chapter 1 (The honey bee gut microbiome: a review from the perspective of bees threatened by agrochemicals) deals with a review of the intestinal microbiome of bees and its changes associated with the use of agrochemicals. In Chapter 2 (Africanized honey bees (*Apis mellifera scutellata* x spp.) gut microbiota: is it similar to African or European bees?), we present a comparative evaluation in silico, using 16S rRNA gene sequencing data from 126 bee intestinal samples European (n=34), African (n=82) and Africanized (n=10). The results demonstrated that the intestinal microbiota of Africanized bees is more similar to that of European bees, although the relative abundance of the genus *Snodgrassella* was higher in Africanized and African bees compared to European bees. The genus *Gilliamella* was more abundant in Africanized bees compared to African bees. Chapter 3 (Tetracycline exposure alters key gut microbiota in africanized honey bees (*Apis mellifera scutellata* x spp.) aimed to experimentally evaluate the effect of exposure to the antibiotic tetracycline in Africanized bees. (CON: fed daily with syrup (10 g) composed of a 1:1 solution of demerara sugar and water, plus a solid protein diet (10 g) composed of 60% soy extract and 40% sugar syrup) and exposed group (TET: fed identically to CON, but with the addition of tetracycline hydrochloride at a dose of 450 μg/g to sugar syrup) were obtained from each group before (day 0) and after exposure to tetracycline (days 3, 6 and 9) After sequencing the V3-V4 region of the 16S rRNA gene in Illumina MiSeq, and processing through QIIME2 and DADA2, the results showed significant differences in the composition (Jaccard) and diversity (Shannon) of the microbiota intestinal, sen such differences become more accentuated as the exposure time increases. TET reduced the relative abundance of *Bombella* and *Fructobacillus*, along with a decrease in essential microbiota such as *Snodgrassella*, *Gilliamella*, Rhizobiaceae and *Apibacter*. As these microorganisms are critical for nutrient metabolism and pathogen defense, it is possible that their decrease could negatively affect bee health. Chapter 3 (The effects of zinc and laboratory emerge date on honey bee gut microbiota) refers to an experimental study on the effect of zinc on the microbiota of European bees. Zinc is often found in agricultural runoff as it is used in fertilizers, insecticides and food additives. Studies on the supposed effects of zinc on bee health are, however, scarce. In this study, bees were exposed to seven concentrations of zinc (0, 50, 100, 250, 500 or 1000 mg/L). Survival was only minimally impacted (>89% survival) at zinc concentrations up to 100 mg/L. To evaluate the effects of zinc on the intestinal microbiota, the bees were exposed to concentrations of 5 or 100 mg/L of zinc. Gut samples were collected before (day 0) and after exposure (days 3, 6 and 9). Abdominal contents were subjected to DNA extraction and sequencing of the V3-V4 region of the 16S rRNA gene (V3-V4) on an Illumina MiSeq. Sequences were filtered and processed through QIIME2 and DADA2. Although exposure to zinc has minimal effects on bacterial DNA concentrations and absolute microorganism counts, a significant difference in concentrations was observed between the different days of emergence (moment that the bee

emerges from the brood comb). In this situation, a decrease in bacterial concentrations was observed. Significant differences in taxonomic diversity and abundance were observed in bees exposed to the highest concentration of zinc (100 mg/L), in which a reduction of several beneficial taxa (*Lactobacillus, Rhizobiaceae, Gilliamella*) and an increase of *Paenibacillus*, a taxon potentially pathogenic. The results indicated that the exposure to zinc, even at relatively low levels, can negatively compromise bee health, even if survival is not drastically affected.

Keywords: bacterial flora of bees; bee subspecies; tetracycline; zinc.

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LISTA DE FIGURAS COMPLEMENTARES

Bee gut microbial composition was significantly altered by treatment (pre-treatment, control, tetracycline) (PERMANOVA: Jaccard R2= 0.115, p = 0.001) and time (D0, D3, D6, D9) (PERMANOVA: Jaccard R2= 0.046, p = 0.001; **Figure 3, Supplementary material. Figure 1).** Notably, the interaction of treatment and time was also significant (Adonis: R2= 0.035, p-value= 0.024), and the effect of treatment increased over time (**Figure 3, Supplementary material. Figure 1**). Microbial diversity also differed significantly by time but not by treatment (Two-way ANOVA: Shannon Index treatment p = 0.295, time p = 0.042; **Figure 4a**). No pairwise comparisons were significant; although, microbial diversity differences on Day 9 (p = 0.081) were greater than at previous timepoints, with the tetracycline group having lower diversity than the control group. Microbial community evenness (Pielou's Index) did not differ

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LISTA DE TABELAS COMPLEMENTARES

LISTA DE ABREVIATURA, SIGLAS E SÍMBOLOS

ANCOM Analysis of composition of microbiomes

ANOVA Análise de variância

ASV Amplicon Sequence Variants

BR Brazil

CAPES Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CCA Centro de ciências Agrárias

CCD Colony Colapse desorder

CON Control

DNA ácido desoxirribonucleico

EUA United States of American

GOX Glucose oxidase

INMET Instituto nacional de meteorologia

KE Kenya

LABE Laboratório de abelhas

LAPOA Laboratório De Avaliação De Pordutos De Origem Animal

MA Massassussets

NCBI National Center for Biotechnology Information

NGS Next Generation Sequence

OIE World Organisation for Animal Health

PB Paraiba

PB Paraíba

PCR Polimerase chain reaction

PD Phylogenetic Diversity

PERMANOVA Permutational analysis of variance

RNA Ácido ribonucleico

SISBIO Sistema de Autorização e Informação em Biodiversidade

SJC São Joaão do Cariri

SRA Sequence Read Archive

SW Switzerland

TET Tetracycline

TX Texas

UFPB Universidade Federal da Paraiba

UK United Kingdom

US United States

SUMÁRIO

INICIAL CONSIDERATIONS

Bees are responsible for ensuring the ecological stability of numerous environments. They have recently been made susceptible by several human activities that have resulted in the extinction of the species due to environmental degradation. The use of agrochemicals is amajor hazard to their survival. Africanized honey bees (*Apis mellifera scutellata* x spp.) play a significant role on the commercialization of apiculture-derived goods in Brazil. These subspecies originated recently in Brazil (1956) as a result of an incidentalcrossbreeding between European (*Apis mellifera* spp.) and African bees (*Apis mellifera scutellata*). Considering that there are significant genetic differences exist between European and African branches, we hypothesize that these variations may have an impact on the composition of the gut microbiome, which is crucial for preserving bee health since the microorganisms are responsible for various tasks such as nutrient breakdown, pesticide tolerance, and pathogen behavior.

Bees may be exposed to tetracycline residues in the environment either directly or indirectly due to the widespread use of antimicrobials, such as tetracyclines, in agriculture, medicine, and beekeeping. Tetracycline exposure in European honey bees has been associated with changes in the gut microbiota that are harmful to bee health. However, antibiotics' impact on the intestinal microbiome of Africanized honey bees has notbeen studied yet. For instance, zinc is frequently used as fertilizers, insecticides, and feed additives and therefore commonly detected in agricultural runoff. By ingesting pollen and nectar contaminated with zinc, honey bees can be exposed to zinc either directly or indirectly. Studies on the purported effects of zinc on honey bee health, however, are scarce.

Considering the critical role of the intestinal microbiota of bees for health and, consequently, pollination of the various biomes, this thesis aimed at investigating the composition of the intestinal microbiota of Africanized bees in relation to European and African bees, in addition to evaluating, experimentally, changes in the microbiota associated with exposure to agrochemicals.

CAPÍTULO 1: REFERENCIAL TEÓRICO - “The honey bee gut microbiome: a review from the perspective of bees threatened by agrochemicals”

The honey bee gut microbiome: a review from the perspective of bees threatened by agrochemicals

Abstract: Although it is very well known that bees play a key role in the stability of several ecosystems globally, anthropogenic activities causing environmental degradation, such as the use of agrochemicals, can ultimately lead to the extinction of native species, with severe ecological consequences. The advances in high throughput sequencing technologies and bioinformatics tools in the last decades have contributed to the understanding of the importance of the microbiome for the health of different animal species. However, there is a paucity of information on the effects of the exposition to agrochemicals on the microbial composition of honeybees and how this can compromise their health. In this systematic review article, we discuss the composition and biological function of native honeybee gut microbiota and how it can be possibly affected by agrochemicals, including antibiotics. We performed a systematic literature review focusing on the effect of agrochemical use on the gut microbiota of honey bees. A bibliographical survey was conducted from 1950 to 2023 in all available databases. We used the keywords “honey bees”, “microbiome” and “agrochemicals”. A total of 94 papers addressing this topic were selected, carefully checked andgrouped into three categories: 1) The bee microbiome and its importance; 2) Functional roles of the bee gut microbiome; and 3) The problem of the anthropic actions that threaten the gut microbiome of bees. The present report can serve future studies addressing the complex ecological aspects of antimicrobial usage in agriculture and livestock in the scope of honeybee preservation.

Keywords: bees, intestines, microbiota, pesticides.

O microbioma intestinal das abelhas melíferas: uma revisão sob a ótica das abelhas ameaçadas por agroquímicos

Resumo: As abelhas garantem a estabilidade ecológica de diversos ecossistemas onde são encontradas. Ultimamente, elas têm sido ameaçadas por muitas atividades antrópicas levando ao desaparecimento de espécies devido à degradação ambiental. Entre as muitas ameaças à sua sobrevivência, está o uso de agrotóxicos. Diante disso, foi realizada uma revisão de literatura com foco no efeito do uso de agroquímicos sobre a microbiota das abelhas. Foi realizado um

levantamento bibliográfico no período de 1950 a 2023 em todas as bases de dados disponíveis. Foram utilizadas as palavras-chave “abelha melíffera”, “microbiota” e “agroquímicos”. Selecionamos 94 artigos abordando o tema. Os artigos selecionados foram agrupados em três: A microbiota das abelhas e sua importância, Funções do microbioma intestinal das abelhas e O problema das ações antrópicas que ameaçam o microbioma intestinal das abelhas. Observou-se que as abelhas estão sujeitas a diversas ameaças que estão causando o declínio de suas populações; o microbioma das abelhas desempenha um papel fundamental na saúde do hospedeiro; muitas atividades humanas ameaçam a microbiota intestinal das abelhas.

Palavras-chave: Abelhas, microbiota intestinal, pesticidas

1. INTRODUCTION

The humankind has shaped and will continue to shape global biodiversity through long- and short-term activities.(ALBUQUERQUE *et al.*, 2018). Human activity is considered the major cause of disturbances on the planet´s ecosystems (FOR; FUTURE, 2019; VITOUSEK *et al.*, 2018) that ultimately affect living native species, including bees. The absence of honeybees in ecosystems is considered a bioindicator of changing global agricultural landscapes or environmental pollution (CELLI; MACCAGNANI, 2003; QUIGLEY; AMDAM; HARWOOD, 2019).

Among all animals, bees are considered the most important insect pollinators because of their foraging behavior and pollination constancy (CORBET; WILLIAMS; OSBORNE, 1991). The lack of honey bees is considered a bioindicator of changing global agricultural landscapes or environmental pollution (CELLI; MACCAGNANI, 2003; QUIGLEY; AMDAM; HARWOOD, 2019). Considering that they play a critical role for both crop production and reproduction of native plant species (BREEZE *et al.*, 2011; GILL *et al.*, 2016; HRISTOV *et al.*, 2020; STAVERT *et al.*, 2020), the decline of bee populations due to the various threats and consequently the reduction in pollination can cause serious imbalances in important biomes. It is predicted that several bee species will face a reduction and three species an increase in their suitable habitat in the coming years (LIMA; MARCHIORO, 2021). According to Smith. *et al.*(2022), it is estimated that about 500,000 human deaths annually could be caused by a 3-5% global loss in fruit and vegetable production due to inadequate pollination.

According to the evolutionary perspective, agrochemicals are considered recent stressors. The exposition of bees to agrochemical compounds, such as fipronil and deltametrin, does not

trigger rapid responses compared to other biological stress factors commonly present in their habitats during their evolution, such as temperature changes and infectious agents (BORDIER *et al.*, 2017).

Currently, there is a great concern about the exposure of bees to chemicalssuch as neonicotinoid compounds including clothianidin, dinotefuran, imidacloprid and its metabolites, thiamethoxam, and nitenpyram, due to their harmful effects on bees physiology even at small doses (DECOURTYE; DEVILLERS, 2010; IWASA *et al.*, 2004; MOTTA; RAYMANN; MORAN, 2018; RAYMANN; SHAFFER; MORAN, 2017; SOARES *et al.*, 2021). In addiction, several recent studies point that other different chemicals can disturb the behavior of bees, such as organophosphate compounds, antimicrobials, herbicides and fungicides (GUSSONI; RIBEIRO, 2017; JOHNSON, Renée, 2013; MOTTA; RAYMANN; MORAN, 2018; RAYMANN; SHAFFER; MORAN, 2017).

Insects are a very diverse group of animals, thriving in many diverse ecosystems. Evolutionarily, these animals have developed adaptations in their digestive systems to feed on a variety of substrates ranging from wood to blood (KAČÁNIOVÁ *et al.*, 2017). Therefore, nutrient digestion depends on many microorganisms living in interaction with the host species. Microbes play a key role in life as ecological interactions with microorganisms are often necessary for the survival of animal species due physiological and biochemical processes that are necessary for the functioning of organic systems. The bee commensal microbiome plays a key role on the insect digestive processes by participating in the metabolism of macronutrients (LEE *et al.*, 2015). Problems associated with disturbances in the normal microbiota of bees can lead to increased mortality (MOTTA; RAYMANN; MORAN, 2018; RAYMANN; SHAFFER; MORAN, 2017).

Considering the importance of the microbiota on the health of bee species, the understanding of the microbial composition and how it is associated with the environment, and the potential dysbiosis caused by agrochemicals exposure and its impact on the physiological and ecological functions of bees, there is a need to develop actions towards animal and vegetal conservation in the different biomes, and to improve sustainable agricultural productivity.

The aim of this review was to provide an overview on potential effects of agrochemical exposure on the gut microbiome and health of honey bees.

1.1. THE BEE MICROBIOME AND ITS IMPORTANCE

Microorganisms have played a crucial role in the evolution of vertebrate and invertebrate animals by means of mutualism, by which both microorganisms and animal hosts benefit from the interaction (FARRER *et al.*, 2022; MORRIS, Sherri J; BLACKWOOD, 2015; WU, Cindy H. *et al.*, 2009). Such interactions are not only necessary for the survival of the species but they are also important in the ecological context. Interactions can occur between phylogenetically distant organisms. For instance, plants depend on a diversity of microorganisms to obtain nutrients, as in the case of the interaction between plants and bacteria of the *Rhizobium* genus responsible for the conversion of atmospheric nitrogen (N2) to ammonia (NH3) by means of the hydrogenase enzyme (MUS *et al.*, 2018).

Animals have co-evolved with a wide range of microorganisms responsible for the development of vital functional host systems, such as digestive, hormonal and immune systems (BLASER, 2014; MCFALL-NGAI *et al.*, 2013; NEGASH, 2022). Animals, even herbivores, do not have the ability to degrade and obtain nutrients from fibrous carbohydrates from the cell wall of plants since they do not produce the necessary enzymes. Therefore, several gut microorganisms such as fungi, protozoa and mainly bacteria are responsible for the degradation of the ingested fiber making the nutrients available to the animals. In return, the animal provides an appropriate habitat for the microorganisms in terms of temperature, pH, nutrients and water activity.

Although insects fed on a wide range of different types of subtracts (KAČÁNIOVÁ *et al.*, 2017), they do not necessarily synthetize the enzymes that are necessary for their degradation. This role is rather played by microorganisms. Termites, for example, depend on the action of various gut microorganisms that degrade cellulose and lignin (WARNECKE *et al.*, 2007)

Bees evolved from a group of solitary, carnivorous, apoid wasps with the rapid rise of angiosperms (ALCOCK *et al.*, 1978; BRANSTETTER *et al.*, 2017; DYER *et al.*, 2012; JOHNSON *et al.*, 2013). The new individuals switched from carnivory to pollen feeding (pollenivory) (BRANSTETTER *et al.*, 2017). This process is believed to have led to the diversification of bees (MURRAY; BOSSERT; DANFORTH, 2018).

It is known that bees live in symbiosis with various microorganisms that are responsible for specific functions ranging from nutrient degradation (LEE *et al.*, 2015) to defense against

pathogenic agents (MOTTA; RAYMANN; MORAN, 2018), as well as bee´sbehavior (VERNIER *et al.*, 2020).

According to Kwong *et al*.(2017), the bacterial species representing the core microbiota of all corbiculate bee, highly social (eusocial) bees, comprising the honey bees, bumble bees, and stingless bees, are: *Gilliamella*, *Bifidobacterium*, *Lactobacillus* Firm-4, and *Lactobacillus* Firm-5. Besides these species, the honey bee (*Apis mellifera* spp.) gut microbiota also presents some lineage-specific phylotypes: *Bartonella apis* (KEŠNEROVÁ; MORITZ; ENGEL, 2016) and *Frischella* (ENGEL; KWONG; MORAN, 2013).

There are remarkable differences in the composition of the microbiome according to the age and functional role of honey bees. The gut of newly emerged worker bees may have no or only a small number of bacteria until the ninth day (POWELL *et al.*, 2014). During this period, they perform an important role on the hive working as nurses, feeding the younger bees (BOMFIM; OLIVEIRA; FREITAS, 2017). Jones *et al*., (2018a) observed different relative abundances of gut microbial taxa among bees depending on their funcions. For instance, bees performing activities within the nest showed a greater abundance of abundance of *Lactobacillus mellis* and *Bifidobacteriaceae* (ENGEL; MARTINSON; MORAN, 2012). In case of nursing bees, the ones that firstly contact the newly emerged brood, are possibly the main inoculators by means of trophallaxis, a process of food transfer by which a given individual pass the contents of its digestive tract to another through regurgitation (SUAREZ; THORNE, 2000).

The contact between older and younger bees ensures the successful transmission of the microbiota (ENGEL *et al*., 2016). Due to the reproductive biology of bees, transmission occurs mainly horizontally. The transmission differs according to the different bacterial species. In *Apis mellifera*, the microbiota transfer can take place both directly, through trophallaxis, (mainly for lactobacilli and *Bifidobacterium*) or by contact with the hive surface, which seems to be particularly relevant for the transfer of Firmicutes. Other Gram-negative bacteria such as *Gilliamella apícola, Frischella perrara,* and *Snodgrassella alvi* depend on the direct contact with fresh feces from other individuals (POWELL *et al.*, 2016).

Once acquired and established, the microbiome plays several functions in the physiology of bees. In terms of functionality, the bee gut microbiome is composed of seven groups according to their functional role: protein metabolism, carbohydrate metabolism, RNA metabolism, respiration, membrane transport, stress response and virulence (LEE *et al.*, 2015). Among these crucial functions, we highlight the ability of some microbes to degrade vegetal carbohydrates and polypeptides by means of glycosidases that breakdown complex polysaccharides and peptidases for protein hydrolysis.

1.2. FUNCTIONAL ROLES OF THE BEE GUT MICROBIOME

Like all other animals, bees need essential nutrients for their survival and reproduction. In many bee species, the diet is basically composed of nectar, which is converted into honey, their main source of carbohydrates, and pollen that is converted into "bee bread" by means of a fermentation process, which provides amino acids, lipids, vitamins and minerals to host bees (AL-KAHTANI *et al.*, 2021; BRODSCHNEIDER; CRAILSHEIM, 2010). Restriction of a number of sugars, such as ramnose, fucose, mannose, sorbose, lactose, melibiosis, galactytol, erythritol and inositol, may compromise bee health (HAYDAK, 1970; TAN *et al.*, 2007). In terms of amino acids, bees require ten essential ones that are all obtained from pollen: arginine, histidine, lysine, tryptophan, felinanima, methionine, threonine, leucine, isoleucine and vanilla (HAYDAK, 1970).

The digestion of pollen itself is very difficult and bees cannot fed on pollen in its natural form (HAYDAK, 1970). Several characteristics of "bee bread" indicate the microbiome to play a key function making nutrients available to the host. For instance, the same lactic acid bacteria found in the bee crop are also found in bee bread suggesting a link between these two microbial habitats (LEE *et al.*, 2015; VÁSQUEZ; OLOFSSON, 2009).

Different members of bee gut microbiota perform specific tasks regarding the digestion of plant tissues. *Lactobacillus mellis* and *Bifidobacteriaceae*, which are involved in the processing of complex carbohydrates, are more abundant in bees performing tasks within the nest, such as food processing and brood feeding, such as the nurse bees (ENGEL; MARTINSON; MORAN, 2012). *Gilliamella* species digest pectin from pollen, and the *Lactobacillus* species inhibit the growth of foulbrood bacteria (REGAN *et al.*, 2018). Bacterial members of the community that produce glycosidases and peptidases (Bacilli, γ- and β-Proteobacteria, Actinobacteria, and Clostridia) play a role on the breakdown of plant polysaccharides and oligopeptides, initiating the processing of complex plant material (LEE *et al.*, 2015). According to Lee *et al*. (2014), functional peptidase encoding-genes have been identified in bacilli, including proline aminopeptidase, dipeptidase, as well as aminopeptidases C. These are fundamental mechanisms that are necessary for the availability of nutrients by the previous degradation of the plant cell wall.

The putative role of the microbiome in the maturation of honey and beebread is still not well understood. Although these processes occur outside the insect, they depend on microbes directly derived from the bee body that are deposited in food cells with digestive enzymes and

organic acids that are present in the secretions of the salivary glands of the bees (DEVEZA *et al.*, 2015). Once sealed, the food cells provide adequate anaerobic environment for the proliferation of microorganisms and the production of important enzymes. (ANDERSON, K. E. *et al.*, 2011; DEGRANDI-HOFFMAN; ECKHOLM; HUANG, 2013; ENGEL; MARTINSON; MORAN, 2012; ENGEL; MORAN, 2013; KIELISZEK *et al.*, 2018; LEE *et al.*, 2015; VÁSQUEZ; OLOFSSON, 2009; WARNECKE *et al.*, 2007). This process improves pollen characteristics by making nutrients available to bees through changes in their composition. There is a significant reduction in complex polysaccharides, changes in the amino acid profile and increased amounts of simple carbohydrates (BEUTLER; OPFINGER, 1948; HERBERT; SHIMANUKI, 1978). The final composition of bee bread is rich in carbohydrates (35 – 61% dry weight), lower in pH than raw pollen, and it contains essential amino acids in appropriate amounts that are necessary for bees development (BRODSCHNEIDER; CRAILSHEIM, 2010; HUMAN; NICOLSON, 2006).

Nectar is collected from flowers by bees, stored in the crop, taken to the colony and then passed on to other bees by trophallaxis. The honey production process continues inside the nest when enzymes (e.g., invertase) and microbes are added to nectar. Trophallaxis in the nest is also important in the reduction of the amount of water in nectar through evaporation (HAYDAK, 1970; NICOLSON; HUMAN; PIRK, 2022; VÁSQUEZ; OLOFSSON, 2009). Once deposited into the food cells, nectar is covered with an operculum, providing anaerobic environment, as happens with pollen. By ingesting and regurgitating nectar, bees inoculate microorganisms including γ-Proteobacteria, Bacilli and Actinobacteria that areresponsible for the breakdown and fermentation of complex macromolecules and the generation of various fermentation products, such as short-chain fatty acids and alcohol (LEE *et al.*, 2015).

The immune defense mechanisms of honeybees, as in other eusocial animals, such as ants and other bees, have important peculiarities in several aspects. Bees present the so called social immunity, characterized by the secretion of antiseptic compounds, such as glucose oxidase (GOX) throughout the colony, thereby providing collective immune protection to other individuals in the hive, comprised by hundreds to many thousands of individuals (GIANNINI *et al.*, 2015; JONES, Ben; SHIPLEY; ARNOLD, 2018; RAVAIANO *et al.*, 2018). Secondly, honeybees also possess an innate immune system, known as individual immunity that is commonly found in other animals. It is charactered by several generalist defense mechanisms, such as physical barriers, as well as cellular and humoral responses, providing immunity against

a wide variety of infectious and parasitic organisms (LARSEN; REYNALDI; GUZMÁN-NOVOA, 2019).

The biological relationship between host and microbiota is usually neglected. Gut microbial communities can greatly affect host health by modulating the host's immune system (KWONG; MANCENIDO; MORAN, 2017). According to Koch and Schmid-Hempel, (2011) the host microbiota can be known as an "extended immune phenotype" in addition to the host immune system itself, providing an unique perspective to understanding bees in health and disease. It is know that the bee gut microbiota provides a protective role against pathogenic agents such as viruses, bacteria and protozoa (DOSCH *et al*., 2021; ENGEL; MARTINSON; MORAN, 2012; FORSGREN *et al*., 2010; KOCH; SCHMID-HEMPEL, 2011; POWELL *et al*., 2016; REGAN *et al*., 2018)

Kwong *et al*. (2017a) observed up-regulation of gene expression of the antimicrobial peptides apidaecin and hymenoptaecin in honeybee gut tissue when inoculated with the normal microbiota compared with bees deprived from normal microbiota.

Gut microbiota can also form biofilms providing a physical barrier in the gut against pathogens. *Snodgrassella* is in direct association with the host tissue followed by a thick layer of *Gilliamella* (ENGEL; MARTINSON; MORAN, 2012). In *Bombus* species *Gilliamella* and *Snodgrassella* were shown to protect against a protozoan parasite(KOCH; SCHMID-HEMPEL, 2011). It was suggested that these bacteria forms a biofilm acting as a protective layer against parasite invasion.

1.3. THE PROBLEM OF THE ANTHROPIC ACTIONS THAT THREATEN THE GUT MICROBIOME OF BEES

The deterioration of the environment caused by deforestation and urbanization of rural areas are the greatest threats to bees. The decrease in forest areas leads to the unavailability of plant species that serve both as housing and as food sources for bees. Disturbances causing the decline of pollinator species can lead to the extinction of several plant species due to the lack of a specific pollinator or seed spreader. This is the case of the extension of the tree species *Sideroxylon grandiflorum* in Mauritius after the extinction of the bird *Raphus cucullatus.* According to Temple (1977), this process occurred due to the absence of a specific seed disseminator.

The small number of colonies in a given region forces queens to mate with related individuals, which leads to inbreeding that contributes significantly to the manifestation of recessive genes (CAIRES; BARCELOS, 2017; NOGUEIRA-NETO, 2002). This condition leads the colonies to death in fifteen to thirty generations (KERR *et al.*, 1996). The colonies die gradually due to the elimination of the queen and lack of worker bees (CAIRES; BARCELOS, 2017).

The indiscriminate use of agrochemicals, such as neonicotinoids, organophosphate compounds, heavy metals or antibiotics that are eventually used in agricultural livestock production systems is another important threat. Conversely to the several natural stressors, such as endemic parasites and increasing global temperature, that contributed to the development of physiological and behavioral mechanisms enabling bees to respond towards the maintenance of homeostasis, agrochemicals are considered new stressors in an evolutionary perspective, recently originated by the intensification of human actions on the ecosystems. Bordier *et al*. (2017) observed that bees exposed to the chemicals fipronil and deltamethrin failed to present a rapid response to stress compared to common stressors such as heat and immune challenge, supporting the hypothesis that damage caused by new chemical stressors can be devastating to bees.

Antimicrobials are widely used in animal production systems. Some of these chemicals are poorly absorbed by animals therapeutically treated with them. Tetracyclines, for example, have 30 to 90% of the administered drug excreted in active forms in urine and feces (CHEE-SANFORD *et al.*, 2009; KHAN; ONGERTH, 2004; WATKINSON *et al.*, 2009). Part of it goes to the environment carried by wastewater or farm runoff (BORRELY *et al.*, 2012; FARIA *et al.*, 2016; HENDRIKSEN *et al.*, 2019).

In some agricultural settings, antibiotics are sprayed on crops at high concentrations(100 to 4,166 ppm) to control bacterial infections in fruit trees (CHANVATIK *et al.*, 2019; HOPKINS, 1979). Antibiotic residues can be found in urban and rural wastewaters (BORRELY *et al.*, 2012; FARIA *et al.*, 2016; HENDRIKSEN *et al.*, 2019) andbees can be exposed to then in both situations (LAU; NIEH, 2016). Tetracycline residues have been detected in irrigation water (0.14 ppm), pig waste lagoons (0.7 ppm), soil (25 ppm), hospital effluents (0.53 ppm), and wastewater treatment plants (0.92 ppm) (MEYER *et al.*, 2000; PENA *et al.*, 2010; WANG *et al.*, 2014).

Glyphosate, an organophosphate compound that has been widely used in crops around the world (LI *et al.*, 2019) is known to negatively affect the microbiome of bees. Glyphosate

can disrupt the beneficial gut microbiota, potentially affecting bee health and their effectiveness as pollinators (MOTTA; RAYMANN; MORAN, 2018).

Zinc is commonly used as soil fertilizers at 5 kg ha-1 for every year or alternate years (SANGEETHA *et al.*, 2022). Zinc is directly sprayed on leaves in many crops worldwide (DANISH TOOR *et al.*, 2020; PATHAK; GUPTA; PANDEY, 2012; SEMIDA *et al.*, 2021; WANG, Jianwei *et al.*, 2012; WASAYA *et al.*, 2017). Zinc can also be combined with pesticides (RAM *et al.*, 2015). It is the active ingredient of a systemic bactericide under development for use against citrus greening (NARANJO *et al.*, 2020). Therefore, bees can be exposed to zinc while foraging in different. However, the effect of zinc on honeybee gut microbiota is still unknown.

2. FINAL CONSIDERATIONS

Bees can be exposed to a number of threats that are causing their populations to declineand therefore compromising pollination.

The gut microbiome plays a fundamental role on the health of bees. Shifts in the composition of thegut microbiota can lead to disturbances in the organic functions of these animals and, consequently, leading to death. The effects of agrochemicals on the gut microbiota of bees should be investigated in order to support sustainable agricultural practices that improve crop productivity associated with preservation of animal species.

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CAPÍTULO 2 - "Africanized honey bees (*Apis mellifera scutellata* x spp.) gut microbiota: is it similar to African or European bees?"

Africanized honey bees (*Apis mellifera scutellata* x spp.) gut microbiota: is it similar to African or European bees?

Abstract: Honey bees are important for many reasons such as ecosystem health, biodiversity maintenance, and crop yield. In Brazil, the Africanized honey bees (*Apis mellifera scutellata* x spp.) have notable importance in the commercialization of products that come from beekeeping. This subspecies was recently originated in Brazil, in 1956, through an accident that lead to the crossbreeding between some European (*Apis mellifera* spp.) with African bees (*Apis mellifera scutellata*). European and African branches present remarkable genetic differences. We assume that these differences can reflect in gut microbiome composition which is very important for the maintenance of bee health. Since the microorganisms are responsible for specific functions ranging from nutrient degradation to pesticide and pathogen tolerance and behavior. To test this we downloaded 126 High-throughput sequencing data of 16s rRNA from NCBI short read archive (SRA) public database. The sequences were collected from different parts of the globe: European subspecies from Austin, Texas – US, Sussex, UK; Laussane, Switzerland. The African ones were collected from five sites in Kenya: Kakamega, Kilifi-Coast, Kwale-Coast, and Nairobi totaling 82 samples. The other 10 samples, the Africanized honey bees, were collected from two different places located in the State of Paraíba – Brazil: in the municipalities Areia and São João do Cariri. These results have shown that the Africanized honey bees present a gut microbiota composition similar to the European honey bees. Some abundance of key bacteria such as *Snodgrassella* were higher in Africanized and African bees compared to European bees and *Gilliamella* were higher in Africanized bees compared to African bees. 4 genera were associated with Africanized bees such as *Snodgrassella, Lactobacillus, Bifidobacterium,* and *Apibacter,* and Orbaceae is associated to African and Africanized honey bees. These results may suggest that pathogen resistance in African and Africanized honey bees can is linked to the gut microbiome composition.

Keywords: Africanized honey bee, Gut, Microbiota, subspecies

Abelhas africanizadas (*Apis mellifera scutellata* x spp.) microbiota intestinal: é semelhante às abelhas africanas ou europeias?

Resumo: As abelhas são muito importantes por muitas razões, como a saúde do ecossistema, a manutenção da biodiversidade e o rendimento das colheitas. No Brasil, as abelhas africanizadas (*Apis mellifera scutellata* x spp.) têm grande importância na comercialização de produtos apícolas. Estas subespécies foram originadas recentemente no Brasil, em 1956, através do cruzamento entre alguns zangões de abelhas européias (*Apis mellifera* spp.) com algumas abelhas rainhas africanas (*Apis mellifera* scutella). Ramos europeus e africanos apresentam diferenças genéticas notáveis. Assumimos que essas diferenças podem refletir na composição do microbioma intestinal, cada uma sendo muito importante para a manutenção da saúde das abelhas. Uma vez que os microrganismos são responsáveis por funções específicas que vão desde a degradação de nutrientes até a tolerância e comportamento a pesticidas e patógenos. Para testar isso, baixamos 126 dados de sequenciamento de alto rendimento de 16s rRNA do banco de dados público do arquivo de leitura curta (SRA) do NCBI. As sequências foram coletadas em diferentes partes do globo: subespécies européias de Austin, Texas – EUA, Sussex, Reino Unido; Laussane, Suíça. Os africanos foram coletados em cinco locais no Quênia: Kakamega, Kilifi-Coast, Kwale- Coast e Nairobi, totalizando 82 amostras. As outras 10 amostras, as abelhas africanizadas, foram coletadas em duas cidades diferentes do Estado da Paraíba – Brasil: Areia e São João do Cariri. Esses resultados mostraram que as abelhas africanizadas apresentam uma composição da microbiota intestinal semelhante às abelhas europeias. Algumas abundâncias de bactérias-chave, como Snodgrassella, foram maiores em abelhas africanizadas e africanas em comparação com abelhas europeias e Gilliamella foi maior em abelhas africanizadas em comparação com abelhas africanas. 4 espécies foram associadas a abelhas africanizadas como Snodgrassella, Lactobacillus, Bifidobacterium e Apibacter, e Orbaceae está associada a abelhas africanas e africanizadas. Esses resultados podem sugerir que a resistência a patógenos em abelhas africanas e africanizadas pode estar ligada à composição do microbioma intestinal.

Palavras-chave: Abelhas, microbioma intestinal, subspecies de abelhas

1. INTRODUCTION

Honey bees are very important for many reasons such as ecosystem health, biodiversity maintenance, and crop yield. Genetic research shows that honey bees originated in Asia and

migrated to Africa and Europe (WALLBERG *et al.*, 2014). Naturally, they occur in Europe, the Middle East, and Africa (MORTENSEN; SCHMEHL; ELLIS, 2013). Thirty-three distinct honey bee subspecies are presented in Africa (11 subspecies), Western Asia and the Middle East (9 subspecies), and Europe (13 subspecies), which subdivided into 5 evolutionary lineages: lineage A (10 subspecies), sublineage Z of the lineage A (3 subspecies), lineage M (3 subspecies), lineage C (10 subspecies), lineage O (3 subspecies), lineage Y (1 subspecies), lineage C or O (3 subspecies) (ILYASOV *et al.*, 2020).

Due to economic benefits such as pollination services and honey and propolis production, the western honey bee has been spread extensively around the world (MORTENSEN; SCHMEHL; ELLIS, 2013). In Brazil, the European branch of honey bees was first introduced in 1839 when the priest Antonio Pinto Carneiro imported European honey bees (*Apis mellifera*) from the countries of Portugal and Spain (DE QUEIROZ ROLIM *et al.*, 2018). Later, between 1845 and 1880, German and Italian immigrants introduced other subspecies of the *Apis mellifera* (DE QUEIROZ ROLIM *et al.*, 2018). This mixture created a European type bee from Brazil to United States. Before Africanization, bees were of mixed Eurasian ancestry, dominated by C group but admixed with M and O groups in bees collected in the United States (WHITFIELD *et al.*, 2006).

More than one century later, in 1956, professor Warwick Estevam Kerr brought from Tanzania and South Africa 26 queens of *A. mellifera scutellata*. The colonies escaped and had become established in the wild and mated with the European branch bees already spreaded in Brazil originating the Africanized Honey bees (*Apis mellifera scutellata* x spp.) (MICHAEL; J. D.; NALEN, 2015).

Bees live in symbiosis with various microorganisms in complex interactions. These host-microbiota interactions are complexes and changes during the life stages, season and castes (DONG *et al.*, 2021; KAPHEIM *et al.*, 2015; KEŠNEROVÁ *et al.*, 2020; POWELL *et al.*, 2018). Some key roles are performed by these microbiota members such nutrient degradation (LEE *et al.*, 2015), defense against pathogenic agents (MOTTA; RAYMANN; MORAN, 2018), pesticide tolerance (WU, Yuqi *et al.*, 2020), behavior by modulatin cuticular odour and the level of aggression in ants and honeybees (TESEO *et al.*, 2019; VERNIER *et al.*, 2020) and inhibit the growth of fungal pathogens (ENGEL *et al.*, 2016; MILLER; SMITH; NEWTON, 2021).

The contact between older and younger bees ensures the transmission of the microbiome (ENGEL *et al.*, 2016). Due to the reproductive biology of bees, transmission occurs mainly horizontally. The transmission differs according to the different bacterial species. In *Apis mellifera*, the microbiota transfer can take place both directly, through trophallaxis, (mainly for lactobacilli and *Bifidobacterium*) or by contact with the hive surface, which seems to be particularly relevant for the transfer of Firmicutes organisms. Other Gram-negative bacteria such as *Gilliamella apicola*, *Frischella perrara,* and *Snodgrassella alvi* depend on the direct contact with fresh feces from other individuals (Powell *et al*.,2014).

European and African branches present remarkable genetic differences (FRANCK *et al.*, 1998; HAN; WALLBERG; WEBSTER, 2012; WALLBERG *et al.*, 2014). Most part of the studies on the effect of agrochemicals are made with European honey bees (CUESTA-MATÉ *et al.*, 2021; DIAZ *et al.*, 2019; HOTCHKISS; POULAIN; FORREST, 2022; KAKUMANU *et al.*, 2016; MOTTA; RAYMANN; MORAN, 2018; NOGRADO *et al.*, 2019; PARIS *et al.*, 2020; RAYMANN *et al.*, 2018). First step to evaluate if the effect of these agrochemicals on the gut microbiota of honey bees is to understand these differences on the phylosymbiosis approach. We hypothesize that host population genetic clusters are reflected by the bacterial community structures. This study aims to identify differences in composition and diversity in the gut microbiota of the African, European and Africanized honey bee branches using 16S rRNA amplicon data and microbial ecology analyses.

2. METHODS

2.1. EXPERIMENTAL DESIGN OF IN SILICO DATA ACQUISITION

126 High-throughput sequencing data of 16s rRNA were downloaded from NCBI short read archive (SRA) public database. We chose the samples based on classification as control groups treatments, workers and in the nursing stage in the manuscripts. It means that the samples were collected from hives in natural field condition. We chose the ones from several countries (**Table 1)**. Sequences of European branch were: 15 samples from Austin, Texas – US (MOTTA; RAYMANN; MORAN, 2018) (https://www.ncbi.nlm.nih.gov/bioproject?term=PRJNA432210 and https://www.ncbi.nlm.nih.gov/bioproject?term=PRJNA480015); 15 from Sussex, UK (JONES, J C *et al.*, 2018) (https://www.ncbi.nlm.nih.gov/bioproject/PRJEB23224); 14 from Laussane, Switzerland (KEŠNEROVÁ *et al.*, 2020)

(https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA578869). The African ones were collected from five sites in Kenya: 17 from Kakamega, 22 from Kilifi-Coast, 20 from Kwale-Coast and 23 from Nairobi totaling 82 (TOLA *et al.*, 2020) (https://zenodo.org/record/4559793#.Y4JUlHbMK5c). The other 10 samples, the Africanized honey bees were collected in steriles tubes containing 70% alcohol from hives raized in two different counties in the State of Paraíba – Brazil: Areia (6° 58′20″ S; 35° 43′16.9″ W; Altitude 545 m) (sequences found in (SOARES *et al.*, 2021) and São João do Cariri (7°22'55.7"S 36°31'40.9"W; Altitude 458m). All bacterial 16s rRNA genes from 136 samples, 126 obtained *in silico* and the 10 from Brazil, were PCR amplified and sequenced in Illumina Miseq. Primers and targeted regions are listed in Table 1. Raw sequences were filtered and processed using the default parameters of the NGS QC Toolkit version 2.3 (PATEL; JAIN, 2012).

2.2. SAMPLING, DNA EXTRACTION, LIBRARY PREPARATION, AND SEQUENCING OF THE AFRICANIZED HONEY BEES

The DNA extraction methods of the other samples are described in Table 1. For Africanized Honey bees, two sets of five samples were collected in the counties of Areia-PB and São João do Cariri-PB.

Prior to extraction, bees were placed on sterile filter paper for 10 min for defrosting and alcohol evaporation. Bee intestines were dissected by using a sterile pair of scissors to make a cross-sectional cut across the last segment of the bee abdomen. With sterile tweezers, abdominal content was collected out of the abdomen and transferred into microtubes.

Prior to extraction, bees were placed on sterile filter paper for 10 min for defrosting and alcohol evaporation. Bee guts were dissected by using a sterile pair of scissors to make a cross-sectional cut across the last segment visible of the bee abdomen avoiding external contamination. With sterile tweezers, abdominal contents were pulled out of the abdomen and transferred into microtubes. Abdominal contents from 20 bees, , representing one sample, were pooled into a single tube for DNA extraction, which was performed using a commercial kit (PowerSoil DNA Isolation kit, Qiagen, Germany) following the manufacturer's protocol. After extraction, DNA was electrophorized in agarose gel for quality analysis. DNA concentrations were quantified by fluorometry (Qubit 2.0. Life Invitrogen, United States) before further processing steps.

The Library Preparation, and Sequencing of the other samples are described in Table 1. For Africanized Honey bees, the V3-V4 region of the microbial 16S rRNA gene was amplified by PCR using 2.5 μL template DNA (5 ng/μL), 5 μL forward primer, 5 μL reverse primer, and

12 μL 2X KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, United States) in a total volume of 25 μL. The following primers were used: 341F (5′–TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC A–3′) and 805R (5′–GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C–3′). PCR reaction conditions were as follows: Initial denaturation at 95°C for 3 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension to 72°C for 5 min (SOARES *et al.*, 2021).

Amplification products were visualized in 1.5% agarose gel before purification using magnetic beads (AMPure XP, Beckman Coulter, United States) to remove excess primer. The dual indices and Illumina sequencing adapters were attached using a Nextera XT Index Kit (Illumina). A second clean up step was then performed using magnetic beads. The purified PCR products were quantified by fluorometry (Qubit 2.0, Life Invitrogen). For sequencing, pooled libraries were denatured with NaOH, diluted with hybridization buffer, then heat denatured. Paired-end sequencing was performed on an Illumina MiSeq with a V2 kit (2 × 250 cycles). At least 5% PhiX DNA was added for sequencing control purposes (Kit PhiX, Illumina).

2.3. SEQUENCE DATA PROCESSING AND STATISTICAL ANALYSIS

The script used for these analyses are found on **Script 1**.

The 136 raw demultiplexed paired-end sequences were processed using QIIME 2-2020.2 (Bolyen *et al*., 2019). Paired-end reads were filtered, denoised, and truncated to a length of 248 base pairs, and then parsed for non-chimeric sequences using DADA2, producing Amplicon Sequence Variants (ASV) (Callahan *et al*., 2016).

Paired-end reads were downloaded separately and then merged using the command “qiime feature-table merge”. Sequences were aligned using “qiime fragment-insertion sepp” for phylogenetic analysis (MATSEN *et al.*, 2012). According to Liu *et al*. (2020), the V3V4 amplicon analysis is compatible with the V4 amplicon analysis after trimming to the same region (**Script 1**). Taxonomic composition of the samples were determined rypla pretrained naive Bayes classifier with a 99% sequence similarity threshold for V3-V4 reference sequences (SILVA-132-99-nb-classifier.qza) and the “qiime feature-classifier classify-sklearn”.

All the alpha diversity analyses were made using QIIME 2-2020.2 (Bolyen *et al*., 2019) accordind to the Sampson *et al*. (2016) script. Microbial diversity was assessed using number of observed ASVs, Shannon (richness and abundance), Faith’s PD (phylogenetic diversity) and

Pielou's (evenness) diversity indices. The indexes were chosed. ANOVAs were used to compare diversity between groups using R version 4.1.0(Ripley, 2001) after to obtain the indexes. After testing for normality using a Shapiro-Wilk test, the means were compered using Tukey test or Kruskal-Wallis Test at 5% probability.

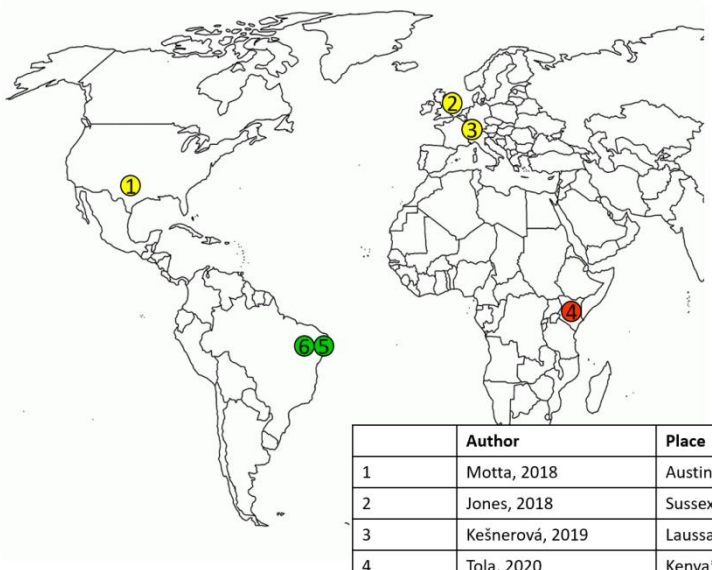
Beta diversity was evaluated using Bray-Curtis, Jaccard, Weighted and Unweighted Unifrac distances in QIIME 2-2020.2. Microbial community similarity and dissimilarity were evaluated by Principle Coordinate Analysis (PCoA) and visualized using the Emperor plugin 2020.2.0 (Vázquez-Baeza *et al*., 2017).Pairwise PERMANOVA was employed as recommended (Anderson, 2001) to test for differences in microbial composition between the branches.

Taxa that are differentially abundant between the treatment groups were identified using an analysis of composition of microbiomes (ANCOM) (Mandal *et al*., 2015) with p values adjusted at 0.05 (Table 2). By means of QIIME, we also identified core taxa, which were present in 90% of the samples using the command "qiime feature-table core-features" (Table 3) **(Script 1)**. Both at the level genera taxonomy. The relative abundances of core microbes were compared by treatment using one-way ANOVA followed by Tukey test or Kruskal-Wallis Rank Sum Tests followed by pairwise comparisons using Pairwise Wilcoxon Rank Sum Tests with adjustment method Bonferroni after normality of data distribution was confirmed by the Shapiro-Wilk test in R 4.1.0 (Ripley, 2001) **(Script 2)**. A *P*-value < 0.05 was used in the statistical tests for significance.

In addition, an multipatt function from the "Indicspecies" analysis package, which assigns a test statistic, or 'indicator value' to each ASV for each group (De Cáceres and Legendre, 2009; de Cáceres *et al*., 2011), was ran, using the "Indicspecies" v.1.7.9 package in R (function "r.g.", with α=0.5), to assess the strength of the relationship between phylotype occurrence and abundance and the branches **(table 4) (Script 3)**.

3. RESULTS

We examined the gut microbiota of 140 samples of three branches of honey bees from six places worldwide (Figure 1, Supplemental table 1).



	Author	Place	Number of samples
1	Motta, 2018	Austin, Texas, US	15
2	Jones, 2018	Sussex, UK	15
3	Kešnerová, 2019	Laussane, Switzerland	14
4	Tola, 2020	Kenya*	(17 + 22 + 20+ 23) = 82*
5	Soares, 2021	S.J. Cariri , PB - Brazil	5
6	Data not published	S.J. Cariri, PB - Brazil	5

*Samples from Kenya comprises fours diferente cities

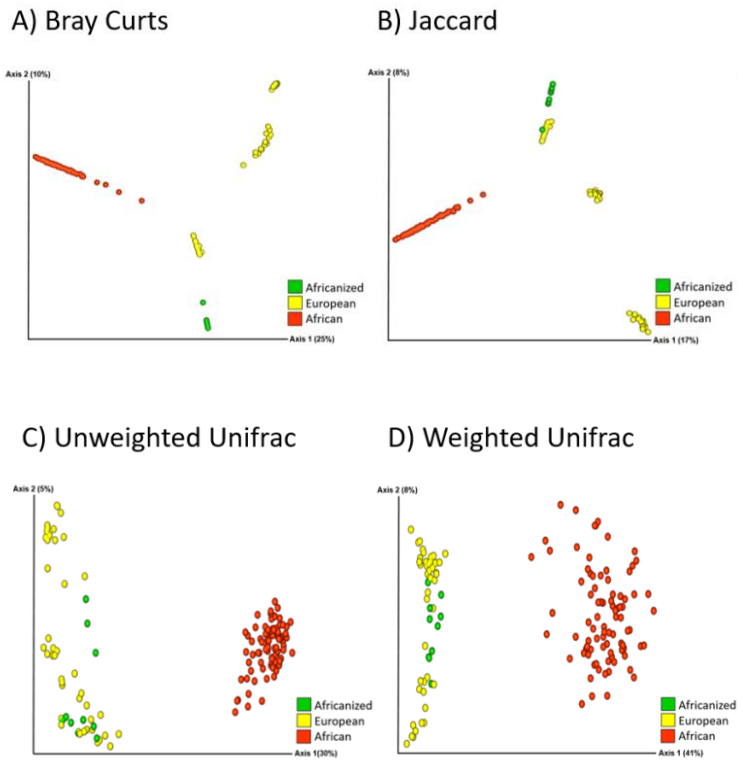
Figure 1. Experimental design with the distribution of the bees, author, sample origin sites and number of samples.

3.1. 16S rRNA SEQUENCING READS

We obtained a total of 919,168 raw reads across all samples, ranging from 1015 (apis-SJC_S92) to 26958 (apis-setor_S25) reads per sample and averaging 6,565.48 reads. Reads identified as chloroplasts, mitochondria, unassigned and eukaryota were removed from all samples.

3.2. MICROBIAL COMPOSITION AND DIVERSITY

Bee gut microbial composition significantly differed between the subspecies (*Apis mellifera scutellata* x spp, *Apis mellifera mellifera*, *Apis mellifera scutellata*) (PERMANOVA: Bray Curts R2 = 0.3186, p-value = 0.001; Jaccard R2 = 0.2197, p-value = 0.001; Unweighted Unifrac R2 = 0.3062, p-value = 0.001; Weighted Unifrac R2 = 0.4178, p-value = 0.001) (Figure 2). The only similarity was observed between European and Africanized honey bees. Africanized and European bees did not differ by weighted Unifrac (Pairwise PERMANOVA Weighted Unifrac pseudo-F = 0.945, q = 0.136) (Figure 2).



233

234 Figure 2. Gut community similarities, compared using PCoA. Bee gut microbial composition
235 significantly differed between the Africanized, European and African subspecies (*Apis mellifera*
236 mellifera, *Apis mellifera scutellata* x spp, *Apis mellifera scutellata*, respectively) trough the indexes: A)
237 Bray Curts, B) Jaccard, C) Unweighted Unifrac and D) Weighted Unifrac. (PERMANOVA: Bray Curts
238 R2 = 0.3186, p-value = 0.001; Jaccard R2 = 0.2197, p-value = 0.001; Unweighted Unifrac R2 = 0.3062,
239 p-value = 0.001; Weighted Unifrac R2 = 0.4178, p-value = 0.001).

240 Microbial diversity also differed significantly between the subspecies (Kruskal-Wallis:
241 Shannon Index p-value = 2.2e-16; Faith's PD p-value = 2.2e-16; Evenness Pielou p-value =
242 1.421e-08; Observed Features p-value = 2.2e-16). African microbiota were more abundant and
243 even followed by Africanized based in Shannon, and more diverse based on Faith's
244 phylogenetic diversity and European less diverse based on the number of ASVs (Figure 3).

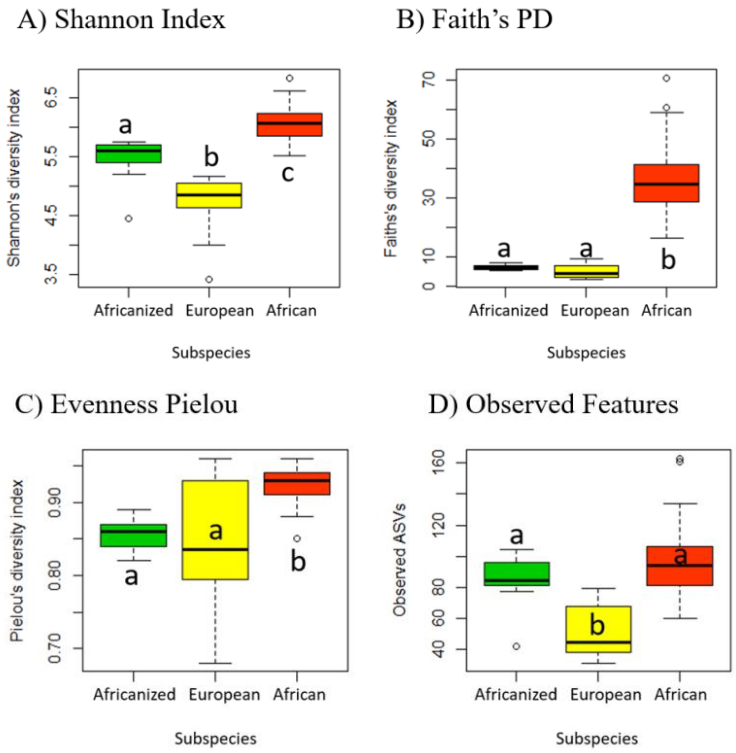


Figure 3. Microbial diversity and evenness by subspecies. Box plot shows outliers, first and third quartiles (lower and upper edges), and highest, lowest, and median values (horizontal black dash) for Africanized, European and African subspecies (*Apis mellifera mellifera, Apis mellifera scutellata x spp, Apis mellifera scutellata*, respectively). Microbial diversity differed significantly between the subspecies (Kruskal-Wallis: Shannon Index, p-value = 2.2e-16; Faith's PD p-value = 2.2e-16; Evenness Pielou p-value = 1.421e-08; Observed Features p-value = 2.2e-16).

3.3. CORE MICROBIOTA AND DIFFERENTIALLY ABUNDANT TAXA

A core microbiota analyses identified three genera that were present in 95% of the samples across all branches including: *Lactobacillus*, *Snodgrassella*, and *Gilliamella.* No features were associated to 100% samples (Table 2).

These taxa accounted for 15% of all genera in the dataset. We then used a Kruskal-Wallis Rank Sum Test to compare relative abundances of these taxa by branches.

Lactobacillus taxa abundances were higher in Africanized compared to African bees (Kruskal-Wallis p-value = 0.453; Pairwise Wilcoxon rank sum test with continuity correction: p-value = 0.00037). *Snodgrassella* taxa abundances were higher in Africanized and African bees compared to European bees (Kruskal-Wallis p-value = 0.3724; Pairwise Wilcoxon rank sum test with continuity correction: Africanized vs European p-value = 0.025; African vs European p-value = 6.2e-07). *Gilliamella* taxa abundances were higher in Africanized bees

compared to African bees (Kruskal-Wallis p-value = 0.4866; Pairwise Wilcoxon rank sum test with continuity correction: p-value = 0.0046) (Figure 4).

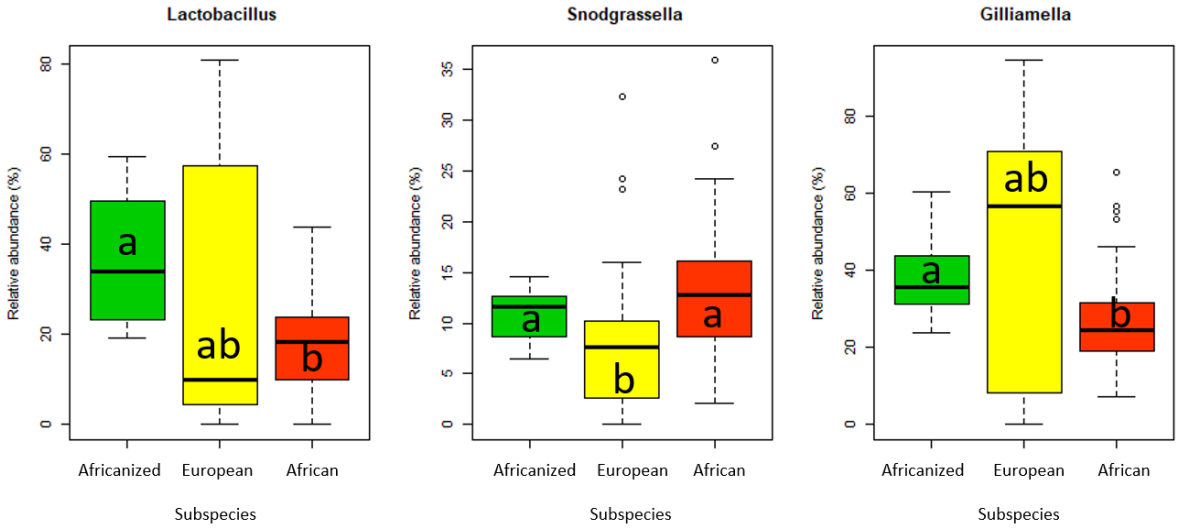


Figure 4. Relative abundances of core microbiota (genera) that were present in 95% of the samples across all subspecies including: A) *Lactobacillus, B) Snodgrassella, and C) Gilliamella*. No features were associated to 100% samples. Box plot shows outliers, first and third quartiles (lower and upper edges), and highest, lowest, and median values (horizontal black dash) for Africanized, European and African subspecies (*Apis mellifera* mellifera, *Apis mellifera scutellata* x spp, *Apis mellifera scutellata*, respectively).

An ANCOM identified nine differentially abundant taxa by branches at genera level, including: members of the family Orbaceae, Rhizobiaceae (Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium) and Enterobacteriaceae, members of the genera *Frischella*, Uncultured gamma proteobacterium, *Pantoea*, *Bombella*, *Gilliamella*, and an unidentified Bacteria (Table 3) (Figure 5).

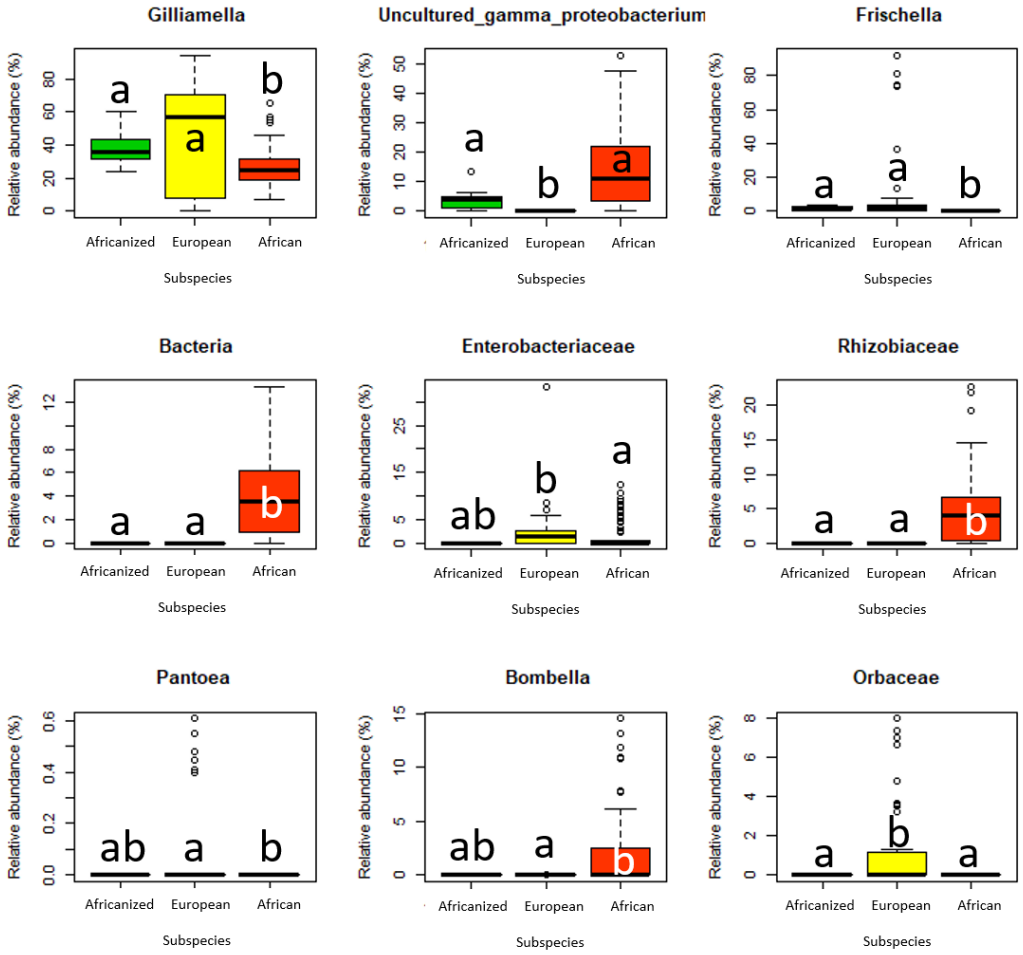


Figure 5. Relative abundances of differentially abundant genera (ANCOM) by subspecies. Box plot shows outliers, first and third quartiles (lower and upper edges), and highest, lowest, and median values (horizontal black dash) for Africanized, European and African subspecies (*Apis mellifera* mellifera, *Apis mellifera scutellata* x spp, *Apis mellifera scutellata*, respectively).

Relative abundances of *Frischella* and *Gilliamella* were lower in African compared to both Africanized and European (*Frischella* relative abundance: Kruskal-Wallis p-value = 1.25e-05; *Gilliamella* relative abundances: Kruskal-Wallis p-value = 0.4866). Relative abundances of an Uncultured gamma proteobacterium from the family Orbaceae was lower in European compared to both African and Africanized (Relative abundance of an uncultured_gamma_ proteobacterium: Kruskal-Wallis p-value = 0.1483). Relative abundances of Enterobacteriaceae, *Pantoea* and Orbaceae were higher in European compared to both African and Africanized (Relative abudance of Enterobacteriaceae: Kruskal-Wallis p-value = 0.05265; *Pantoea*: Kruskal-Wallis p-value = 0.07521; Orbaceae: Kruskal-Wallis p-value = 0.005403). Relative abundances of Rhizobiaceae and *Bombella* were higher in African compared to European and Africanized (Relative abudance of Rhizobiaceae: Kruskal-Wallis p-value = 0.9999; Bombella: Kruskal-Wallis p-value = 0.9983). A ASVs identified at level of

Bacteria was higher in African compared to both European and Africanized (Kruskal-Wallis p-value = 0.9996) (Figure 6).

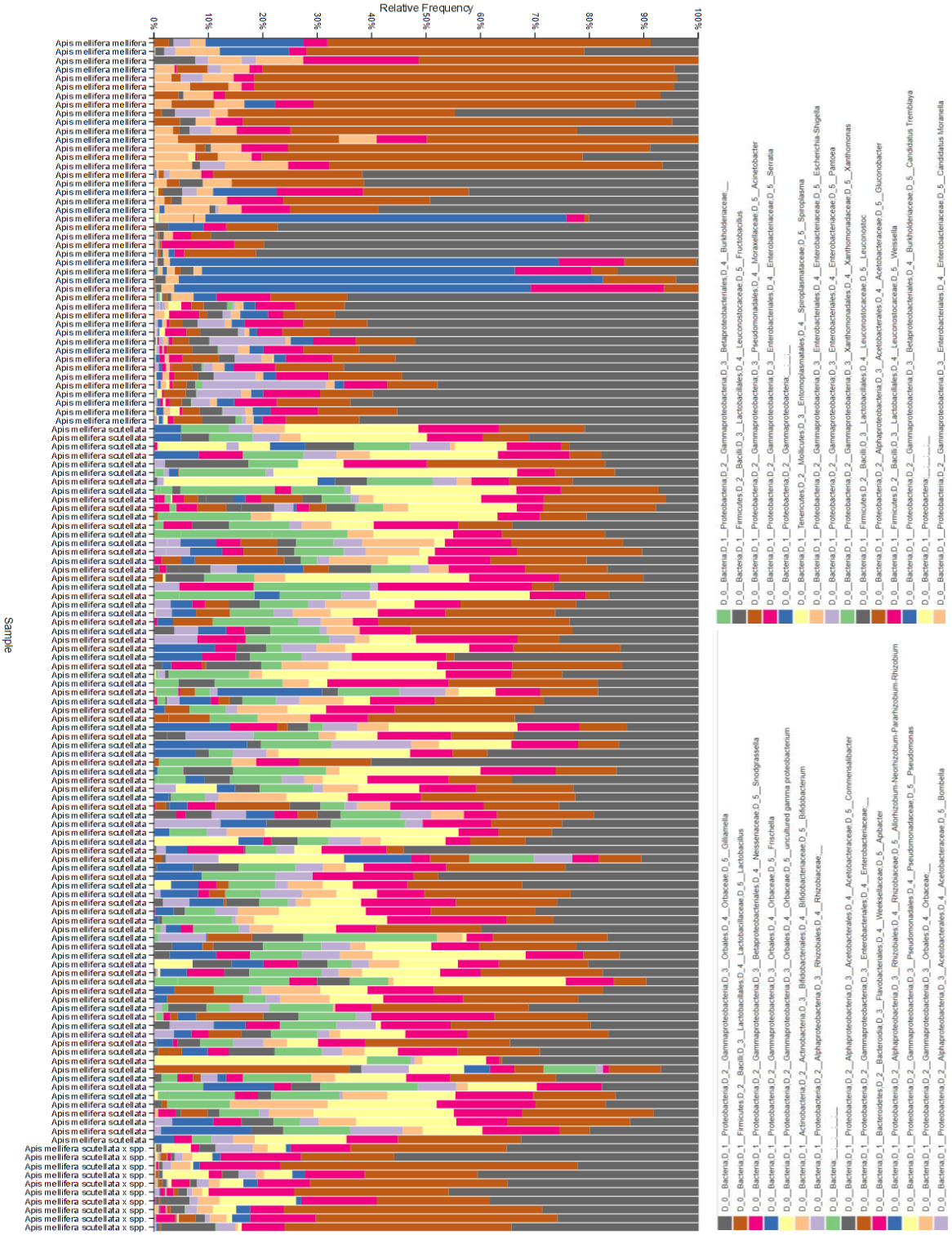


Figure 6. Stacked column graph showing the relative and absolute abundances of gut bacterial species in diferent groups of honey bees.

3.4. INDICATOR SPECIES ANALYSIS

We carried out indicator species analysis to explore whether ASVs were representative between the branches. Taxa with an indicator value of >0.4 and $p \leq 0.05$ were considered indicator species. Total number of species of 169 where 41 were selected being 36 species associated to one group and 5 species to two groups (Table 4).

4 species were associated to African subspecies: one at Bacteria level (statistics = 0.732, p-value = 0.0001), one of the family Rhizobiaceae that belongs to the genus *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (statistics = 0.621 p-value = 0.0001), one of the genus *Bombella* (statistics = 0.406 p-value = 0.0032) and one from the family Burkholderiaceae (statistics = 0.276 p-value = 0.0286). 4 species were associated to Africanized subspecies: *Snodgrassella* (statistics = 0.592, p-value = 0.0002), *Lactobacillus* (statistics = 0.588, p-value = 0.0001), *Bifidobacterium* (statistics = 0.523, p-value = 0.0002) and *Apibacter* (statistics = 0.306, p-value = 0.0173), all at genus level. 4 species were associated to European subspecies: two from the families Orbaceae (statistics = 0.515, p-value = 0.0001) and Enterobacteriaceae (statistics = 0.509, p-value = 0.0001) and two from the genera *Pantoea* (statistics = 0.308, p-value = 0.0089) and *Frischella* (statistics = 0.273, p-value = 0.0265). One group associated to Group African and Africanized: from the family Orbaceae and genera uncultured gamma proteobacterium (statistics = 0.489, p-value = 0.0001), and finally 1 genera associated to Africanized and European: D_5__Gilliamella (statistics = 0.546, p-value = 0.0003).

4. DISCUSSION

4.1. MICROBIAL COMPOSITION AND DIVERSITY

Gut microbiote composition differed among host species. As Weighted UniFrac distances ordinated onto a PCoA plot showed no clear visual separation based on branches, although by PERMANOVA, Africanized and European bees did not differs by Weighted Unifrac. Weighted Unifrac measures the evolutionary distances between microbial communities. African queen bees that were introduced in European hives and African colonies living in the wild were collected by beekeepers to their apiaries favoring the contact between the two subspecies. The contact between eusocial beings such as a bee hive community guarantees the maintenance of the gut microbiota by transferring microorganisms within the population (ENGEL *et al.*, 2016; POWELL *et al.*, 2014).

Shannon's index accounts for both abundance and evenness of the branches (KIM, Bo-Ra *et al.*, 2017). African honey bees showed to be more abundant and even followed by Africanized based on this index. The same pathern was observed in Evenness's Pielou. Pielou's evenness, is a measure of diversity that is the ratio of observed diversity to the maximum possible in a sample having the same number of species (PIELOU, 1966). In African honey bees there was no great variation of gut microbiota across different locations. It indicates a conserved honey bee gut microbiota even in very different ecosystems. However, all samples of African bees came from a unique country. In another hand, samples from European bees are from different sites in Europe and United States. Considering that the maintenance of the gut microbitoa is more influenced by genetics than geographic location (ALMEIDA *et al.*, 2023; KWONG *et al.*, 2017), it may indicates that the African sites are more suitable environments for the growth of beneficial bacteria instead of environmental or opportunistic bacteria, according to Tola *et al*. (2020).

African showed the greatest phylogenetic diversity through Faith's Phylogenetic Diversity. Africanized and Europeans did not differed in Faith´s phylogenetic diversity confirming the result for Weighted Unifrac. Phylogenetic differences among species can be based directly on their evolutionary histories (FAITH, 1992). It is no surprise considering that all African bees are from the same subspecies. In another hand European bees came from different sites and probably compriseed different subspecies from Europe.

Surprisingly, European bees showed the lowest number of observed features. Low diversity seems to be a common characteristic in all corbiculate bee gut microbiome, the microbiome of each host remains distinctive when compared against other species (KWONG *et al.*, 2017).

4.2. CORE MICROBIAL TAXA AND DIFFERENTIALLY ABUNDANT MICROBES BY TREATMENT

Three microbial species were considered belonging the core microbiome. They were present in 90% of the samples in genus level. Our results are supported by Kwong *et al*. (2017) that says the emergence of the eusocial corbiculate bees likely coincided with their acquisition of five core gut bacterial genera, *Snodgrassella, Gilliamella, Lactobacillus Firm-4, LactobacillusFirm-5 e Bifidobacterium* (KWONG *et al.*, 2017). In our study, *Bifidobacterium* was present only in 80 % of the samples. The genus was not present in 14 samples of African

bees. *Bifidobacterium* genus is consistently found in the honeybee gut microbiota, although at lower abundances (MORAN *et al.*, 2012).

Gillamella and *Snodgrassella* have a key participation against parasites as they produce a biofilm on the gut wall (Raymann and Moran, 2018). *Snodgrassella* as well works in digestion and energy production through the oxidation of fermentated products. *Gilliamella* is involved in nutrient metabolism and is the major degrader of monosaccharides, pectin, and hemicellulose in the bee gut (ENGEL; MARTINSON; MORAN, 2012; FOUAD *et al.*, 2016b; ZHENG *et al.*, 2019).

Nosemosis is an important disease that affects honey bees and can be caused by *Nosema apis* and *N. ceranae* (GUIMARÃES-CESTARO *et al.*, 2020), these are found on all continents and have often been associated with the colapse of honey bee colonies (Colony Collapse Disorder) and other reports of high bee losses (TEIXEIRA *et al.*, 2013). *Snodgrassella* relative abundances were smaller in European honey bees. Africanized bees appear to have greater resistance to pathogens than European bees (MAGGI *et al.*, 2016; TIBATÁ *et al.*, 2021). Compared African and European bee populations and reported that africanized bees had a lower rate of *N. ceranae* infection (MENDOZA *et al.*, 2014). It can indicate that the gut microbiome can confere resistence to these pathogens in African and Africanized honey bees.

Gilliamella were higher in Africanized compared to African. Gram negative species such as *Gilliamella apicola, Frischella perrara and Snodgrassella alvi* are horizontally acquired through fresh feces on the surface of the hive (POWELL *et al.*, 2014) and seasonal variation influences *Gilliamella* abundance in Europe and North America (BLEAU *et al.*, 2020; KEŠNEROVÁ *et al.*, 2020; LUDVIGSEN *et al.*, 2015). Typical communities, resembling those of workers within hives, were established in the presence of nurse workers or nurse worker fecal material (Powell *et al*., 2014). *Gilliamella* and *Lactobacillus* are able to enzymatically break down and ferment the sugars found in pollen, honey, and nectar (ENGEL; MARTINSON; MORAN, 2012; KWONG *et al.*, 2014). *Lactobacillus* spp. acts protecting food stores and inhibit pathogenic microbes by lowering pH levels or producing secondary metabolites (PAIN *et al.*, 1966).

Frischella presented lower relative abundances in African compared to both European and Africanized. This genera is a Gammaproteobacteria that resides in the bee pylorus (ENGEL; BARTLETT; MORAN, 2015). A representant of this genera, the *Frischella perrara*,

is a bacterium associated with impaired development (MAES *et al.*, 2016) because it can causes scab formation in the gut of its honey bee host (ENGEL; BARTLETT; MORAN, 2015).

A genera of the family Orbaceae, uncultured gamma proteobacterium (Orbaceae) were lower in European compared to both African and Africanized. Members of this family can beneficially contribute to the innate immune system of the honey bee by stimulating the production of antimicrobial peptides (KWONG; MANCENIDO; MORAN, 2017).

Enterobacteriaceae relative abundances was higher in African compared to European. This family is considered beneficial to healthy gut microbiota in humans, insects and other animals as they are the fermenters of sugar and are involved in the host defence mechanism (ANDERSON, K. E. *et al.*, 2011). A representant of the family Enterobacteriaceae, the *Enterobacter* genus is mainly involved in lactose fermentation (CABRAL, 2010). Honey bees forages in sources rich in carbohydrates, such as sucrose, glucose, and fructose (HAYDAK, 1970). However, other carbohydrates can be present in their diet in lesser quantities, such as mannose, galactose, xylose, arabinose, rhamnose, lactose, melibiose, raffinose, and melezitose) and may be toxic to bees as they do not have specific enzymatic activity for their metabolization (BARKER; LEHNER, 1974; JOHNSON, Reed M., 2015). *Enterobacter* genus is putative, in case of bee intoxication, mitigate the effects by metabolizing lactose. Again, African and Africanized honey bees seems to present a gut microbiota that present key function in honey bee health.

Rhizobiaceae higher in African compared to both Africanized and European. This taxon is frequently found in the honey bee gut, but its function on the health of the host remains unknown (RAYMANN; SHAFFER; MORAN, 2017).

Bombella relative abundances were higher in African compared to European. All members of the genus *Bombella* share unique characteristic for acetic acid producing (KIM, Minji; KIM; PARK, 2022). The drop in pH caused by lactic and acetic acid fermentation and alcoholic fermentation, is important to ensuring a defensive role and spoilage avoidance of the "bee bread"(BARTA *et al.*, 2022), fermented pollen and the most important source of protein for bees. Curiositly, Africanized honey bees collect more pollen and less nectar compared to Europeans because they allocate less bees for nectar collection (DANKA *et al.*, 1987; PESANTE; RINDERER; COLLINS, 1987). This fact can be related either to the environment where they live or the gut microbiota of the host. *Bombella* is also associated with larvae

protection against microbial pathogens (HÄRER; HILGARTH; EHRMANN, 2022; MILLER; SMITH; NEWTON, 2021).

Our resusts sugest that the African gut microbiota composition presents some taxa that assures the mantainance of the bee health (HAMIDUZZAMAN *et al.*, 2015) and is putative to be includes in the reasons why African and Africanized honey bees are superior in pathogen resistence than European honey bees.

5. CONCLUSION

The bee gut microbiota present great differences between the branches. The composition of the Africanized honey bees presented similarities with the European Honey bees. Some taxa present in African honey bees may help to explain some peculiar characteristics of the African bees such as behavior, pathogen resistence and nutrient use. More studies have to be done to explain the direct effect of bee gut microbiota on these characteristics.

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716 **SUPPLEMENTARY MATERIAL**

717 **Supplementary tables**

718 *Table 1. Information about the sequencing data.*

Sample_number	Sample_ID	Region	Coordenates	Subspecies	16s region	Illumina plataform	DNA extraction	Type of DNA extraction
1	apis-setor_S1	Areia, PB - Brazil	6° 58′20″ S; 35° 43′16.9″ W	*Apis mellifera scutellata* x spp	v3-v4	Illumina miseq	Soares *et al*., 2021	PowerSoil DNA Isolation kit
2	apis-setor_S13	Areia, PB - Brazil	6° 58′20″ S; 35° 43′16.9″ W	*Apis mellifera scutellata* x spp	v3-v4	Illumina miseq	Soares *et al*., 2021	PowerSoil DNA Isolation kit
3	apis-setor_S25	Areia, PB - Brazil	6° 58′20″ S; 35° 43′16.9″ W	*Apis mellifera scutellata* x spp	v3-v4	Illumina miseq	Soares *et al*., 2021	PowerSoil DNA Isolation kit
4	apis-setor_S37	Areia, PB - Brazil	6° 58′20″ S; 35° 43′16.9″ W	*Apis mellifera scutellata* x spp	v3-v4	Illumina miseq	Soares *et al*., 2021	PowerSoil DNA Isolation kit
5	apis-setor_S49	Areia, PB - Brazil	6° 58′20″ S; 35° 43′16.9″ W	*Apis mellifera scutellata* x spp	v3-v4	Illumina miseq	Soares *et al*., 2021	PowerSoil DNA Isolation kit
6	apis-SJC_S12	S,J, Cariri , PB - Brazil	7°22'55.7"S 36°31'40.9"W	*Apis mellifera scutellata* x spp	v3-v4	Illumina miseq	Soares *et al*., 2021	PowerSoil DNA Isolation kit

7	apis-SJC_S24	S,J, Cariri , PB - Brazil	7°22'55.7"S 36°31'40.9"W	*Apis mellifera scutellata* x spp	v3-v4	Illumina miseq	Soares *et al*., 2021	PowerSoil DNA Isolation kit
8	apis-SJC_S36	S,J, Cariri , PB - Brazil	7°22'55.7"S 36°31'40.9"W	*Apis mellifera scutellata* x spp	v3-v4	Illumina miseq	Soares *et al*., 2021	PowerSoil DNA Isolation kit
9	apis-SJC_S48	S,J, Cariri , PB - Brazil	7°22'55.7"S 36°31'40.9"W	*Apis mellifera scutellata* x spp	v3-v4	Illumina miseq	Soares *et al*., 2021	PowerSoil DNA Isolation kit
10	apis-SJC_S92	S,J, Cariri , PB - Brazil	7°22'55.7"S 36°31'40.9"W	*Apis mellifera scutellata* x spp	v3-v4	Illumina miseq	Soares *et al*., 2021	PowerSoil DNA Isolation kit
11	HiveDay0_Control1_S1	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based
12	HiveDay0_Control2_S2	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based
13	HiveDay0_Control3_S3	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based
14	HiveDay0_Control4_S4	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based
15	HiveDay0_Control5_S10	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based
16	HiveDay0_Control5_S11	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based
17	HiveDay0_Control5_S12	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based

18	HiveDay0_Control5_S13	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based
19	HiveDay0_Control5_S14	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based
20	HiveDay0_Control5_S15	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based
21	HiveDay0_Control5_S16	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based
22	HiveDay0_Control5_S5	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based
23	HiveDay0_Control5_S6	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based
24	HiveDay0_Control5_S7	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based
25	HiveDay0_Control5_S8	Austin, Texas - US	30°17'05.7"N 97°44'02.1"W	*Apis mellifera* mellifera	V4	Illumina*	Raymann *et al*., 2017	CTAB/phenol-based
26	APM30	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and Insect DNA MiniPrep
27	APM41	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and Insect DNA MiniPrep

28	APM42	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and Insect DNA MiniPrep
29	APM43	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and Insect DNA MiniPrep
30	APM45	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and Insect DNA MiniPrep
31	APM56	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and Insect DNA MiniPrep
32	APM57	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and Insect DNA MiniPrep
33	APM58	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and

								Insect DNA MiniPrep
34	APM59	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and Insect DNA MiniPrep
35	APM60	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and Insect DNA MiniPrep
36	APM71	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and Insect DNA MiniPrep
37	APM72	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and Insect DNA MiniPrep
38	APM73	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and Insect DNA MiniPrep

39	APM74	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and Insect DNA MiniPrep
40	APM75	Sussex, UK	50°51'55.3"N 0°04'34.6"W	*Apis mellifera* mellifera	V4	Illumina miseq	(Jones *et al*., 2018a)	Zymo Research Tissue and Insect DNA MiniPrep
41	BL_H_S4	Laussane, Switzerland	46°31'26.9"N 6°34'55.1"E	*Apis mellifera* mellifera	V4	Illumina miseq	Kešnerová *et al*., 2017	CTAB/phenol-based
42	CB_H_S8	Laussane, Switzerland	46°31'26.9"N 6°34'55.1"E	*Apis mellifera* mellifera	V4	Illumina miseq	Kešnerová *et al*., 2017	CTAB/phenol-based
43	DM_H_S15	Laussane, Switzerland	46°31'26.9"N 6°34'55.1"E	*Apis mellifera* mellifera	V4	Illumina miseq	Kešnerová *et al*., 2017	CTAB/phenol-based
44	FR_H_S2	Laussane, Switzerland	46°31'26.9"N 6°34'55.1"E	*Apis mellifera* mellifera	V4	Illumina miseq	Kešnerová *et al*., 2017	CTAB/phenol-based
45	GC_H_S3	Laussane, Switzerland	46°31'26.9"N 6°34'55.1"E	*Apis mellifera* mellifera	V4	Illumina miseq	Kešnerová *et al*., 2017	CTAB/phenol-based
46	LD_H_S1	Laussane, Switzerland	46°31'26.9"N 6°34'55.1"E	*Apis mellifera* mellifera	V4	Illumina miseq	Kešnerová *et al*., 2017	CTAB/phenol-based
47	LG_H_S9	Laussane, Switzerland	46°31'26.9"N 6°34'55.1"E	*Apis mellifera* mellifera	V4	Illumina miseq	Kešnerová *et al*., 2017	CTAB/phenol-based
48	QT_H_S7	Laussane, Switzerland	46°31'26.9"N 6°34'55.1"E	*Apis mellifera* mellifera	V4	Illumina miseq	Kešnerová *et al*., 2017	CTAB/phenol-based

49	T1_H_S14	Laussane, Switzerland	46°31'26.9"N 6°34'55.1"E	*Apis mellifera* mellifera	V4	Illumina miseq	Kešnerová *et al*., 2017	CTAB/phenol-based
50	T2_H_S13	Laussane, Switzerland	46°31'26.9"N 6°34'55.1"E	*Apis mellifera* mellifera	V4	Illumina miseq	Kešnerová *et al*., 2017	CTAB/phenol-based
51	T3_H_S12	Laussane, Switzerland	46°31'26.9"N 6°34'55.1"E	*Apis mellifera* mellifera	V4	Illumina miseq	Kešnerová *et al*., 2017	CTAB/phenol-based
52	T4_H_S11	Laussane, Switzerland	46°31'26.9"N 6°34'55.1"E	*Apis mellifera* mellifera	V4	Illumina miseq	Kešnerová *et al*., 2017	CTAB/phenol-based
53	T5_H_S10	Laussane, Switzerland	46°31'26.9"N 6°34'55.1"E	*Apis mellifera* mellifera	V4	Illumina miseq	Kešnerová *et al*., 2017	CTAB/phenol-based
54	YS_H_S6	Laussane, Switzerland	46°31'26.9"N 6°34'55.1"E	*Apis mellifera* mellifera	V4	Illumina miseq	Kešnerová *et al*., 2017	CTAB/phenol-based
55	Kakamega-101	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
56	Kakamega-102	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
57	Kakamega-103	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
58	Kakamega-105	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
59	Kakamega-106	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
60	Kakamega-108	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based

61	Kakamega-111	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
62	Kakamega-114	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
63	Kakamega-115	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
64	Kakamega-116	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
65	Kakamega-92	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
66	Kakamega-93	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
67	Kakamega-94	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
68	Kakamega-96	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
69	Kakamega-97	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
70	Kakamega-98	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
71	Kakamega-99	Kakamega-Kenya	0°16'52.0"N 34°44'48.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
72	Kilifi-Coast-61	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based

73	Kilifi-Coast-62	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
74	Kilifi-Coast-63	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
75	Kilifi-Coast-64	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
76	Kilifi-Coast-65	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
77	Kilifi-Coast-66	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
78	Kilifi-Coast-67	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
79	Kilifi-Coast-68	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
80	Kilifi-Coast-69	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
81	Kilifi-Coast-71	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
82	Kilifi-Coast-72	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
83	Kilifi-Coast-74	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
84	Kilifi-Coast-75	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based

85	Kilifi-Coast-76	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
86	Kilifi-Coast-77	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
87	Kilifi-Coast-78	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
88	Kilifi-Coast-79	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
89	Kilifi-Coast-84	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
90	Kilifi-Coast-85	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
91	Kilifi-Coast-86	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
92	Kilifi-Coast-87	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
93	Kilifi-Coast-88	Kilifi-Coast-Kenya	3°30'54.0"S 39°54'02.7"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
94	Kwale-Coast-31	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
95	Kwale-Coast-35	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
96	Kwale-Coast-36	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based

97	Kwale-Coast-37	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
98	Kwale-Coast-38	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
99	Kwale-Coast-39	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
100	Kwale-Coast-41	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
101	Kwale-Coast-42	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
102	Kwale-Coast-43	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
103	Kwale-Coast-45	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
104	Kwale-Coast-46	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
105	Kwale-Coast-47	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
106	Kwale-Coast-48	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
107	Kwale-Coast-51	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
108	Kwale-Coast-52	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based

109	Kwale-Coast-55	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
110	Kwale-Coast-56	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
111	Kwale-Coast-57	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
112	Kwale-Coast-58	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
113	Kwale-Coast-59	Kwale-Coast-Kenya	4°10'35.3"S 39°27'24.6"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
114	Nairobi-1	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
115	Nairobi-11	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
116	Nairobi-12	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
117	Nairobi-13	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
118	Nairobi-17	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
119	Nairobi-18	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
120	Nairobi-19	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based

121	Nairobi-2	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
122	Nairobi-21	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
123	Nairobi-22	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
124	Nairobi-23	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
125	Nairobi-24	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
126	Nairobi-25	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
127	Nairobi-27	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
128	Nairobi-28	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
129	Nairobi-29	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
130	Nairobi-3	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
131	Nairobi-4	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
132	Nairobi-5	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based

133	Nairobi-6	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
134	Nairobi-7	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
135	Nairobi-8	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based
136	Nairobi-9	Nairobi-Kenya	1°19'46.7"S 36°50'44.4"E	*Apis mellifera scutellata*	V4	Illumina miseq	Tola *et al*., 2020	CTAB/phenol-based

719

720

721

Table 2.Analysis of composition of microbiomes (ANCOM).

Feature ID	W	Reject null hypothesis
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Orbales;D_4__Orbaceae;D_5__uncultured gamma proteobacterium	18	True
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Orbales;D_4__Orbaceae;D_5__Frischella	18	True
D_0__Bacteria;__;__;__;__;__	17	True
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;__	17	True
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Orbales;D_4__Orbaceae;__	17	True
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Rhizobiaceae;D_5__Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	17	True
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Pantoea	16	True
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Orbales;D_4__Orbaceae;D_5__Gilliamella	15	True
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Acetobacterales;D_4__Acetobacteraceae;D_5__Bombella	14	True

Table 3.able 3. Identified core taxa, which were present in 90% of the samples.

Feature ID	2%	9%	25%	50%	75%	91%	98%
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Lactobacillaceae;D_5__Lactobacillus;__	0	65.6	159.25	316	812.5	1561	3874.7
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Betaproteobacteriales;D_4__Neisseriaceae;D_5__Snodgrassella;__	0	67.2	171	226.5	346.25	1156.8	1710.6
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Orbales;D_4__Orbaceae;D_5__Gilliamella;D_6__uncultured gamma proteobacterium	27.3	61.3	80	153	1194.75	6244.7	8791.3

Table 4. Indic species results.

	Stat	p.value	Significance
Group African #sps. 4			
D_0__Bacteria;__;__;__;__;__	0.732	0.0001	***
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Rhizobiaceae;D_5__Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	0.621	0.0001	***
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Acetobacterales;D_4__Acetobacteraceae;D_5__Bombella	0.406	0.0032	**
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Betaproteobacteriales;D_4__Burkholderiaceae;__	0.276	0.0286	*
Group Africanized #sps. 4			
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Betaproteobacteriales;D_4__Neisseriaceae;D_5__Snodgrassella	0.592	0.0002	***
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Lactobacillaceae;D_5__Lactobacillus	0.588	0.0001	***
D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Bifidobacteriales;D_4__Bifidobacteriaceae;D_5__Bifidobacterium	0.523	0.0002	***
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacteriales;D_4__Weeksellaceae;D_5__Apibacter	0.306	0.0173	*
Group European #sps. 4			
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Orbales;D_4__Orbaceae;__	0.515	0.0001	***
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;__	0.509	0.0001	***
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Pantoea	0.308	0.0089	**
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Orbales;D_4__Orbaceae;D_5__Frischella	0.273	0.0265	*
Group African+Africanized #sps. 1			
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Orbales;D_4__Orbaceae;D_5__uncultured gamma proteobacterium	0.489	0.0001	***
Group Africanized+European #sps. 1			
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Orbales;D_4__Orbaceae;D_5__Gilliamella	0.546	0.0003	***

Scripts

Script 1

Qiime script

Protocolo de analise *in silico* do artigo

"Africanized honey bees (*Apis mellifera scutellata* x spp.) gut microbiota: is it close to African or European bees and what it implicates in bee health?"

```
##PBS -N ondemand/sys/myjobs/default
#PBS -A PAS1331
#PBS -l walltime=48:00:00
#PBS -l nodes=1:ppn=10
#PBS -j oe
#PBS -m abe
#
# Move to the directory where the job was submitted
#
# Run script
#
sh myscript.sh > my_results
#
# Now, copy data (or move) back once the simulation has completed
# Run sequential job
#
/usr/bin/time ./myscript.sh
#
# Now, copy data (or move) back once the simulation has completed
```

```
#

cp message.out $SLURM_SUBMIT_DIR

##

source activate qiime2-2020.2

 #cd /users/PAS1331/kilmersoares/ondemand/data/sys/myjobs/projects/default/23

#Download and installing the sratoolki for ubuntu system

# wget --output-document sratoolkit.tar.gz http://ftp-
trace.ncbi.nlm.nih.gov/sra/sdk/current/sratoolkit.current-ubuntu64.tar.gz

 #tar -vxzf sratoolkit.tar.gz

 #export
PATH=$PATH:/users/PAS1331/kilmersoares/ondemand/data/sys/myjobs/projects/default/23/
sratoolkit.2.10.9-ubuntu64/bin

 #which fastq-dump

 #download the sra files

 #STEP 1. Download a table of the metadata into a CSV file “SraRunInfo.csv”:

#From SRA web page :

#click on “Send to (top right corner)” Select “File” Select format “RunInfo” Click on “Create
File”

#STEP 2. Read this CSV file “SraRunInfo.csv” into R:

#The SRA files are automatically download in the current working directory using the
following R script:

#testing

#fastq-dump --stdout SRR10322593.1 | head -n 8

#mkdir readsSRA

#cd /users/PAS1331/kilmersoares/ondemand/data/sys/myjobs/projects/default/23/readsSRA

#save the file in a separeted directory
```

```
#cd /users/PAS1331/kilmersoares/ondemand/data/sys/myjobs/projects/default/23/(THE SEPARETED DIRECTORY CHOSEN)

#replace "texas.csv"for the name you Chosen

#R

#re

#Motta, 2018

 #Read SRA file infos

#sri<-read.csv("texas.csv", stringsAsFactors=FALSE)

#head(sri)

#files<-basename(sri$download_path)

#for(i in 1:length(files)) download.file(sri$download_path[i], files[i])

# Assure that all the files has been downloaded successfully

# Remember, the R object files has been created in the previous code chunk

#stopifnot( all(file.exists(files)) )

#for(f in files) {

 # cmd = paste("fastq-dump --split-3", f)

 #cat(cmd,"\n")#print the current command

 #system(cmd) # invoke command

#Jones, 2018

 #Read SRA file infos

#sri<-read.csv("uk.csv", stringsAsFactors=FALSE)

#head(sri)

#files<-basename(sri$download_path)

#for(i in 1:length(files)) download.file(sri$download_path[i], files[i])

# Assure that all the files has been downloaded successfully
```

```
# Remember, the R object files has been created in the previous code chunk
#stopifnot( all(file.exists(files)) )
#for(f in files) {
# cmd = paste("fastq-dump --split-3", f)
# cat(cmd,"\n")#print the current command
 # system(cmd) # invoke command
#}
#JKešnerová, 2019
 #Read SRA file infos
#sri<-read.csv("Switzer.csv", stringsAsFactors=FALSE)
#head(sri)
#files<-basename(sri$download_path)
#for(i in 1:length(files)) download.file(sri$download_path[i], files[i])
# Assure that all the files has been downloaded successfully
# Remember, the R object files has been created in the previous code chunk
#stopifnot( all(file.exists(files)) )
#for(f in files) {
 # cmd = paste("fastq-dump --split-3", f)
 # cat(cmd,"\n")#print the current command
 # system(cmd) # invoke command
#}
#q()
cd /users/PAS1331/kilmersoares/ondemand/data/sys/myjobs/projects/default/23
```

```
#Importing data

#qiime tools import \

 #--type 'SampleData[PairedEndSequencesWithQuality]' \

# --input-path manifest_BR.csv \

# --output-path demux_seqs_BR.qza \

# --input-format PairedEndFastqManifestPhred33

# qiime demux summarize \

# --i-data demux_seqs_BR.qza \

# --o-visualization demux_seqs_BR.qzv


# qiime tools import \

# --type 'SampleData[PairedEndSequencesWithQuality]' \

# --input-path manifest_TX.csv \

# --output-path demux_seqs_TX.qza \

# --input-format PairedEndFastqManifestPhred33

# qiime demux summarize \

 # --i-data demux_seqs_TX.qza \

 # --o-visualization demux_seqs_TX.qzv


 # qiime tools import \

# --type 'SampleData[PairedEndSequencesWithQuality]' \

# --input-path manifest_UK.csv \

# --output-path demux_seqs_UK.qza \

# --input-format PairedEndFastqManifestPhred33
```

```
# qiime demux summarize \
 # --i-data demux_seqs_UK.qza \
 # --o-visualization demux_seqs_UK.qzv


 # qiime tools import \
# --type 'SampleData[PairedEndSequencesWithQuality]' \
# --input-path manifest_SW.csv \
# --output-path demux_seqs_SW.qza \
# --input-format PairedEndFastqManifestPhred33
# qiime demux summarize \
# --i-data demux_seqs_SW.qza \
 # --o-visualization demux_seqs_SW.qzv


 # qiime tools import \
# --type 'SampleData[PairedEndSequencesWithQuality]' \
 #--input-path manifest_KEcsv \
 #--output-path demux_seqs_KE.qza \
 #--input-format PairedEndFastqManifestPhred33
 #qiime demux summarize \
 # --i-data demux_seqs_KE.qza \
 # --o-visualization demux_seqs_KE.qzv


  #Sequence quality control and feature table
# This method denoises paired-end sequences, dereplicates them, and filters chimeras.
```

```
#qiime dada2 denoise-paired \
 # --i-demultiplexed-seqs ./demux_seqs_BR.qza \
 # --p-trunc-len-f 250 \
 # --p-trunc-len-r 250 \
 # --o-table ./dada2_table_BR.qza \
 # --o-representative-sequences ./dada2_rep_set_BR.qza \
 # --o-denoising-stats ./dada2_stats_BR.qza

#qiime dada2 denoise-paired \
# --i-demultiplexed-seqs ./demux_seqs_TX.qza \
# --p-trunc-len-f 250 \
# --p-trunc-len-r 250 \
# --o-table ./dada2_table_TX.qza \
# --o-representative-sequences ./dada2_rep_set_TX.qza \
# --o-denoising-stats ./dada2_stats_TX.qza

#qiime dada2 denoise-paired \
# --i-demultiplexed-seqs ./demux_seqs_UK.qza \
# --p-trunc-len-f 220 \
# --p-trunc-len-r 200 \
# --o-table ./dada2_table_UK.qza \
# --o-representative-sequences ./dada2_rep_set_UK.qza \
# --o-denoising-stats ./dada2_stats_UK.qza
```

```
#qiime dada2 denoise-paired \
# --i-demultiplexed-seqs ./demux_seqs_SW.qza \
# --p-trunc-len-f 250 \
# --p-trunc-len-r 250 \
# --o-table ./dada2_table_SW.qza \
# --o-representative-sequences ./dada2_rep_set_SW.qza \
# --o-denoising-stats ./dada2_stats_SW.qza

#qiime dada2 denoise-paired \
# --i-demultiplexed-seqs ./demux_seqs_KE.qza \
# --p-trunc-len-f 250 \
# --p-trunc-len-r 250 \
# --o-table ./dada2_table_KE.qza \
# --o-representative-sequences ./dada2_rep_set_KE.qza \
# --o-denoising-stats ./dada2_stats_KE.qza

 #METADATA DADA TABULATE####
 qiime metadata tabulate \
 --m-input-file metadata.tsv \
 --o-visualization metadata.qzv

 qiime metadata tabulate \
 --m-input-file ./dada2_stats_BR.qza \
 --o-visualization ./dada2_stats_BR.qzv
```

```
qiime feature-table summarize \
 --i-table ./dada2_table_BR.qza \
 --m-sample-metadata-file ./metadata_BR.tsv \
 --o-visualization ./dada2_table_BR.qzv
qiime metadata tabulate \
 --m-input-file ./dada2_stats_TX.qza \
 --o-visualization ./dada2_stats_TX.qzv
qiime feature-table summarize \
 --i-table ./dada2_table_TX.qza \
 --m-sample-metadata-file ./metadata_TX.tsv \
 --o-visualization ./dada2_table_TX.qzv
qiime metadata tabulate \
 --m-input-file ./dada2_stats_UK.qza \
 --o-visualization ./dada2_stats_UK.qzv
qiime feature-table summarize \
 --i-table ./dada2_table_UK.qza \
 --m-sample-metadata-file ./metadata_UK.tsv \
 --o-visualization ./dada2_table_UK.qzv
qiime metadata tabulate \
 --m-input-file ./dada2_stats_SW.qza \
 --o-visualization ./dada2_stats_SW.qzv
qiime feature-table summarize \
 --i-table ./dada2_table_SW.qza \
 --m-sample-metadata-file ./metadata_SW.tsv \
```

```
 --o-visualization ./dada2_table_SW.qzv

qiime metadata tabulate \

 --m-input-file ./dada2_stats_KE.qza \

 --o-visualization ./dada2_stats_KE.qzv

qiime feature-table summarize \

 --i-table ./dada2_table_KE.qza \

 --m-sample-metadata-file ./metadata_KE.tsv \

 --o-visualization ./dada2_table_KE.qzv

 #Alpha Rarefaction, Selecting a Rarefaction Depth


 #Alpha Rarefaction, Selecting a Rarefaction Depth

# Generate interactive alpha rarefaction curves by computing rarefactions between `min_depth` and `max_depth`. The number of intermediate depths to compute is controlled by the `steps` parameter, with n `iterations` being computed at each rarefaction depth. If sample metadata is provided, samples may be grouped based on distinct values within a metadata column

#We have chosen the values based on min and max the maximum sample total frequency of the feature_table dada2_table.qzv for each set of samples.

qiime diversity alpha-rarefaction \

 --i-table ./dada2_table?.qza \

 --m-metadata-file ./metadata_BR.tsv \

 --o-visualization ./alpha_rarefaction_curves_BR.qzv \

 --p-min-depth 1000 \

 --p-max-depth          26837

qiime diversity alpha-rarefaction \

 --i-table ./dada2_table_TX.qza \
```

```
 --m-metadata-file ./metadata_TX.tsv \
 --o-visualization ./alpha_rarefaction_curves_TX.qzv \
 --p-min-depth 1000 \
 --p-max-depth          26837
qiime diversity alpha-rarefaction \
 --i-table ./dada2_table_UK.qza \
 --m-metadata-file ./metadata_UK.tsv \
 --o-visualization ./alpha_rarefaction_curves_UK.qzv \
 --p-min-depth 5000 \
 --p-max-depth          19000

qiime diversity alpha-rarefaction \
 --i-table ./dada2_table_SW.qza \
 --m-metadata-file ./metadata_SW.tsv \
 --o-visualization ./alpha_rarefaction_curves_SW.qzv \
 --p-min-depth 10000 \
 --p-max-depth          18000
qiime diversity alpha-rarefaction \
 --i-table ./dada2_table_KE.qza \
 --m-metadata-file ./metadata_KE.tsv \
 --o-visualization ./alpha_rarefaction_curves_KE.qzv \
 --p-min-depth 1000 \
 --p-max-depth          13000
#Generating a phylogenetic tree for diversity analysis
```

The following single command will produce two outputs: 1) phylogeny.qza is the Phylogeny[Rooted] and 2) placements.qza provides placement distributions for the fragments. Sequences which are not at least 75% similar by sequence identity to any record in the tree to insert into are not inserted into the tree.

We chose SEPP among the available phylogenetic insertion pipelines because of its scalable divide-and-conquer algorithm. recursively breaks down a problem into two or more sub-problems of the same or related type, until these become simple enough to be solved directly. The divide-and-conquer paradigm is often used to find an optimal solution of a problem.

#Generating a phylogenetic tree for diversity analysis BR

qiime fragment-insertion sepp \

--i-representative-sequences ./dada2_rep_set_BR.qza \

--i-reference-database sepp-refs-gg-13-8.qza \

--o-tree ./tree_BR.qza \

--o-placements ./tree_placements_BR.qza \

--p-threads 4

#creating core metrics results

Method, which rarefies a FeatureTable[Frequency] to a user-specified depth, computes several alpha and beta diversity metrics, and generates principle coordinates analysis (PCoA) plots using Emperor for each of the beta diversity metrics.

Generating a phylogenetic tree for diversity analysis BR

qiime diversity core-metrics-phylogenetic \

--i-table ./dada2_table_BR.qza \

--i-phylogeny ./tree_BR.qza \

--m-metadata-file ./metadata_BR.tsv \

--p-sampling-depth 1000 \

--output-dir ./core-metrics-results_BR

```
#Generating a phylogenetic tree for diversity analysis TX
qiime fragment-insertion sepp \
 --i-representative-sequences ./dada2_rep_set_TX.qza \
 --i-reference-database sepp-refs-gg-13-8.qza \
 --o-tree ./tree_TX.qza \
 --o-placements ./tree_placements_TX.qza \
 --p-threads 4
qiime diversity core-metrics-phylogenetic \
 --i-table ./dada2_table_TX.qza \
 --i-phylogeny ./tree_TX.qza \
 --m-metadata-file ./metadata_TX.tsv \
 --p-sampling-depth 1600 \
 --output-dir ./core-metrics-results_TX
#Generating a phylogenetic tree for diversity analysis UK
qiime fragment-insertion sepp \
 --i-representative-sequences ./dada2_rep_set_UK.qza \
 --i-reference-database sepp-refs-gg-13-8.qza \
 --o-tree ./tree_UK.qza \
 --o-placements ./tree_placements_UK.qza \
 --p-threads 4
qiime diversity core-metrics-phylogenetic \
 --i-table ./dada2_table_UK.qza \
 --i-phylogeny ./tree_UK.qza \
 --m-metadata-file ./metadata_UK.tsv \
```

```
 --p-sampling-depth 5000 \
 --output-dir ./core-metrics-results_UK
#Generating a phylogenetic tree for diversity analysis SW
qiime fragment-insertion sepp \
 --i-representative-sequences ./dada2_rep_set_SW.qza \
 --i-reference-database sepp-refs-gg-13-8.qza \
 --o-tree ./tree_SW.qza \
 --o-placements ./tree_placements_SW.qza \
 --p-threads 4
qiime diversity core-metrics-phylogenetic \
 --i-table ./dada2_table_SW.qza \
 --i-phylogeny ./tree_SW.qza \
 --m-metadata-file ./metadata_SW.tsv \
 --p-sampling-depth 10000 \
 --output-dir ./core-metrics-results_SW
#Generating a phylogenetic tree for diversity analysis KE
qiime fragment-insertion sepp \
 --i-representative-sequences ./dada2_rep_set_KE.qza \
 --i-reference-database sepp-refs-gg-13-8.qza \
 --o-tree ./tree_KE.qza \
 --o-placements ./tree_placements_KE.qza \
 --p-threads 4
qiime diversity core-metrics-phylogenetic \
 --i-table ./dada2_table_KE.qza \
```

```
 --i-phylogeny ./tree_KE.qza \
 --m-metadata-file ./metadata_KE.tsv \
 --p-sampling-depth 1200 \
 --output-dir ./core-metrics-results_KE
 #Diversity analysis
 #Alpha diversity
##We used the alpha group significance plugin to test for differences in alpha diversity
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_BR/faith_pd_vector.qza \
 --m-metadata-file ./metadata_BR.tsv \
 --o-visualization ./core-metrics-results_BR/faiths_pd_statistics_BR.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_BR/evenness_vector.qza \
 --m-metadata-file ./metadata_BR.tsv \
 --o-visualization ./core-metrics-results_BR/evenness_statistics_BR.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_BR/shannon_vector.qza \
 --m-metadata-file ./metadata_BR.tsv \
 --o-visualization ./core-metrics-results_BR/shannon_statistics_BR.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_BR/observed_otus_vector.qza \
 --m-metadata-file ./metadata_BR.tsv \
 --o-visualization ./core-metrics-results_BR/observed_otus_vector_BR.qzv
qiime diversity alpha-group-significance \
```

```
 --i-alpha-diversity ./core-metrics-results_TX/faith_pd_vector.qza \
 --m-metadata-file ./metadata_TX.tsv \
 --o-visualization ./core-metrics-results_TX/faiths_pd_statistics_TX.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_TX/evenness_vector.qza \
 --m-metadata-file ./metadata_TX.tsv \
 --o-visualization ./core-metrics-results_TX/evenness_statistics_TX.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_TX/shannon_vector.qza \
 --m-metadata-file ./metadata_TX.tsv \
 --o-visualization ./core-metrics-results_TX/shannon_statistics_TX.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_TX/observed_otus_vector.qza \
 --m-metadata-file ./metadata_TX.tsv \
 --o-visualization ./core-metrics-results_TX/observed_otus_vector_TX.qzv
 #Alpha diversity
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_UK/faith_pd_vector.qza \
 --m-metadata-file ./metadata_UK.tsv \
 --o-visualization ./core-metrics-results_UK/faiths_pd_statistics_UK.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_UK/evenness_vector.qza \
 --m-metadata-file ./metadata_UK.tsv \
 --o-visualization ./core-metrics-results_UK/evenness_statistics_UK.qzv
```

```
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_UK/shannon_vector.qza \
 --m-metadata-file ./metadata_UK.tsv \
 --o-visualization ./core-metrics-results_UK/shannon_statistics_UK.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_UK/observed_otus_vector.qza \
 --m-metadata-file ./metadata_UK.tsv \
 --o-visualization ./core-metrics-results_UK/observed_otus_vector_UK.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_SW/faith_pd_vector.qza \
 --m-metadata-file ./metadata_SW.tsv \
 --o-visualization ./core-metrics-results_SW/faiths_pd_statistics_SW.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_SW/evenness_vector.qza \
 --m-metadata-file ./metadata_SW.tsv \
 --o-visualization ./core-metrics-results_SW/evenness_statistics_SW.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_SW/shannon_vector.qza \
 --m-metadata-file ./metadata_SW.tsv \
 --o-visualization ./core-metrics-results_SW/shannon_statistics_SW.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_SW/observed_otus_vector.qza \
 --m-metadata-file ./metadata_SW.tsv \
 --o-visualization ./core-metrics-results_SW/observed_otus_vector_SW.qzv
```

```
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_KE/faith_pd_vector.qza \
 --m-metadata-file ./metadata_KE.tsv \
 --o-visualization ./core-metrics-results_KE/faiths_pd_statistics_KE.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_KE/evenness_vector.qza \
 --m-metadata-file ./metadata_KE.tsv \
 --o-visualization ./core-metrics-results_KE/evenness_statistics_KE.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_KE/shannon_vector.qza \
 --m-metadata-file ./metadata_KE.tsv \
 --o-visualization ./core-metrics-results_KE/shannon_statistics_KE.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results_KE/observed_otus_vector.qza \
 --m-metadata-file ./metadata_KE.tsv \
 --o-visualization ./core-metrics-results_KE/observed_otus_vector_KE.qzv
#MERGING for overall analyses
##We merged all tables for downstream analyses
qiime feature-table merge \
 --i-tables dada2_table_BR.qza \
 --i-tables dada2_table_TX.qza \
 --i-tables dada2_table_UK.qza \
 --i-tables dada2_table_SW.qza \
 --i-tables dada2_table_KE.qza \
```

```
 --o-merged-table dada2_table.qza


 qiime feature-table merge-seqs \
 --i-data dada2_rep_set_BR.qza \
 --i-data dada2_rep_set_TX.qza \
 --i-data dada2_rep_set_UK.qza \
 --i-data dada2_rep_set_SW.qza \
 --i-data dada2_rep_set_KE.qza \
 --o-merged-data dada2_rep_set.qza
 qiime feature-table summarize \
 --i-table ./dada2_table.qza \
 --m-sample-metadata-file ./metadata.tsv \
 --o-visualization ./dada2_table.qzv


 #Generating a phylogenetic tree for diversity analysis
qiime fragment-insertion sepp \
 --i-representative-sequences ./dada2_rep_set.qza \
 --i-reference-database sepp-refs-gg-13-8.qza \
 --o-tree ./tree.qza \
 --o-placements ./tree_placements.qza \
 --p-threads 4 # update to a higher number if you can


 ##I have chosen I have chosen 1000 and 26959 based on min and max the maximum sample
total frequency of the feature_table dada2_table.qzv. The minimum rarefaction depth. The
maximum rarefaction depth must be greater than min-depth.
```

```
 Range(1, None)  min-depth.
qiime diversity alpha-rarefaction \
--i-table ./dada2_table.qza \
--m-metadata-file ./metadata.tsv \
--o-visualization ./alpha_rarefaction_curves.qzv \
--p-min-depth 1000 \
--p-max-depth 26959
#Diversity analysis

 #I have chosen p-sampling-depth 1000 based on the min Feature Count on dada2_table.qzv
qiime diversity core-metrics-phylogenetic \
 --i-table ./dada2_table.qza \
 --i-phylogeny ./tree.qza \
 --m-metadata-file ./metadata.tsv \
 --p-sampling-depth 1000 \
 --output-dir ./core-metrics-results

 #Alpha Rarefaction group significance (after core metrics).
## We will test for associations between categories in the sample metadata file and alpha diversity data.
qiime diversity alpha-group-significance \
 --i-alpha-diversity core-metrics-results/shannon_vector.qza \
 --m-metadata-file metadata.tsv \
 --o-visualization core-metrics-results/shannon_vector-group-significance.qzv
```

```
#Alpha diversity
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results/faith_pd_vector.qza \
 --m-metadata-file ./metadata.tsv \
 --o-visualization ./core-metrics-results/faiths_pd_statistics.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results/evenness_vector.qza \
 --m-metadata-file ./metadata.tsv \
 --o-visualization ./core-metrics-results/evenness_statistics.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results/shannon_vector.qza \
 --m-metadata-file ./metadata.tsv \
 --o-visualization ./core-metrics-results/shannon_statistics.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results/observed_otus_vector.qza \
 --m-metadata-file ./metadata.tsv \
 --o-visualization ./core-metrics-results/observed_otus_statistic.qzv

#Beta diversity OVERALL ANALISES
# Determine whether groups of samples are significantly different from one another using a permutation-based statistical test.
#For biome
qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
```

```
 --m-metadata-column biome \
 --p-pairwise \
 --o-visualization core-metrics-results/unweighted-unifrac-biome-significance.qzv
qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --m-metadata-column biome \
 --p-pairwise \
 --o-visualization core-metrics-results/weighted-unifrac-biome-significance.qzv

qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --m-metadata-column biome \
 --p-pairwise \
 --o-visualization core-metrics-results/bray_curtis-biome-significance.qzv
qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/jaccard_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --m-metadata-column biome \
 --p-pairwise \
 --o-visualization core-metrics-results/jaccard-biome-significance_pairwise.qzv

 #For Subspecie
```

```
qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --m-metadata-column species \
 --o-visualization core-metrics-results/unweighted-unifrac-species-significance.qzv \
 --p-pairwise
qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --m-metadata-column species \
 --p-pairwise \
 --o-visualization core-metrics-results/weighted-unifrac-species-significance.qzv \

qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --m-metadata-column species \
 --p-pairwise \
 --o-visualization core-metrics-results/bray_curtis-species-significance.qzvI
qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/jaccard_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --m-metadata-column species \
 --p-pairwise \
```

--o-visualization core-metrics-results/jaccard-species-significance_pairwise.qzv

#Adonis (PERMANOVA)

It is a non-parametric multivariate statistical permutation test. PERMANOVA is used to compare groups of objects and test the null hypothesis that the centroids and dispersion of the groups as defined by measure space are equivalent for all groups. We used Adonis to look at a multivariate model. to see the effect of more than one grouping variable.

qiime diversity adonis \

--i-distance-matrix core-metrics-results/jaccard_distance_matrix.qza \

--m-metadata-file metadata.tsv \

--o-visualization core-metrics-results/jaccard_adonis.qzv \

--p-formula species

qiime diversity adonis \

--i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza \

--m-metadata-file metadata.tsv \

--o-visualization core-metrics-results/bray_curtis_adonis.qzv \

--p-formula species

qiime diversity adonis \

--i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \

--m-metadata-file metadata.tsv \

--o-visualization core-metrics-results/unweighted_unifrac_distance_adonis.qzv \

--p-formula species

```
qiime diversity adonis \
--i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza \
--m-metadata-file metadata.tsv \
--o-visualization core-metrics-results/weighted_unifrac_distance_adonis.qzv \
--p-formula species

#Taxonomic classification
#alinhar e classificar
#Plugin for taxonomic classification of sequences. Contains multiple methods for sequence classification, including methods to train and employ scikit-learn classifiers for sequence classification.
#The naive Bayes algorithm for multinomially distributed data, and is one of the two classic naive Bayes variants used in text classification (where the data are typically represented as word vector counts, although tf-idf vectors are also known to work well in practice).
#We use silva-132-99-nb-classifier.qza
qiime feature-classifier classify-sklearn \
 --i-reads ./dada2_rep_set.qza \
 --i-classifier ./silva-132-99-nb-classifier.qza \
 --o-classification ./taxonomy.qza
qiime metadata tabulate \
 --m-input-file ./taxonomy.qza \
 --o-visualization ./taxonomy.qzv
qiime feature-table tabulate-seqs \
 --i-data ./dada2_rep_set.qza \
 --o-visualization ./dada2_rep_set.qzv
```

```
#Filter the table of ASVs . Filtering can be applied to retain only specific taxa.
#Mitochondria
qiime taxa filter-table \
 --i-table dada2_table.qza \
 --i-taxonomy taxonomy.qza \
 --p-exclude     Mitochondria \
 --o-filtered-table table-no-mitochondria.qza
#Chloroplast
qiime taxa filter-table \
 --i-table table-no-mitochondria.qza \
 --i-taxonomy taxonomy.qza \
 --p-exclude     Chloroplast \
 --o-filtered-table table-no-chloroplast.qza
#Unassigned
qiime taxa filter-table \
 --i-table table-no-chloroplast.qza \
 --i-taxonomy taxonomy.qza \
 --p-exclude     Unassigned \
 --o-filtered-table table-no-Unassigned.qza
#Eukaryota
qiime taxa filter-table \
 --i-table table-no-Unassigned.qza \
 --i-taxonomy taxonomy.qza \
 --p-exclude     Eukaryota \
```

```
 --o-filtered-table table-final.qza

 # reattribute taxonomy

qiime feature-table filter-seqs \

 --i-data dada2_rep_set.qza \

 --i-table table-final.qza \

 --o-filtered-data seqs-final.qza


 #Taxonomy barchart (5000 based on the min Feature Count on dada2_table.qzv

qiime feature-table filter-samples \

 --i-table ./table-final.qza \

 --p-min-frequency 1000 \

 --o-filtered-table ./table_2kfiltered.qza

qiime taxa barplot \

 --i-table ./table_2kfiltered.qza \

 --i-taxonomy ./taxonomy.qza \

 --m-metadata-file ./metadata.tsv \

 --o-visualization ./taxa_barplotfiltered.qzv

 #Generates the table with the number of asvs per sample And the taxa in it and with the first table. We use the taxa table collapsed to the ASV level

# table dada2 with taxonomy

qiime taxa collapse \

 --i-table table-final.qza \

 --i-taxonomy taxonomy.qza \

 --p-level 7 \

 --o-collapsed-table table-l7.qza
```

```
#Generates the table with the number of ASVs per sample

qiime tools export \

 --input-path table-l7.qza \

 --output-path table_exported-feature-table_L7

cd table_exported-feature-table_L7

biom convert --to-tsv -i feature-table.biom -o bee_sub_table_L7.tsv

cd /users/PAS1331/kilmersoares/ondemand/data/sys/myjobs/projects/default/23

#Differential abundance with ANCOM Analysis of composition of microbiomes. Filter features from table based on frequency and/or metadata. Any samples with a frequency of zero after feature filtering will also be removed.

## p-min-frequency: The minimum total frequency that a feature must have o be retained; --p-max-frequency: The maximum total frequency that a feature can have to be retained. If no value is provided this will default to infinity (i.e., no maximum frequency filter will be applied).

qiime feature-table filter-features \

 --i-table table_2kfiltered.qza \

 --p-min-frequency 50 \

 --p-min-samples 5 \

 --o-filtered-table table_2k_abund.qza


# table with taxonomy

 qiime taxa collapse \

 --i-table table_2k_abund.qza \

 --i-taxonomy taxonomy.qza \

 --p-level 6 \
```

```
 --o-collapsed-table table-l62.qza


 #Differential abundance with ANCOM

## ANCOM accounts for the underlying structure in the data and can be used for comparing the composition of microbiomes in two or more populations.

# First, incrementing all counts in table by pseudocount.

qiime composition add-pseudocount \

 --i-table table-l62.qza \

 --o-composition-table comp-table-l62.qza

 #Ancon biome

qiime composition ancom \

 --i-table comp-table-l62.qza \

 --m-metadata-file metadata.tsv \

 --m-metadata-column biome \

 --o-visualization ancom-subject_biome.qzv


 #Ancon species

qiime composition ancom \

 --i-table comp-table-l62.qza \

 --m-metadata-file metadata.tsv \

 --m-metadata-column species \

 --o-visualization ancom-subject_species.qzv


 #Core features
```

```
# Identify "core" features, which are features observed in a user-defined fraction of the samples.

#We considerered ASVs present in at least 95% of the samples

qiime feature-table core-features \

 --i-table table-l6.qza \

 --o-visualization core-features


#Machine-learning classifiers for predicting sample characteristics

# Predicts a categorical sample metadata column using a supervised learning classifier. Uses nested stratified k-fold cross validation for automated hyperparameter optimization and sample prediction. Outputs predicted values for each input sample, and relative importance of each feature for model accuracy.

qiime sample-classifier classify-samples \

 --i-table ./table-l6.qza \

 --m-metadata-file ./metadata.tsv \

 --m-metadata-column species \

 --p-random-state 666 \

 --p-n-jobs 1 \

 --output-dir ./sample-classifier-results/

#Heat map

## Generate a heatmap of important features. Features are filtered based on importance scores; samples are optionally grouped by metadata; and a heatmap is generated that displays (normalized) feature abundances per sample.

qiime sample-classifier heatmap \

 --i-table ./table-l6.qza \

 --i-importance ./sample-classifier-results/feature_importance.qza \
```

```
 --m-sample-metadata-file metadata.tsv \
 --m-sample-metadata-column species \
 --p-group-samples \
 --p-feature-count 100 \
 --o-heatmap ./sample-classifier-results/heatmap100.qzv \
 --o-filtered-table ./sample-classifier-results/filtered-table100.qza


qiime sample-classifier heatmap \
 --i-table ./table-l6.qza \
 --i-importance ./sample-classifier-results/feature_importance.qza \
 --m-sample-metadata-file metadata.tsv \
 --m-sample-metadata-column species \
 --p-group-samples \
 --p-feature-count 50 \
 --o-heatmap ./sample-classifier-results/heatmap50.qzv \
 --o-filtered-table ./sample-classifier-results/filtered-table50.qza


qiime sample-classifier heatmap \
 --i-table ./table-l6.qza \
 --i-importance ./sample-classifier-results/feature_importance.qza \
 --m-sample-metadata-file metadata.tsv \
 --m-sample-metadata-column species \
 --p-group-samples \
```

```
 --p-feature-count 10 \
 --o-heatmap ./sample-classifier-results/heatmap10.qzv \
 --o-filtered-table ./sample-classifier-results/filtered-table10.qza


qiime sample-classifier heatmap \
 --i-table ./table-l6.qza \
 --i-importance ./sample-classifier-results/feature_importance.qza \
 --m-sample-metadata-file metadata.tsv \
 --m-sample-metadata-column species \
 --p-group-samples \
 --p-feature-count 20 \
 --o-heatmap ./sample-classifier-results/heatmap20.qzv \
 --o-filtered-table ./sample-classifier-results/filtered-table20.qza

qiime sample-classifier heatmap \
 --i-table ./table-l6.qza \
 --i-importance ./sample-classifier-results/feature_importance.qza \
 --m-sample-metadata-file metadata.tsv \
 --m-sample-metadata-column species \
 --p-group-samples \
 --p-feature-count 15 \
 --o-heatmap ./sample-classifier-results/heatmap15.qzv \
 --o-filtered-table ./sample-classifier-results/filtered-table15.qza
```

Script 2

R studio Post hoc analysis

Protocolo de analise *in silico* do artigo

"Africanized honey bees (*Apis mellifera scutellata* x spp.) gut microbiota: is it close to African or European bees and what it implicates in bee health?"

```
#importdata

library(readr)

library(readr)

data_sub <-
read_csv("~/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Subspecies/Sub_R/dat
a_sub.csv")

View(data_sub)

#Tukey test

library(laercio)

require(laercio)

#Subspecies

#diversity

par(mfrow = c(1,2), oma = c(2,1,1,1))

# shannon

shapiro.test(data_sub$shannon) #not signifcant, data are normal

#modelo

#anova <- aov(shannon ~ Abrev, data=data_sub)

#summary(anova)

#tukey_ctrl <- LTukey(anova, which="Abrev", conf.level=0.95)

#kruskal.test

kruskal.test(shannon ~ Abrev, data=data_sub)
```

```
pairwise.wilcox.test(data_sub $ shannon,data_sub $Abrev, p.adj = "bonferroni",
exact=FALSE)

#PLOT

boxplot(shannon ~ Abrev, data=data_sub, cex = 1,  main=" Shannon's
Index",xlab="Subspecies", ylab="Shannon's diversity index", col =
c("#00CC00","#ffff00","#ff3300"))


#faith

shapiro.test(data_sub$faith) #not signifcant, data are normal

 #modelo

#anova <- aov(faith ~ Abrev, data=data_sub)

#summary(anova)

#tukey_ctrl <- LTukey(anova, which="Abrev", conf.level=0.95)


#kruskal.test

kruskal.test(faith ~ Abrev, data=data_sub)

pairwise.wilcox.test(data_sub $ faith,data_sub $Abrev, p.adj = "bonferroni")

#PLOT

boxplot(faith ~ Abrev, data=data_sub, cex = 1,  main= "Faith", xlab="Subspecies", ylab=
"Faiths's diversity index",col = c("#00CC00","#ffff00","#ff3300"))

# pielou

shapiro.test(data_sub$pielou) #not signifcant, data are normal

#modelo

#anova <- aov(pielou ~ Abrev, data=data_sub)

#summary(anova)

#tukey_ctrl <- LTukey(anova, which="Abrev",conf.level=0.95)
```

```
#kruskal.test

kruskal.test(pielou ~ Abrev, data=data_sub)

pairwise.wilcox.test(data_sub $ pielou,data_sub $Abrev, p.adj = "bonferroni", exact=FALSE)

#PLOT

boxplot(pielou ~ Abrev, data=data_sub, cex = 1,  main="Eveness Pielou",xlab="Subspecies",
ylab="Pielou's diversity index", col = c("#00CC00","#ffff00","#ff3300"))


#observed_otus

shapiro.test(data_sub$observed_otus) #not signifcant, data are normal

##modelo

#anova <- aov(observed_otus ~ Abrev, data=data_sub)

#summary(anova)

#tukey_ctrl <- LTukey(anova, which="Abrev", conf.level=0.95)

#kruskal.test

kruskal.test(observed_otus ~ Abrev, data=data_sub)

pairwise.wilcox.test(data_sub $observed_otus, data_sub $Abrev, p.adj = "bonferroni",
exact=FALSE)

#PLOT

boxplot(observed_otus ~ Abrev, data=data_sub, cex = 1,  main=" Observed
ASVs",xlab="Subspecies", ylab="Observed ASVs", col = c("#00CC00","#ffff00","#ff3300"))

#relative abundances

#CORE

par(mfrow = c(1,3), oma = c(2,1,1,1))

#Lactobacillus

shapiro.test(data_sub$Lactobacillus) #not signifcant, data are normal
```

```
#modelo

#anova <- aov(Lactobacillus ~ Abrev, data=data_sub)

#summary(anova)

#tukey_ctrl <- LTukey(anova, which="Abrev", conf.level=0.95)

#kruskal.test

kruskal.test(species ~ Lactobacillus, data=data_sub)

pairwise.wilcox.test(data_sub $ Lactobacillus, data_sub $Abrev, p.adj = "bonferroni",
exact=FALSE)


#PLOT

boxplot(Lactobacillus ~ Abrev, data=data_sub, cex = 1, main="
Lactobacillus",xlab="Subspecies", ylab="Relative abundance (%)", col =
c("#00CC00","#ffff00","#ff3300"))

# Snodgrassella

shapiro.test(data_sub$Snodgrassella) #not signifcant, data are normal

#modelo

#anova <- aov(Snodgrassella ~ Abrev, data=data_sub)

#summary(anova)

#tukey_ctrl <- LTukey(anova, which="Abrev", conf.level=0.95)

#kruskal.test

kruskal.test(species ~ Snodgrassella, data=data_sub)

pairwise.wilcox.test(data_sub $ Snodgrassella, data_sub $Abrev, p.adj = "bonferroni",
exact=FALSE)

#PLOT
```

```
boxplot(Snodgrassella ~ Abrev, data=data_sub, cex = 1, main="
Snodgrassella",xlab="Subspecies", ylab="Relative abundance (%)", col =
c("#00CC00","#ffff00","#ff3300"))

# Gilliamella ####CORE AND ANCOM

shapiro.test(data_sub$Gilliamella) #not signifcant, data are not normal

#modelo

#anova <- aov(Gilliamella ~ Abrev, data=data_sub)

#summary(anova)

#tukey_ctrl <- LTukey(anova, which="Abrev", conf.level=0.95)

#kruskal.test

kruskal.test(species ~ Gilliamella, data=data_sub)

pairwise.wilcox.test(data_sub $ Gilliamella, data_sub $Abrev, p.adj = "bonferroni",
exact=FALSE)

#PLOT

boxplot(Gilliamella ~ Abrev, data=data_sub, cex = 1, main="
Gilliamella",xlab="Subspecies", ylab="Relative abundance (%)", col =
c("#00CC00","#ffff00","#ff3300"))

#ANCOM

par(mfrow = c(1,1), oma = c(2,1,1,1))

#

# Frischella

shapiro.test(data_sub$Frischella) #not signifcant, data are normal

#modelo

#anova <- aov(Frischella ~ Abrev, data=data_sub)

#summary(anova)

#tukey_ctrl <- LTukey(anova, which="Abrev", conf.level=0.95)
```

```
#kruskal.test

kruskal.test(species ~ Frischella, data=data_sub)

pairwise.wilcox.test(data_sub $ Frischella, data_sub $Abrev, p.adj = "bonferroni",
exact=FALSE)


#PLOT

boxplot(Frischella ~ Abrev, data=data_sub, cex = 1, main=" Frischella",xlab="Subspecies",
ylab="Relative abundance (%)", col = c("#00CC00","#ffff00","#ff3300"))


# Uncultured gamma proteobacterium

shapiro.test(data_sub$Uncultured_gamma_proteobacterium) #not signifcant, data are normal

#modelo

#anova <- aov(Uncultured_gamma_proteobacterium  ~ Abrev, data=data_sub)

#summary(anova)

#tukey_ctrl <- LTukey(anova, which="Abrev", conf.level=0.95)

#kruskal.test

kruskal.test(species ~ Uncultured_gamma_proteobacterium  , data=data_sub)

pairwise.wilcox.test(data_sub $ Uncultured_gamma_proteobacterium  , data_sub $Abrev,
p.adj = "bonferroni", exact=FALSE)

#PLOT

boxplot(Uncultured_gamma_proteobacterium   ~ Abrev, data=data_sub, cex = 1,
main="Uncultured_gamma_proteobacterium ", xlab="Subspecies", ylab="Relative abundance
(%)", col = c("#00CC00","#ffff00","#ff3300"))


# Enterobacteriaceae

shapiro.test(data_sub$Enterobacteriaceae) #not signifcant, data are normal
```

```
#modelo

#anova <- aov(Enterobacteriaceae ~ Abrev, data=data_sub)

#summary(anova)

#tukey_ctrl <- LTukey(anova, which="Abrev", conf.level=0.95)

#kruskal.test

kruskal.test(species ~ Enterobacteriaceae, data=data_sub)

pairwise.wilcox.test(data_sub $ Enterobacteriaceae, data_sub $Abrev, p.adj = "bonferroni", exact=FALSE)

#PLOT

boxplot(Enterobacteriaceae ~ Abrev, data=data_sub, cex = 1, main=" Enterobacteriaceae",xlab="Subspecies", ylab="Relative abundance (%)", col = c("#00CC00","#ffff00","#ff3300"))

# Rhizobiaceae

shapiro.test(data_sub$Rhizobiaceae) #not signifcant, data are normal

#modelo

#anova <- aov(Rhizobiaceae ~ Abrev, data=data_sub)

#summary(anova)

#tukey_ctrl <- LTukey(anova, which="Abrev", conf.level=0.95)

#kruskal.test

kruskal.test(species ~ Rhizobiaceae, data=data_sub)

pairwise.wilcox.test(data_sub $ Rhizobiaceae,data_sub $Abrev, p.adj = "bonferroni", exact=FALSE)

#PLOT

boxplot(Rhizobiaceae ~ Abrev, data=data_sub, cex = 1, main=" Rhizobiaceae",xlab="Subspecies", ylab="Relative abundance (%)", col = c("#00CC00","#ffff00","#ff3300"))
```

```
# Pantoea

shapiro.test(data_sub$Pantoea) #not signifcant, data are normal

#modelo

anova <- aov(Pantoea ~ Abrev, data=data_sub)

summary(anova)

tukey_ctrl <- LTukey(anova, which="Abrev", conf.level=0.95)

#kruskal.test

kruskal.test(species ~ Pantoea, data=data_sub)

pairwise.wilcox.test(data_sub $ Pantoea, data_sub $Abrev, p.adj = "bonferroni",
exact=FALSE)

#PLOT

boxplot(Pantoea  ~ Abrev, data=data_sub, cex = 1, main=" Pantoea ",xlab="Subspecies",
ylab="Relative abundance (%)", col = c("#00CC00","#ffff00","#ff3300"))

# Bombella

shapiro.test(data_sub$Bombella) #not signifcant, data are normal

#modelo

anova <- aov(Bombella ~ Abrev, data=data_sub)

summary(anova)

tukey_ctrl <- LTukey(anova, which="Abrev", conf.level=0.95)

#kruskal.test

kruskal.test(species ~ Bombella, data=data_sub)

pairwise.wilcox.test(data_sub $ Bombella,data_sub $Abrev, p.adj = "bonferroni",
exact=FALSE)

#PLOT
```

```
boxplot(Bombella ~ Abrev, data=data_sub, cex = 1,  main=" Bombella",xlab="Subspecies",
ylab="Relative abundance (%)", col = c("#00CC00","#ffff00","#ff3300"))


Gilliamella ####CORE AND ANCOM

shapiro.test(data_sub$Gilliamella) #not signifcant, data are not normal

modelo

anova <- aov(Gilliamella ~ Abrev, data=data_sub)

summary(anova)

tukey_ctrl <- LTukey(anova, which="Abrev", conf.level=0.95)

#kruskal.test

kruskal.test(species ~ Gilliamella, data=data_sub)

pairwise.wilcox.test(data_sub $ Gilliamella, data_sub $Abrev, p.adj = "bonferroni",
exact=FALSE)

#PLOT

boxplot(Gilliamella ~ Abrev, data=data_sub, cex = 1, main="
Gilliamella",xlab="Subspecies", ylab="Relative abundance (%)", col =
c("#00CC00","#ffff00","#ff3300"))


# Bacteria

shapiro.test(data_sub$ Bacteria) #not signifcant, data are normal

#modelo

anova <- aov(Bacteria ~ Abrev, data=data_sub)

summary(anova)

tukey <- LTukey(anova, which="Abrev", conf.level=0.95)

#kruskal.test

kruskal.test(species ~ Bacteria, data=data_sub)
```

```
pairwise.wilcox.test(data_sub $ Bacteria, data_sub $Abrev, p.adj = "bonferroni",
exact=FALSE)

#PLOT

boxplot(Bacteria ~ Abrev, data=data_sub, cex = 1, main=" Bacteria",xlab="Subspecies",
ylab="Relative abundance (%)", col = c("#00CC00","#ffff00","#ff3300"))


# Orbaceae

shapiro.test(data_sub$Orbaceae) #not signifcant, data are normal

#modelo

anova <- aov(Orbaceae ~ Abrev, data=data_sub)

summary(anova)

tukey_ctrl <- LTukey(anova, which="Abrev", conf.level=0.95)

#kruskal.test

kruskal.test(species ~ Orbaceae, data=data_sub)

pairwise.wilcox.test(data_sub $ Orbaceae, data_sub $Abrev, p.adj = "bonferroni",
exact=FALSE)

#PLOT

boxplot(Orbaceae  ~ Abrev, data=data_sub, cex = 1, main=" Orbaceae ",xlab="Subspecies",
ylab="Relative abundance (%)", col = c("#00CC00","#ffff00","#ff3300"))
```

Script 3

R studio Indic species

Protocolo de analise *in silico* do artigo

"Africanized honey bees (*Apis mellifera scutellata* x spp.) gut microbiota: is it close to African or European bees and what it implicates in bee health?"

```
install.packages("indicspecies")

library(indicspecies)

library(readr)

library(phyloseq)

packageVersion("phyloseq")


table <- read_csv("indicspecies/table_L6.csv")

View(table)

#indicspeaces

#Subspecies

abund = table[,3:ncol(table)]

subspecies = table$Subspecies

inv = multipatt(abund, subspecies, func = "r.g", control = how(nperm=9999))

summary(inv)

#Site

abund = table[,3:ncol(table)]

Site = table$Site

inv = multipatt(abund, Site, func = "r.g", control = how(nperm=9999))

summary(inv)
```

CAPÍTULO 3 - Tetracycline Exposure Alters Key Gut Microbiota in Africanized Honey Bees (*Apis mellifera scutellata* x spp.)

Tetracycline exposure alters key gut microbiota in Africanized honey bees (*Apis mellifera scutellata* x spp.)

Abstract: Honey bees play a critical role in ecosystem health, biodiversity maintenance, and crop yield. Antimicrobials, such as tetracyclines, are used widely in agriculture, medicine, and in bee keeping, and bees can be directly or indirectly exposed to tetracycline residues in the environment. In European honey bees, tetracycline exposure has been linked with shifts in the gut microbiota that negatively impact bee health. However, the effects of antimicrobials on Africanized honey bee gut microbiota have not been examined. The aim of this study was to investigate the effects of tetracycline exposure on the gut microbial community of Africanized honey bees (*Apis mellifera scutellata* x spp), which are important pollinators in South, Central, and North America. Bees (n=1,000) were collected from hives in Areia-PB, Northeastern Brazil, placed into plastic chambers and kept under controlled temperature and humidity conditions. The control group (CON) was fed daily with syrup (10g) consisting of a 1:1 solution of demerara sugar and water, plus a solid protein diet (10g) composed of 60% soy extract and 40% sugar syrup. The tetracycline group (TET) was fed identically but with the addition of tetracycline hydrochloride (450 µg/g) to the sugar syrup. Bees were sampled from each group before (day 0), and after tetracycline exposure (days 3, 6 and 9). Abdominal contents dissected out of each bee underwent DNA extraction and 16S rRNA sequencing (V3-V4) on an Illumina MiSeq. Sequences were filtered and processed through QIIME2 and DADA2. Microbial community composition and diversity and differentially abundant taxa were evaluated by treatment and time. Bee gut microbial composition (Jaccard) and diversity (Shannon) differed significantly and increasingly over time and between CON and TET groups. Tetracycline exposure was associated with decreased relative abundances of *Bombella* and *Fructobacillus*, along with decreases in key core microbiota such as *Snodgrassella*, *Gilliamella*, Rhizobiaceae, and *Apibacter*. These microbes are critical for nutrient metabolism and pathogen defense, and it is possible that decreased abundances of these microbes could negatively affect bee health. Considering the global ecological and economic importance of honey bees as pollinators, it is critical to understand the effects of agrochemicals including antimicrobials on honey bees.

Keywords: Bees, antibiotics, intestinal, microbiome

A exposição à tetraciclina altera a microbiota intestinal em abelhas africanizadas (*Apis mellifera scutellata* x spp.)

Resumo: As abelhas desempenham um papel crítico na saúde do ecossistema, manutenção da biodiversidade e rendimento das culturas. Antimicrobianos, como as tetraciclinas, são amplamente utilizados na agricultura, na medicina e na apicultura, e as abelhas podem ser expostas direta ou indiretamente a resíduos de tetraciclina no meio ambiente. Nas abelhas europeias, a exposição à tetraciclina tem sido associada a mudanças na microbiota intestinal que afetam negativamente a saúde das abelhas. No entanto, os efeitos dos antimicrobianos na microbiota intestinal das abelhas africanizadas não foram examinados. O objetivo deste estudo foi investigar os efeitos da exposição à tetraciclina na comunidade microbiana intestinal de abelhas africanizadas (*Apis mellifera scutellata* x spp.), que são importantes polinizadores nas Américas do Sul, Central e do Norte. As abelhas (n = 1.000) foram coletadas de colmeias em Areia-PB, Nordeste do Brasil, colocadas em câmaras plásticas e mantidas em condições controladas de temperatura e umidade. O grupo controle (CON) foi alimentado diariamente com xarope (10 g) composto por uma solução 1:1 de açúcar demerara e água, além de uma dieta protéica sólida (10 g) composta por 60% de extrato de soja e 40% de xarope de açúcar. O grupo tetraciclina (TET) foi alimentado de forma idêntica, mas com a adição de cloridrato de tetraciclina (450 μg/g) ao xarope de açúcar. As abelhas foram amostradas de cada grupo antes (dia 0) e após a exposição à tetraciclina (dias 3, 6 e 9). O conteúdo abdominal dissecado de cada abelha foi submetido à extração de DNA e sequenciamento de 16S rRNA (V3-V4) em um Illumina MiSeq. As sequências foram filtradas e processadas através de QIIME2 e DADA2. A composição e diversidade da comunidade microbiana e os táxons diferencialmente abundantes foram avaliados por tratamento e tempo. A composição microbiana do intestino de abelha (Jaccard) e a diversidade (Shannon) diferiram significativamente e cada vez mais ao longo do tempo e entre os grupos CON e TET. A exposição à tetraciclina foi associada à diminuição da abundância relativa de *Bombella* e *Fructobacillus*, juntamente com a diminuição da microbiota essencial, como *Snodgrassella*, *Gilliamella*, *Rhizobiaceae* e *Apibacter*. Esses micróbios são críticos para o metabolismo de nutrientes e defesa de patógenos, e é possível que a diminuição da abundância desses micróbios possa afetar negativamente a saúde das abelhas. Considerando a importância ecológica e econômica global das abelhas como polinizadores, é fundamental entender os efeitos dos agroquímicos, incluindo antimicrobianos nas abelhas.

Palavras-chave: Abelhas, antibióticos, intestino, microbioma

1. INTRODUCTION

Bees play a critical role as pollinators in ecosystems across the globe, contributing to the maintenance of biodiversity on Earth (KEVAN; VIANA, 2003; MICHENER, 2022). In addition to this important ecological function, bees are also essential as pollinators in agriculture systems (GISDER; GENERSCH, 2017; HUNG *et al.*, 2018). Honey bees (*Apis* spp.), specifically, are the top crop pollinators and directly enhance crop yields (GISDER; GENERSCH, 2017). The Africanized honey bee (*Apis mellifera scutellata* x spp.), a crossbreed between European honey bees (*Apis mellifera* spp.) and African honey bees (*Apis mellifera scutellata*), emerged in the late 1950's in Brazil (WINSTON, 1992). African honey bees adapted and spread widely across the Americans because of their reproductive traits and superior ability to colonize tropical ecosystems compared with European bees. Some of the traits include improved thermoregulation capacity, greater resistance to diseases, increased egg-laying rates, more frequent queen replacement, and shorter developmental time (GUZMÁN-NOVOA *et al.*, 2011).

In spite of their great economic and biological importance, bee populations across the planet have been under increasing threat due to human population expansion, habitat destruction, and the use of agrochemicals including pesticides and antimicrobials. The use of such compounds has been associated with an increased occurrence of Colony Collapse Disorder (CCD), a phenomenon characterized by the disappearance of worker bees and compromise of the honey bee colony (CAIRES; BARCELOS, 2017; MOTTA; RAYMANN; MORAN, 2018; RAYMANN; SHAFFER; MORAN, 2017). According to a recent report on global antimicrobial use in livestock (OIE, 2022), tetracyclines were the most commonly used antimicrobial class among the 116 countries that provided data. Moreover, tetracyclines represented approximately 35% of the antimicrobial use in these countries, including use for growth promotion in feed animals, which is an ongoing practice in many countries. Recently, tetracyclines were also highlighted as an option for the treatment and prophylaxis of COVID-19, and tetracycline use has increased significantly in some hospitals during the pandemic (Sodhi and Etminan, 2020; Peñalva *et al.*, 2021).

Importantly, tetracycline is poorly absorbed by mammalian hosts and 30 to 90% of the drug is excreted in active forms in urine and feces (CHEE-SANFORD *et al.*, 2009; KHAN; ONGERTH, 2004; WATKINSON *et al.*, 2009). This can result in increased antimicrobial contamination in wastewater and farm runoff (BORRELY *et al.*, 2012; FARIA *et al.*, 2016;

HENDRIKSEN *et al.*, 2019). Tetracycline residues have been detected in irrigation water (0.14 ppm), pig waste lagoons (0.7 ppm), soil (25 ppm), hospital effluents (0.53 ppm), and at wastewater treatment plants (0.92 ppm) (MEYER *et al.*, 2000; PENA *et al.*, 2010; WANG, Feng-Hua *et al.*, 2014). Although prohibited in Brazil and Europe, oxytetracycline is also used to control bacterial infections in fruit trees including *Candidatus Liberibacter* spp., the causative agent of Citrus Greening Disease (CHANVATIK *et al.*, 2019), and *Xylella fastidiosa*, which causes Pierce's disease in grapevines (HOPKINS, 1979). In these cases, oxytetracycline is sprayed over orchards or vineyards, and oxytetracycline concentrations on plant tissues can range from 100 to 4,166 ppm (Chanvatik *et al*., 2019).

Bees can be indirectly exposed to antimicrobials while foraging in these agricultural or urban environments that contain tetracycline residues (LAU; NIEH, 2016). Bees can also be directly exposed to tetracyclines in the course of treatment for European and American foulbrood, bacterial diseases that cause severe losses in hives and honey production (MARTEL *et al.*, 2006; MASOOD *et al.*, 2022). To treat foulbrood, oxytetracycline is applied directly onto the hives at doses ranging from 500 (DINKOV; KANELOV; ZHELYAZKOVA, 2005) to 5900 ppm (KOCHANSKY, 2000). Antimicrobials can disturb gut microbial communities and affect their overall structure and function (BLASER, 2014). Gut microbes are critical to host health (CLARK; MACH, 2017; MONDA *et al.*, 2017; PESSIONE, 2012) and play a role in immune system development, biosynthesis of vitamins (LEBLANC *et al.*, 2013) and hormones (CLARKE *et al.*, 2014), and cellulose degradation (WARNECKE *et al.*, 2007). Antimicrobial-induced alterations in the gut microbiota compromise nutrient metabolism (LEE *et al.*, 2015) and pathogen defense mechanisms in European honey bees (ENGEL; MARTINSON; MORAN, 2012; KOCH; SCHMID-HEMPEL, 2011; KWONG; MANCENIDO; MORAN, 2017; MARTINSON; MOY; MORAN, 2012; MOTTA; RAYMANN; MORAN, 2018; WANG, Kai *et al.*, 2021).

Considering the widespread prevalence of tetracycline in the environment due to its use in agriculture, medicine, and in relation hive health, and evidence of gut microbiome disturbances in European honey bees due to antimicrobial exposure, the aim of this study was to investigate the effects of tetracycline on the gut microbiota of Africanized honey bees (*Apis mellifera scutellata* x spp.) in tropical conditions. While there are studies in African and European bees (RAYMANN; SHAFFER; MORAN, 2017; TIAN *et al.*, 2012; TOLA *et al.*, 2020; WU, Yuqi *et al.*, 2020), to our knowledge, this is the first report on gut microbiota and on antimicrobial use in Africanized honey bees.

2. METHODS

2.1. EXPERIMENTAL DESIGN AND SAMPLING

The study was carried out in December 2019 at the Bee Laboratory (LABE) of the Federal University of Paraiba, Areia -PB (6° 58'20" S; 35° 43'16.9" W; Altitude 545 m). The average annual temperature of Paraiba is 22.54 º C; the average relative humidity is 83.65%; and the annual precipitation in 2019 was 1360.2 mm (INMET, 2020).

On Day 0 (D0), approximately one thousand nurse bees (6-12 days old) (200 bees per hive) were collected from five outdoor hives at LABE. Nurse bees were identified based on their behavior and location on the brood comb. Bees were divided into 10 plastic chambers with 100 bees placed in each chamber. Each chamber contained bees and a 9 cm^2-piece of brood comb all from the same hive. Bees of different hives were not mixed together, and bees were only exposed to brood comb from their own hive. Five chambers (representing all 5 hives) were assigned to the control (CON) group, and the remaining 5 chambers (representing all 5 hives) were assigned to the tetracycline (TET) group. The plastic chambers measured 176.71 cm^2 and were covered with a nylon screen. Chambers were kept in an incubator at 32°C and 66% relative humidity (TE-371, Tecnal, Piracicaba, Brazil) (**Figure 1**). The control group (CON), was fed daily with 10g of syrup consisting of a 1:1 solution of demerara sugar and water. Sterile cotton balls were soaked into the syrup and then placed into the bee chambers daily. Bees were also fed a solid protein diet (10g) composed of 60% soy extract and 40% demerara syrup solution. The tetracycline group (TET) was fed identically except that syrup contained 450 µg/g (equivalent to 450 ppm) tetracycline hydrochloride (Tetramed, Medquímica, Brazil). This dose reflects what honey bees may be exposed to within some agricultural environments and is similar to the range of hive dosing (500-5900 ppm) for the treatment of foulbrood (RAYMANN; SHAFFER; MORAN, 2017).

Five replicates of 20 bees each were collected from each group at each sampling point including: day 0 (D0, pre-treatment) and days three (D3), six (D6), and nine (D9) (**Figure 2**). Bees were placed in sterile tubes containing 70% alcohol, transported to the lab and stored at -20°C until extraction. All procedures performed were approved by the Biodiversity Authorization and Information System – SISBIO (Protocol #: 71750-1, approved on 09/19/2019).

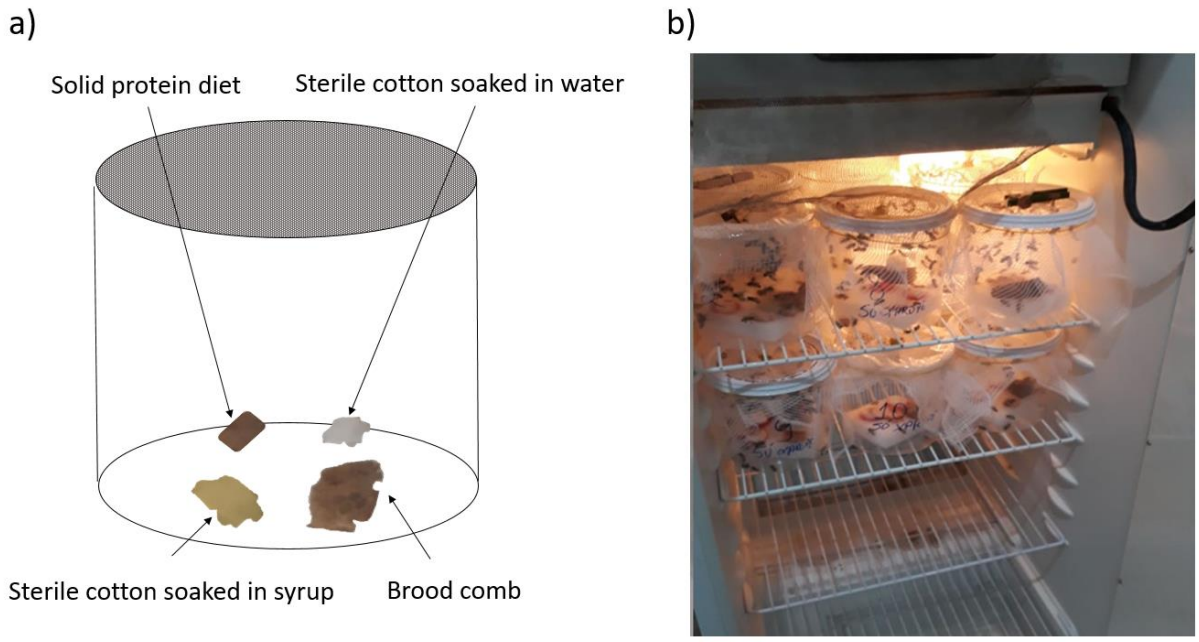

Figure 1. a) Approximately 100 bees were housed in each plastic chamber along with a piece of brood comb. Sterile cotton soaked in water or sugar syrup and a solid protein diet were also included in each chamber, and chambers were covered with Nylon screen. B) All chambers were placed in an incubator that was maintained at 32°C ± 1.45 temp and 66% ± 5.34 relative humidity for the duration of the experiment.

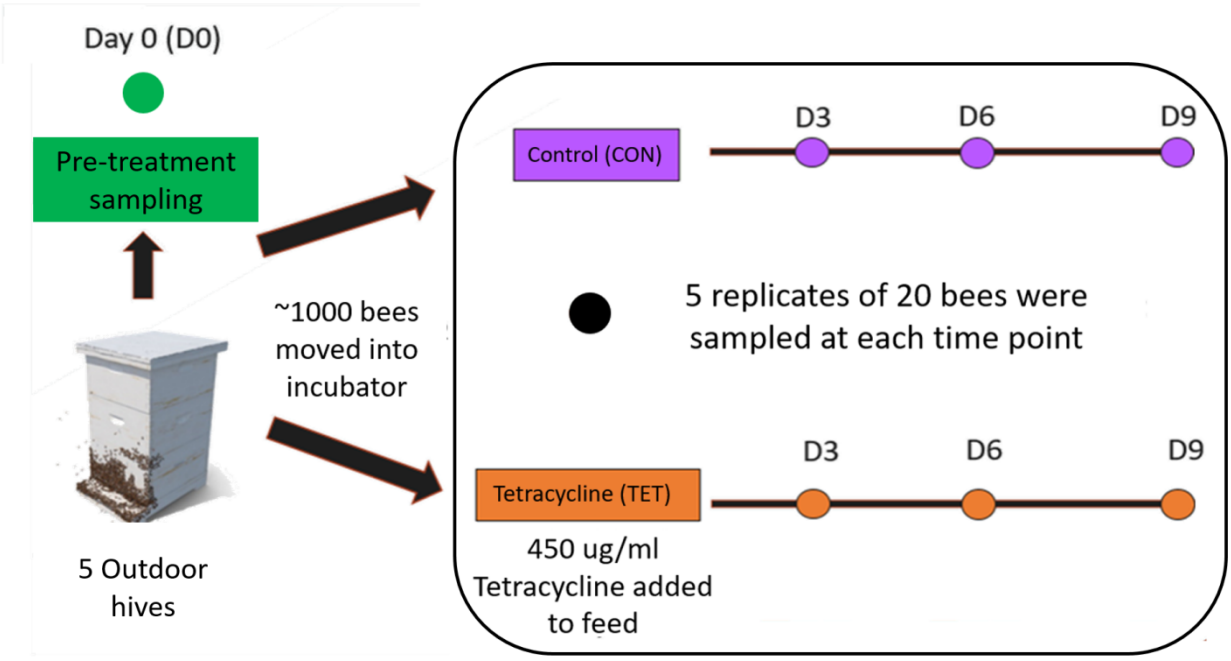

Figure 2. Experimental design. Five replicates of 20 bees were collected from the control (CON) and tetracycline (TET) groups at each time point including Day 0 (D0 – Pre-treatment), and Days 3 (D3), 6 (D6), and 9 (D9).

2.2. DNA EXTRACTION, LIBRARY PREPARATION, AND SEQUENCING

Prior to extraction, bees were placed on sterile filter paper for 10 minutes for defrosting and alcohol evaporation. Bee intestines were dissected by using a sterile pair of scissors to make a cross-sectional cut across the last segment of the bee abdomen. With sterile tweezers, abdominal content was collected out of the abdomen and transferred into microtubes. Abdominal contents from 20 bees were pooled into a single tube for DNA extraction, which was performed using a commercial kit (PowerSoil DNA Isolation kit, Qiagen, Germany) following the manufacturer's protocol. After extraction, DNA was electrophorized in agarose gel for quality analysis. DNA concentrations were quantified by fluorometry (Qubit 2.0, Life Invitrogen, USA) before further processing steps.

The V3-V4 region of the microbial 16S rRNA gene was amplified by PCR using 2.5 μL template DNA (5 ng/μL), 5 μL forward primer, 5 μL reverse primer, and 12 μL 2X KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA) in a total volume of 25 μL. The following primers were used: 341F (5′–TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC A–3′) and 805R (5′–GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C–3′). PCR reaction conditions were as follows: Initial denaturation at 95°C for 3 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30s and a final extension to 72°C for 5 min.

Amplification products were visualized in 1.5% agarose gel before purification using magnetic beads (AMPure XP, Beckman Coulter, USA) to remove excess primer. The dual indices and Illumina sequencing adapters were attached using a Nextera XT Index Kit (Illumina). A second clean up step was then performed using magnetic beads. The purified PCR products were quantified by fluorometry (Qubit 2.0, Life Invitrogen). For sequencing, pooled libraries were denatured with NaOH, diluted with hybridization buffer, then heat denatured. Paired-end sequencing was performed on an Illumina MiSeq with a V2 kit (2 x 250 cycles). At least 5% PhiX DNA was added for sequencing control purposes (Kit PhiX, Illumina). Negative controls including blanks (no template) that underwent the extraction along with all of the other samples and samples from each of the feeds.

2.3. SEQUENCE PROCESSING AND STATISTICAL ANALYSES

The raw demultiplexed paired-end sequences were processed using QIIME 2-2020.2 (BOLYEN *et al.*, 2019). Reads were filtered, denoised, and truncated to a length of 248 base

pairs, and then parsed for non-chimeric sequences using DADA2, producing Amplicon Sequence Variants (ASV) (CALLAHAN *et al.*, 2016). Sequences were aligned using "qiime fragment-insertion sepp" for phylogenetic analysis (MATSEN *et al.*, 2012). Taxonomic composition of the samples were determined rypla pretrained naive Bayes classifier with a 99% sequence similarity threshold for V3-V4 reference sequences (SILVA-132-99-nb-classifier.qza) and the "qiime feature-classifier classify-sklearn". Negative control samples were examined for potential contaminant taxa. No taxa overlapped between negative control and true samples. Microbial diversity was quantified using Pielou's (evenness) and Shannon (richness and abundance) diversity indices. ANOVAs were used to compare diversity between groups in R 4.1.0 (RIPLEY, 2001) after testing for normality using a Shapiro-Wilk test.

Beta diversity was evaluated using Bray-Curtis and Jaccard distances in QIIME 2-2020.2 (BOLYEN *et al.*, 2019). Microbial community composition was evaluated by Principle Coordinate Analysis (PCoA) and visualized using the Emperor plugin 2020.2.0 (VÁZQUEZ-BAEZA *et al.*, 2017). PERMANOVAs were employed as recommended (ANDERSON, Marti J, 2001) to test for differences in microbial composition between experimental groups (Pre-treatment vs. CON vs. TET) and over time (Day 0 – pre-treatment, and Days 3, 6, 9).

Differentially abundant taxa between groups were identified using an analysis of composition of microbiomes (ANCOM) (MANDAL *et al.*, 2015). We also performed a core microbiota analysis in QIIME2, to identify taxa present in 95% of the samples. The relative abundances of core microbes were then compared by treatment and time using two-way ANOVAa after testing for normality using a Shapiro-Wilk test. A P-value < 0.05 was used in the statistical tests for significance.

3. RESULTS

3.1. 16S rRNA SEQUENCING READS

We obtained a total of 3,575,254 raw reads across all samples, ranging from 10,268 to 459,284 reads per sample and averaging 102,150 reads per sample. After the denoising process, 3,346,889 (93.61%) were retained for downstream analyses. Reads were classified into 2,140 features which were aligned to 131 different taxa. Reads identified as chloroplasts, mitochondria, unassigned and eukaryota were removed from all samples.

3.2. MICROBIAL COMPOSITION AND DIVERSITY IN TETRACYCLINE-TREATED BEES

Bee gut microbial composition was significantly altered by treatment (pre-treatment, control, tetracycline) (PERMANOVA: Jaccard R2= 0.115, p = 0.001) and time (D0, D3, D6, D9) (PERMANOVA: Jaccard R2= 0.046, p = 0.001; **Figure 3, Supplementary material. Figure 1).** Notably, the interaction of treatment and time was also significant (Adonis: R2= 0.035, p-value= 0.024), and the effect of treatment increased over time (**Figure 3, Supplementary material. Figure 1**). Microbial diversity also differed significantly by time but not by treatment (Two-way ANOVA: Shannon Index treatment p = 0.295, time p = 0.042; **Figure 4a**). No pairwise comparisons were significant; although, microbial diversity differences on Day 9 (p = 0.081) were greater than at previous timepoints, with the tetracycline group having lower diversity than the control group. Microbial community evenness (Pielou's Index) did not differ significantly by treatment or time (Two-way ANOVA: Pielou's Index treatment p = 0.457, time p = 0.061; **Figure 4b**).

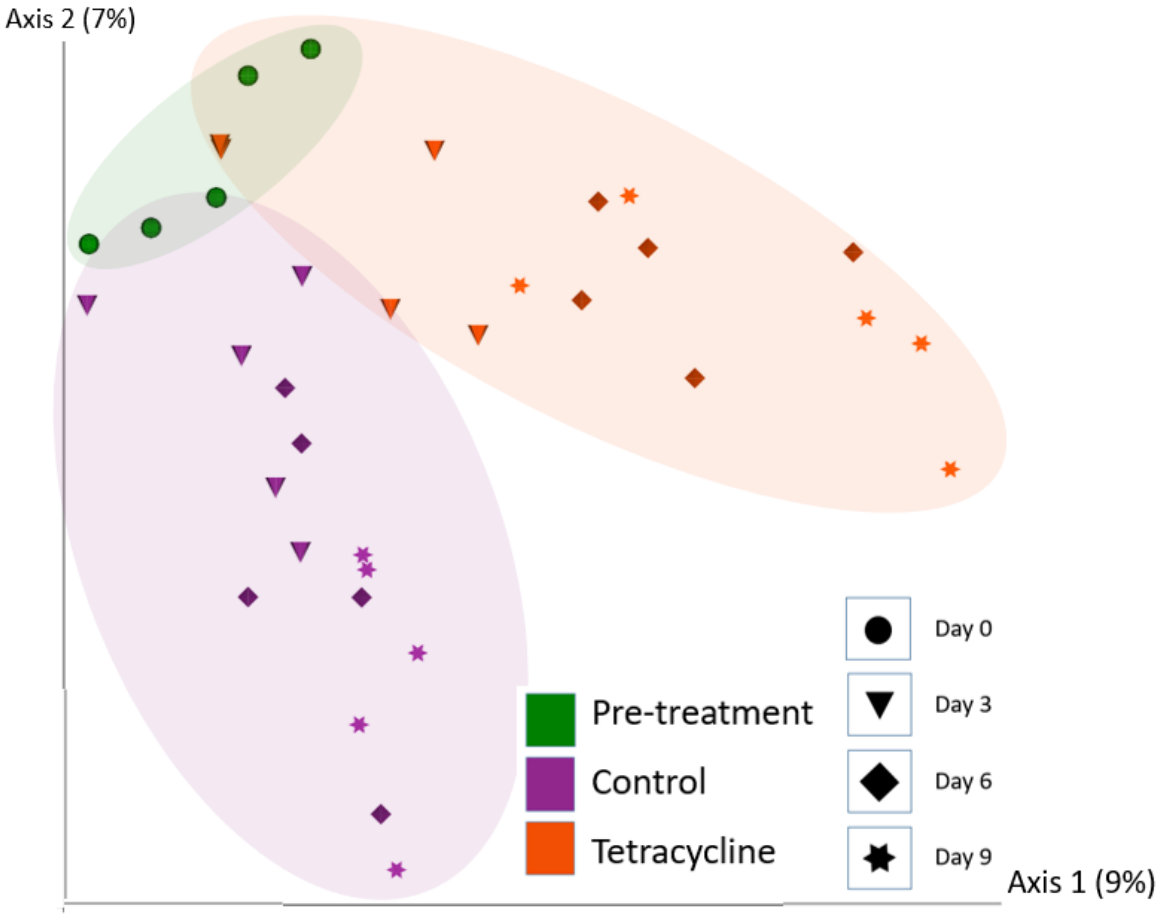

Figure 3. Bee gut microbial composition (Jaccard) based on treatment (Pre-treatment, Control, Tetracycline) and time (Day 0 – pre-treatment, Days 3, 6, 9). Microbial composition was significantly altered by treatment (PERMANOVA: $p = 0.001$) and time (PERMANOVA: $p = 0.001$; also **Supp. Figure 1**).

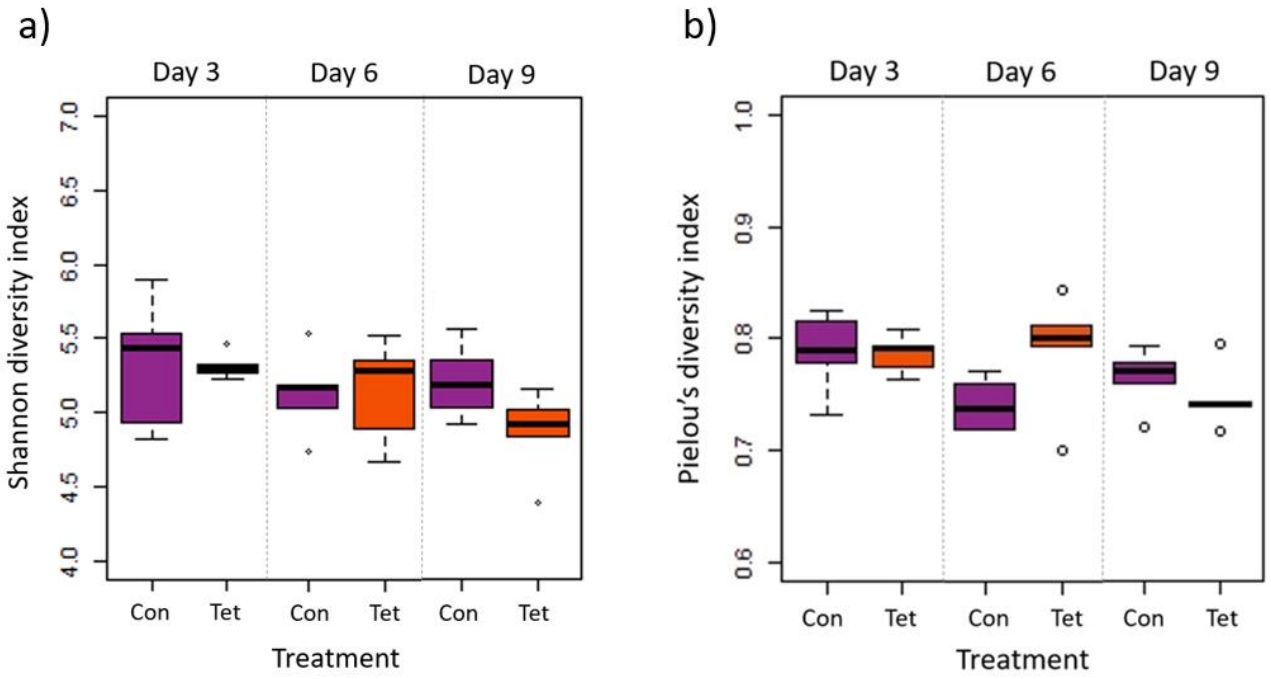

Figure 4. Microbial diversity and evenness by treatment and time. Box plot shows outliers, first and third quartiles (lower and upper edges), and highest, lowest and median values (horizontal black dash) for Control (Con) and Tetracycline (Tet) groups. a) There were significant differences in diversity (Shannon Index) by time (p = 0.042) but not treatment (p = 0.295); although, no pairwise comparisons were significant. b) There were no significant differences in evenness (Pielou's Index) by time (p = 0.061) or by treatment (p = 0.457).

3.3. CORE MICROBIOTA AND DIFFERENTIALLY ABUNDANT TAXA

A core microbiota analyses identified eight genera that were present in 95% of the samples across all treatments and times including: *Lactobacillus*, a taxon from the class Gammaproteobacteria, *Bifidobacterium*, *Snodgrassella*, *Gilliamella*, a taxon from the family Rhizobiaceae, *Apibacter*, and *Commensalibacter* (**Figure 5**). These taxa accounted for 22% of all genera in the dataset. We then used a two-way ANOVA to compare relative abundances of these taxa by treatment and time.

Lactobacillus and the Gammaproteobacteria taxa abundances increased in the tetracycline group over time (Two way ANOVA: *Lactobacillus* treatment $p < 0.0001$, time $p = 0.684$, interaction $p = 0.049$; Gammaproteobacteria treatment $p = 0.0003$, time $p = 0.0001$, interaction Gammaproteobacteria $p = 0.01$; **Figure 5a,b**). *Bifidobacterium* was also increased in the tetracycline group ($p = 0.029$); although, abundances did not change over time (**Figure 5c**). Abundances of *Snodgrassella*, *Gilliamella* and a taxon from the Rhizobiaceae family all decreased over time in the tetracycline group (*Snodgrassella* treatment $p = 0.007$, time $p = 0.006$; *Gilliamella* treatment $p = 0.01$, time $p = 0.065$; Rhizobiaceae treatment $p < 0.0001$, time

p = 0.98; **Figure 5d,e,f**). *Apibacter* was also significantly decreased in the tetracycline group; although only at the early time points (treatment *p* < 0.004; time *p* = 0.834; interaction *p* = 0.004; **Figure 5g**). There were no significant differences in the relative abundances of *Commensalibacter* between groups or over time (p > 0.05; **Figure 5h**).

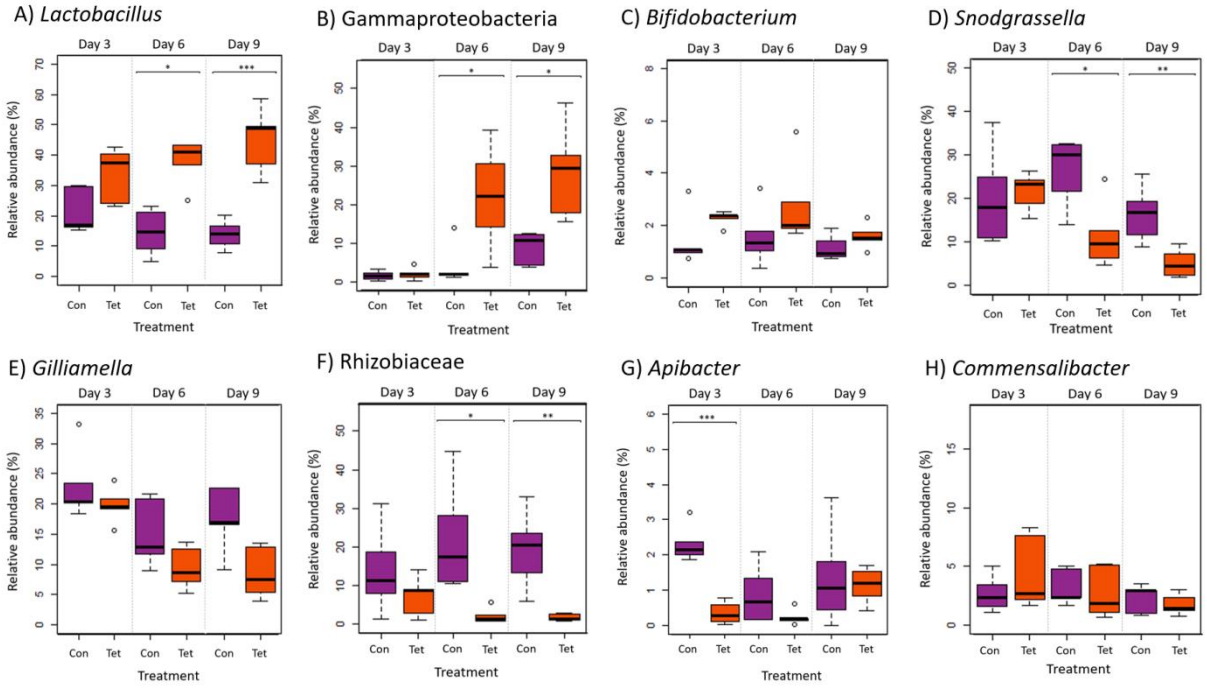

Figure 5. Relative abundances of core microbiota (genera) that were present in 95% of all samples: A) Lactobacillus, B) an unidentified genera in the Gammaproteobacteria class, C) Bifidobacterium, D) Snodgrassella, E) Gilliamella, F) one taxon from the family Rhizobiaceae, G) Apibacter and H) Commensalibacter. Box plot shows outliers, first and third quartiles (lower and upper edges), and highest, lowest and median values (horizontal black dash) for Control (Con) and Tetracycline (Tet) groups. (ANOVA: *p < 0.05; ** p < 0.01 and *** p < 0.001).

An ANCOM identified five differentially abundant taxa by treatment at the genera level, including *Bombella* and *Fructobacillus*, an unidentified taxon in the family Enterobacteriaceae, *Idiomarina,* and an unidentified taxon in the class Gammaproteobacteria (**Figure 6, 5b**). The relative abundances of *Bombella*, *Fructobacillus*, and the Enterobacteriaceae family taxa differed significantly by treatment (Two-way ANOVA: *Bombella p* = 0.000986; *Fructobacillus p* = 0.0002; Enterobacteriaceae taxa *p* < 0.0001) but not by time (*Bombella p* = 0.115; *Fructobacillus p* = 0.107; Enterobacteriaceae taxa *p* = 0.186), and were decreased in the tetracycline group at all time points (**Figure 6a,b,c**). *Idiomarina* differed significantly by treatment (Idiomarina *p* = 0.0002) and by time (*Idiomarina p* < 0.0001;), and there was a significant interaction between treatment and time (*Idiomarina p* = 0.005;) as both *Idiomarina*

and the Gammaproteobacteria taxa increased over time particularly in the tetracycline group (**Figure 6d, 5b**). We also performed an ANCOM analysis at the L7 (roughly species) and amplicon sequencing variant levels and produced similar results in terms of differentially abundant microbes: *Fructobacillus* (W=30) and *Bombella* (W=30) species were decreased in the tetracycline group, while 2 Gammaproteobacteria ASVs (W=194, W=179) increased over time in the tetracycline group.

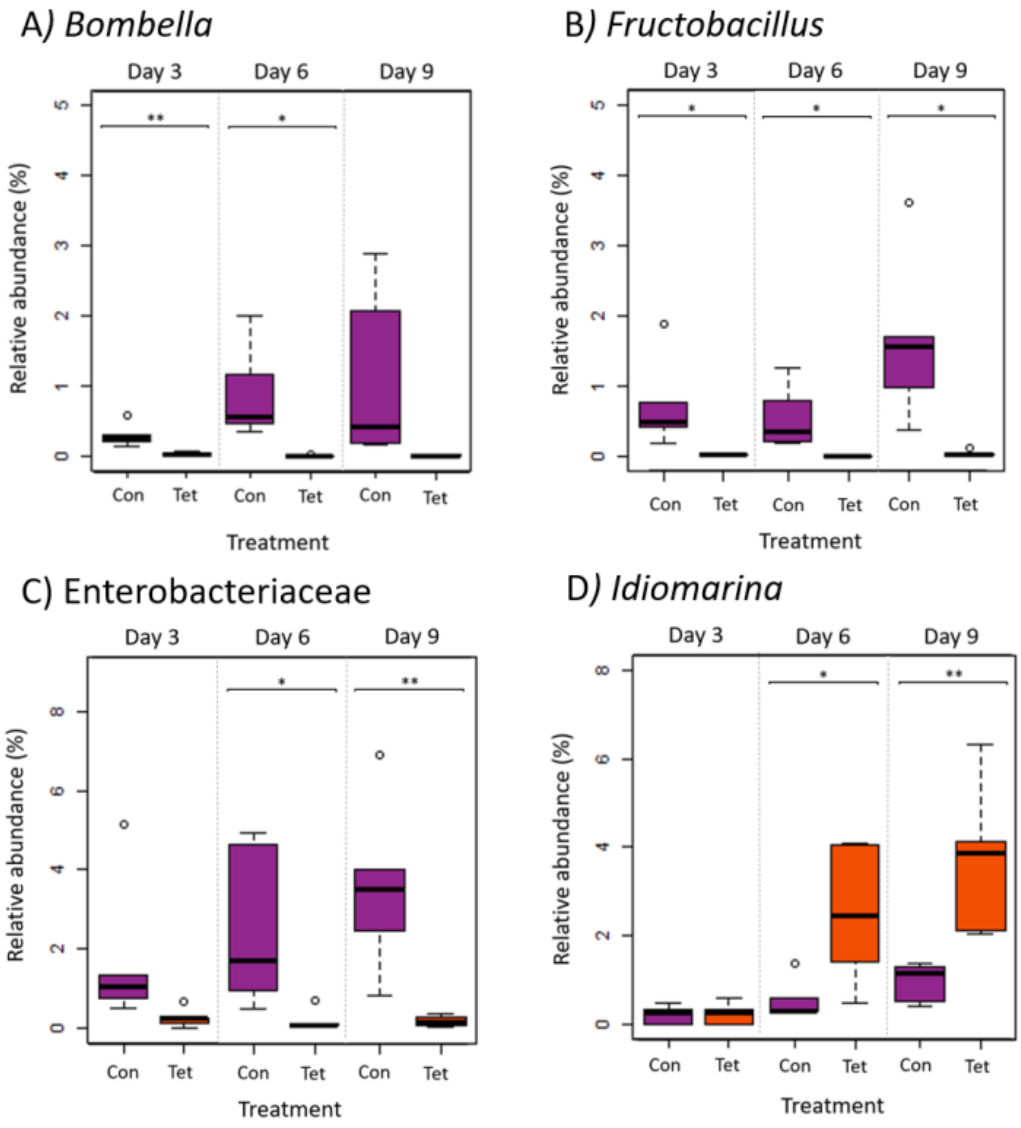

Figure 6. Relative abundances of differentially abundant genera (ANCOM) by treatment and by time. A) *Bombella*, B) *Fructobacillus*, C) a taxon in the family Enterobacteriaceae and D) Idiomarina. A Gammaproteobacteria taxa was also identified as a core microbe and a differentially abundant microbe between Con and Tet groups and is shown in **Figure 5b**. Box plot shows outliers, first and third quartiles (lower and upper edges), and highest, lowest and median values (horizontal black dash) for Control (Con) and Tetracycline (Tet) groups. (ANOVA: * $p < 0.05$; ** $p < 0.01$ and *** significant at $p < 0.001$).

4. DISCUSSION

Our results demonstrated that tetracycline exposure was associated with alterations in Africanized honey bee gut microbial composition but not diversity over time. We further identified shifts in core and non-core microbiota by treatment and time. These tetracycline-linked gut microbial changes could have negative implications for honey bee nutrient metabolism and pathogen resistance.

4.1. CORE MICROBIAL TAXA AND TETRACYCLINE TREATMENT

All 8 core microbial taxa identified in this this study (*Lactobacillus,* a taxon of the class Gammaproteobacteria, *Bifidobacterium*, *Snodgrassella*, *Gilliamella*, a taxon of the family Rhizobiaceae, *Apibacter*, and *Commensalibacter*) have been previously reported as core microbiota in European honey bees (Engel *et al*., 2012; Powell *et al*., 2014; Kwong and Moran, 2016; Raymann *et al*., 2017; Motta *et al*., 2018). The increased relative abundance of three core microbes - *Lactobacillus*, *Bifidobacterium,* and a taxon of the Gammaproteobacteria class – in bees exposed to tetracycline has also been observed in previous studies on bees exposed to chemical compounds or in compromised hives. For instance, increased relative abundances of *Lactobacillales and Gammaproteobacteria* were reported in hives showing colony collapse disorder (CCD) (Cornman *et al*., 2012), while increased abundances of Bifidobacteriaceae have been reported in bees exposed to coumaphos, an organophosphate insecticide used to control ectoparasites on cattle (BLEAU *et al.*, 2020). Taken together, these results indicate that *Lactobacillus, Bifidobacterium,* and Gammaproteobacteria may be positively associated with exposure to agrochemicals. It is also possible that these changes in microbial abundance may actually represent changes in environmental microbes (source microbes) associated with agrochemicals. However, the *Lactobacillus* in this study are specifically identified as *Lactobacillus melliventris*, *Lactobacillus kunkeei*, *Lactobacillus apis, Lactobacillus helsingborgensis, Lactobacillus sp., Lactobacillus kimbladii strain Dan46* all taxon that have been isolated from the honeybee gastrointestinal tract (ARREDONDO *et al.*, 2018; KILLER *et al.*, 2014; MUDROŇOVÁ *et al.*, 2011; OLOFSSON *et al.*, 2014). Moreover, in this study, bees were exposed to tetracycline through their feed in a controlled environment as opposed to foraging in a natural environment broadly contaminated with tetracycline. This leads us to speculate that the microbial abundance changes we observed are likely to be bee-associated as opposed to environmental. Notably, our results differ from two studies on European honey bees

that reported decreases in *Lactobacillius* and *Bifidobacterium* following exposure to oxytetracycline or tetracycline (Daisley *et al*., 2020; Raymann *et al*. 2017).

While *Lactobacillus*, *Bifidobacterium,* and a Gammaproteobacteria taxon increased in response to tetracycline exposure, four core taxa decreased in relative abundance under the same treatment: *Snodgrassella*, *Gilliamella*, *Apibacter,* and a taxon of the Rhizobiaceae family. *Snodgrassella*, *Gilliamella*, *Apibacter,* and a taxon of the Rhizobiaceae family. A decrease in *Snodgrassella* has been observed in previous studies on European bees after exposure to tetracycline or glyphosate (MOTTA; RAYMANN; MORAN, 2018; RAYMANN; SHAFFER; MORAN, 2017). *Snodgrassella* and *Gillamella* synergistically produce a biofilm on the gut wall (RAYMANN; MORAN, 2018) that serves as barrier against pathogen colonization and translocation (ENGEL; MARTINSON; MORAN, 2012; MARTINSON; MOY; MORAN, 2012; MOTTA; RAYMANN; MORAN, 2018). Moreover, *Snodgrassella* plays an important role in digestion and energy production through the oxidation of fermentation products. *Gilliamella* is involved in nutrient metabolism and is the major degrader of monosaccharides, pectin, and hemicellulose in the bee gut (ENGEL; MARTINSON; MORAN, 2012; FOUAD *et al.*, 2016a; ZHENG *et al.*, 2019). Pectin-rich pollen is large part of the honey bee diet, but bees do not produce pectinases and must rely on gut microbes like *Gilliamella* for pectin metabolism. Like *Snodgrassella* and *Gilliamella*, *Apibacter* also colonizes the gut wall (KWONG; STEELE; MORAN, 2018), and some strains of *Apibacter* encode a type VI secretion system (T6SS) (KWONG; STEELE; MORAN, 2018), which promotes colonization resistance through the delivery of toxic antibacterial proteins into neighboring cells (STEELE *et al.*, 2017). Decreased abundances of *Snodgrassella*, *Gilliamella,* and *Apibacter* could impact nutrient metabolism and pathogen defense in Africanized honey bees.

Tetracyclines are broad-spectrum antibiotics with activity against both gram-positive and gram-negative bacteria. However, it is possible that slight differences in sensitivity to tetracycline could explain the taxonomic shifts we observed with tetracycline exposure. Gram positive and gram negative bee gut bacteria reportedly have different sensitivities to host-produced antimicrobial peptides including apidaecin and hymenoptaecin. In a previous study by Kwong *et al*. (2017), gram-positive species (*Lactobacillus* Firm-5, *Bifidobacterium* sp.) were highly resistant to apidaecin and hymenoptaecin, while gram-negative species, particularly *Snodgrassella alvi*, were more sensitive to hymenoptaecin. It is possible that gram positive bacteria, such as *Lactobacillus* and *Bifidobacterium* are less sensitive to tetracycline, while gram negative bacteria – such as *Snodgrassella*, *Gilliamella*, *Apibacter,* and

Rhizobiaceae, are more sensitive to tetracycline and therefore decreased in abundance following tetracycline exposure while *Lactobacillus* and *Bifidobacterium* increased (Powell *et al*., 2014; Kwong and Moran, 2016; Kešnerová *et al*., 2020). It is also possible that these differences in sensitivity may be linked to gut location: Microbial taxa common in the ileum (*Snodgrassella, Gilliamella, Apibacter,* and *Rhizobiaceae*) may be exposed earlier or to greater concentrations of tetracycline than bacterial species that dominate the hindgut (*Lactobacillus* and *Bifidobacterium*). *Commensalibacter* was the only core microbe that did not vary in relative abundance after tetracycline exposure; however, these bacteria do vary by season and age in honey bees (Ellegaard and Engel, 2019). In sum, alterations in the core microbiota following tetracycline exposure, and particularly decreased abundances of *Snodgrassella*, *Gilliamella*, Rhizobiaceae, and *Apibacter*, suggest a reduced capacity for pathogen defense and nutrient metabolism which could potentially increase the susceptibility of Africanized honey bees to parasites or infections.

4.2. DIFFERENTIALLY ABUNDANT MICROBES BY TREATMENT

Among the five differentially abundant taxa identified between treatment groups, three (*Bombella*, *Fructobacillus*, an Enterobacteriaceae taxon) were decreased in abundance in bees exposed to tetracycline, while two were increased (*Idiomarina,* and a Gammaproteobacteria taxon, which was also identified as a core bacteria). *Bombella,* formerly *Parasaccharibacter apium* (SMITH, Eric A *et al.*, 2020), is positively associated with bee larval development and protection against *Nosema apis* infection (CORBY-HARRIS *et al.*, 2016; MILLER; SMITH; NEWTON, 2021). A previous study also showed that exposure to thiacloprid (insecticide) led to *Bombella* reductions in a dose-dependent manner (Liu *et al*., 2020). The decreased abundance of *Fructobacillus* observed in our study was expected, as these bacteria are known to be highly sensitive to tetracycline (ROKOP; HORTON; NEWTON, 2015). *Fructobacillus* is found throughout bee hives (ENDO *et al.*, 2011) and it colonizes brood cells, bee bread, and nectar, creating a niche that promotes the growth and inoculation of core microbes into larvae and developing worker bees (ROKOP; HORTON; NEWTON, 2015). As such, decreased abundances of *Bombella* and *Fructobacillus* due to tetracycline exposure could negatively affect Africanized honey bee larval development.

To our knowledge, this is the first study characterizing the gut microbiota of Africanized honey bees in relation to tetracycline exposure. However, this study had several limitations. While the microbial shifts we observed suggest possible negative implications for bee health,

we do not have associated immunological, behavioral, fitness, or production data to explicitly support these implications. Secondly, in this study, we selected a tetracycline concentration consistent with that reported in some agricultural or hive applications. However, quantifying the concentration of tetracycline to which bees are actually exposed under natural conditions is challenging and likely highly variable. Third, we observed a shift in the gut microbiota between pre-treatment and *both* the CON and TET groups, suggesting either an age or "incubator effect" due to an altered diet and environment; although, we attempted to replicate natural temperature and humidity conditions as closely as possible within the incubator. Despite this, there were still clear differences between the CON and TET groups over time. Fourth, we did not perform absolute quantification (qPCR) of bacterial abundances in this study. As such, the changes in relative abundance of bacterial taxa we observed between groups may or may not be significant in terms of absolute abundances. Finally, the function of some of the differentially abundant microbes we identified, such as *Apibacter*, have yet to be elucidated. As such, deeper sequencing and associated studies with metabolomics or transcriptomics are necessary to clarify the role of these microbes in the bee gut.

5. CONCLUSION

Tetracycline exposure altered gut microbial composition in Africanized honey bees (*Apis mellifera scutellata* x spp), and was specifically associated with decreased abundances of *Bombella*, *Fructobacillus, Snodgrassella*, *Gilliamella*, Rhizobiaceae, and *Apibacter*. These microbes play a key role in nutrient metabolism and pathogen defense, and reduced abundances of these microbes could potentially have negative impacts on bee health. Considering the global ecological and economic importance of honey bees as pollinators, it is critical to understand the effects of widely used antimicrobials on honey bee health, as bees can be directly or indirectly exposed to these drugs throughout the environments in which they forage. Future studies assessing bee fitness, behavior, immune response, and disease susceptibility in relation to agrochemical exposure will further elucidate the impacts of these gut microbial changes. Understanding how chemicals, like antimicrobials, affect bees is essential to guide agricultural practices that effectively support ecosystem health.

Additional Requirements

All experimental procedures were previously approved by the Biodiversity Authorization and Information System – SISBIO (Protocol #: 71750-1, approved on 09/19/2019).

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Data Availability Statement

Sequencing data is available at NCBI (PRJNA732391).

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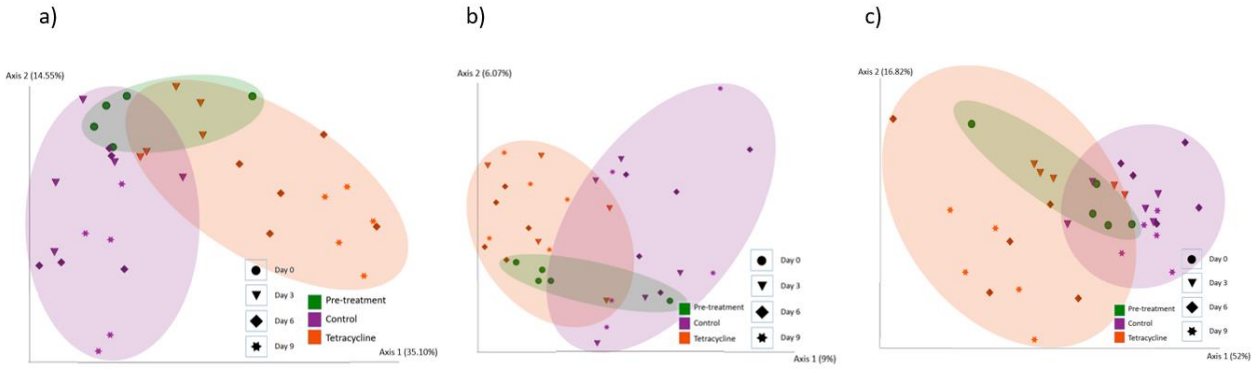
Supplementary material



Supplementary material. Figure 2. Bee gut microbial composition by treatment (Pre-treatment, Control, Tetracycline) and time (Day 0 – pre-treatment, Days 3, 6, 9) by a) Bray-Curtis, b) Unweighted UniFrac and c) Weighted UniFrac. Microbial composition was significantly altered by treatment (PERMANOVA: Bray Curtis p = 0.001; Unweighted UniFrac p = 0.001; Weighted UniFrac. p = 0.001) and by time (PERMANOVA: Bray-Curtis p = 0.001; Unweighted UniFrac p = 0.376; Weighted UniFrac p = 0.013). Also see **Figure 3**.

SUPPLEMENTARY MATERIAL

Qiime 2 scripts

Script adapted from Sampson *et al*. (2016)

```
#PBS -N ondemand/sys/myjobs/default
#PBS -A PAS1331
#PBS -l walltime=24:00:00
#PBS -l nodes=1:ppn=10
#PBS -j oe
#PBS -m abe
#
# Move to the directory where the job was submitted
#
cd /users/PAS1331/kilmersoares/ondemand/data/sys/myjobs/projects/default/17
source activate qiime2-2020.2
#
# Run script
#
sh myscript.sh > my_results
#
# Now, copy data (or move) back once the simulation has completed
#
###cp my_results $PBS_O_WORKDIR
qiime metadata tabulate \
```

```
  --m-input-file metadata.tsv \
  --o-visualization metadata.qzv
    #Importing data into QIIME 2
qiime tools import \
  --type 'SampleData[PairedEndSequencesWithQuality]' \
  --input-path readsgz/ \
  --input-format CasavaOneEightLanelessPerSampleDirFmt \
  --output-path demux_seqs.qza
      qiime demux summarize \
  --i-data ./demux_seqs.qza \
  --o-visualization ./demux_seqs.qzv
   #Sequence quality control and feature table (#ONLY WORKS IN THE QUEUE
qiime dada2 denoise-single \
  --i-demultiplexed-seqs ./demux_seqs.qza \
  --p-trunc-len 248 \
  --o-table ./dada2_table.qza \
  --o-representative-sequences ./dada2_rep_set.qza \
  --o-denoising-stats ./dada2_stats.qza
qiime metadata tabulate \
  --m-input-file ./dada2_stats.qza  \
  --o-visualization ./dada2_stats.qzv
qiime feature-table summarize \
  --i-table ./dada2_table.qza \
  --m-sample-metadata-file ./metadata.tsv \
```

```
 --o-visualization ./dada2_table.qzv

  #Generating a phylogenetic tree for diversity analysis

qiime fragment-insertion sepp \

 --i-representative-sequences ./dada2_rep_set.qza \

 --i-reference-database sepp-refs-gg-13-8.qza \

 --o-tree ./tree.qza \

 --o-placements ./tree_placements.qza \

 --p-threads 4  # update to a higher number if you can

  #Alpha Rarefaction, Selecting a Rarefaction Depth

qiime diversity alpha-rarefaction --i-table ./dada2_table.qza  --m-metadata-file ./metadata.tsv  --o-visualization ./alpha_rarefaction_curves.qzv  --p-min-depth 10 --p-max-depth 10268

 #Diversity analysis

qiime diversity core-metrics-phylogenetic \

 --i-table ./dada2_table.qza \

 --i-phylogeny ./tree.qza \

 --m-metadata-file ./metadata.tsv \

 --p-sampling-depth 5600 \

 --output-dir ./core-metrics-results

   #Alpha Rarefaction group significance (after core metrics)

qiime diversity alpha-group-significance \

 --i-alpha-diversity core-metrics-results/shannon_vector.qza \

 --m-metadata-file metadata.tsv \

 --o-visualization core-metrics-results/shannon_vector-group-significance.qzv

   #Alpha diversity

qiime diversity alpha-group-significance \
```

```
  --i-alpha-diversity ./core-metrics-results/faith_pd_vector.qza \
  --m-metadata-file ./metadata.tsv \
  --o-visualization ./core-metrics-results/faiths_pd_statistics.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results/evenness_vector.qza \
 --m-metadata-file ./metadata.tsv \
 --o-visualization ./core-metrics-results/evenness_statistics.qzv
   #alpha-correlation for continuous covariate
qiime diversity alpha-correlation \
--i-alpha-diversity core-metrics-results/evenness_vector.qza \
--m-metadata-file metadata.tsv \
--p-method spearman \
--output-dir alpha_correlation/
qiime diversity alpha-correlation \
--i-alpha-diversity core-metrics-results/shannon_vector.qza \
--m-metadata-file metadata.tsv \
--p-method shannon \
--output-dir alpha_correlation_shannon/
  #Alpha diversity longitudinal anova
qiime longitudinal anova \
 --m-metadata-file ./core-metrics-results/faith_pd_vector.qza \
 --m-metadata-file ./metadata.tsv \
 --p-formula 'faith_pd ~ treatment_FCT * time_days' \
 --o-visualization ./core-metrics-results/faiths_pd_anova.qzv
```

```
    #Beta diversity
   #For treatment_FCT
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column treatment_FCT \
  --o-visualization core-metrics-results/unweighted-unifrac-treatment_FCT-significance.qzv
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column treatment_FCT \
  --o-visualization core-metrics-results/weighted-unifrac-treatment_FCT-significance.qzv
    #For treatment
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column treatment \
  --o-visualization core-metrics-results/unweighted-unifrac-treatment-significance.qzv \
  --p-pairwise
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column treatment \
  --o-visualization core-metrics-results/weighted-unifrac-treatment-significance.qzv \
```

```
  --p-pairwise
    #For time
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column time_days \
  --o-visualization core-metrics-results/unweighted-unifrac-time_days-significance.qzv \
  --p-pairwise
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column time_days \
  --o-visualization core-metrics-results/weighted-unifrac-time_days-significance.qzv \
  --p-pairwise
   #see a statistically significant difference even if it is caused by variation within one group
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column treatment \
  --o-visualization core-metrics-results/unweighted-unifrac-treatment-significance_disp.qzv \
  --p-method permdisp
    #Adonis to look at a multivariate model
 qiime diversity adonis \
  --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
```

```
  --m-metadata-file metadata.tsv \
  --o-visualization core-metrics-results/unweighted_adonis.qzv \
  --p-formula treatment_FCT+time_days

  #Taxonomic classification
    #alinhar e classificar
qiime feature-classifier classify-sklearn \
  --i-reads ./dada2_rep_set.qza \
  --i-classifier ./silva-132-99-nb-classifier.qza \
  --o-classification ./taxonomy.qza
qiime metadata tabulate \
  --m-input-file ./taxonomy.qza \
  --o-visualization ./taxonomy.qzv
qiime feature-table tabulate-seqs \
  --i-data ./dada2_rep_set.qza \
  --o-visualization ./dada2_rep_set.qzv
  #Filter the table of AVSs
#Mitochondria
qiime taxa filter-table \
--i-table dada2_table.qza \
--i-taxonomy taxonomy.qza \
--p-exclude      Mitochondria \
--o-filtered-table table-no-mitochondria.qza
#Chloroplast
```

```
qiime taxa filter-table \
 --i-table table-no-Unassigned.qza \
 --i-taxonomy taxonomy.qza \
 --p-exclude     Chloroplast \
 --o-filtered-table table-no-mitochondria.qza
#Unassigned
qiime taxa filter-table \
 --i-table table-no-Unassigned.qza \
 --i-taxonomy taxonomy.qza \
 --p-exclude     Unassigned \
 --o-filtered-table table-no-mitochondria.qza
#Eukaryota
qiime taxa filter-table \
 --i-table table-no-Unassigned.qza \
 --i-taxonomy taxonomy.qza \
 --p-exclude     Unassigned \
 --o-filtered-table table-final.qza
  #Realign taxonomia
qiime feature-table filter-seqs \
 --i-data dada2_rep_set.qza \
 --i-table table-final.qza \
 --o-filtered-data seqs-final \
qiime feature-classifier classify-sklearn \
 --i-reads ./seqs-final.qza \
```

```
 --i-classifier ./silva-132-99-nb-classifier.qza \
 --o-classification ./taxonomyfinal.qza
qiime metadata tabulate \
 --m-input-file ./taxonomyfinal.qza \
 --o-visualization ./taxonomyfinal.qzv
  #Taxonomy barchart qiime feature-table filter-samples \
 --i-table ./table-final.qza \
 --p-min-frequency 5000 \
 --o-filtered-table ./table_2kfiltered.qza
qiime taxa barplot \
 --i-table ./table_2kfiltered.qza \
 --i-taxonomy ./taxonomyfinal.qza \
 --m-metadata-file ./metadata.tsv \
 --o-visualization ./taxa_barplotfiltered.qzv
  #genereates the table with the number of reads per sample
qiime tools export \
 --input-path table_2kfiltered.qza \
 --output-path table_exported-feature-table
cd table_exported-feature-table
biom convert --to-tsv -i feature-table.biom -o bee_tet_table.tsv
#Differential abundance with ANCOM
qiime feature-table filter-features \
  --i-table ./table_2kfiltered.qza \
  --p-min-frequency 50 \
```

```
  --p-min-samples 3 \
  --o-filtered-table ./table_2k_abund.qza
  # table with taxonomy
 qiime taxa collapse \
  --i-table table_2k_abund.qza \
  --i-taxonomy taxonomy.qza \
  --p-level 6 \
  --o-collapsed-table table-l6.qza
    #Differential abundance with ANCOM
qiime composition add-pseudocount \
  --i-table table-l6.qza \
  --o-composition-table comp-table-l6.qza
#Ancon treatment
qiime composition ancom \
  --i-table comp-table-l6.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column treatment \
  --o-visualization ancom-subject.qzv
  #Ancon treatment
 qiime composition ancom \
  --i-table comp-table-l6.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column treatment_FCT \
  --o-visualization ancom-subject_FCT.qzv
```

```
   #core features
qiime feature-table core-features --i-table table-l6.qza  --o-visualization core-features
#Longitudinal analysis
#PCoA-based analyses
qiime longitudinal volatility \
--m-metadata-file ./metadata.tsv \
--m-metadata-file ./core-metrics-results/unweighted_unifrac_pcoa_results.qza \
--p-state-column time_hours \
--p-individual-id-column samples \
--p-default-group-column 'treatment' \
--p-default-metric 'Axis 2' \
--o-visualization ./pc_vol.qzv
#Machine-learning classifiers for predicting sample characteristics
qiime sample-classifier classify-samples \
--i-table ./table-l6.qza \
--m-metadata-file ./metadata.tsv \
--m-metadata-column treatment \
--p-random-state 666 \
--p-n-jobs 1 \
--output-dir ./sample-classifier-results/
qiime sample-classifier heatmap \
 --i-table ./table-l6.qza \
 --i-importance ./sample-classifier-results/feature_importance.qza \
 --m-sample-metadata-file metadata.tsv \
```

--m-sample-metadata-column treatment \

--p-group-samples \

--p-feature-count 100 \

--o-heatmap ./sample-classifier-results/heatmap100.qzv \

--o-filtered-table ./sample-classifier-results/filtered-table100.qza

R Script

library(readr)

shannon_ct <- read_tsv("D:/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Dados pra valer dos Antimicrobianos/Tetracycline effect RESULTS/alpha_correlation/shannon_ct.tsv")

View(shannon_ct)

library(readr)

shannon_ct_d3 <- read_tsv("D:/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Dados pra valer dos Antimicrobianos/Tetracycline effect RESULTS/alpha_correlation/shannon_ct_d3.tsv")

View(shannon_ct_d3)

library(readr)

shannon_ct_d6 <- read_tsv("D:/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Dados pra valer dos Antimicrobianos/Tetracycline effect RESULTS/alpha_correlation/shannon_ct_d6.tsv")

View(shannon_ct_d6)

library(readr)

shannon_ct_d9 <- read_tsv("D:/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Dados pra valer dos Antimicrobianos/Tetracycline effect RESULTS/alpha_correlation/shannon_ct_d9.tsv")

View(shannon_ct_d9)

library(readr)

```
shannon_ct_control <- read_tsv("D:/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Dados pra valer dos Antimicrobianos/Tetracycline effect RESULTS/alpha_correlation/shannon_ct_control.tsv")

View(shannon_ct_control)

library(readr)

shannon_ct_tet <- read_tsv("D:/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Dados pra valer dos Antimicrobianos/Tetracycline effect RESULTS/alpha_correlation/shannon_ct_tet.tsv")

View(shannon_ct_tet)

#importdata

library(readr)

reads_ct <- read_tsv("D:/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Dados pra valer dos Antimicrobianos/Tetracycline effect RESULTS/table_exported-feature-table/reads_ct.tsv")

View(reads_ct)

#reads_ct_ctrl.tsv

library(readr)

reads_ct_ctrl <- read_tsv("D:/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Dados pra valer dos Antimicrobianos/Tetracycline effect RESULTS/table_exported-feature-table/reads_ct_ctrl.tsv")

View(reads_ct_ctrl)

#reads_ct_tet.tsv

library(readr)

reads_ct_tet <- read_tsv("D:/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Dados pra valer dos Antimicrobianos/Tetracycline effect RESULTS/table_exported-feature-table/reads_ct_tet.tsv")
```

```
View(reads_ct_tet)

#reads_ct_d3.tsv

library(readr)

reads_ct_d3 <- read_tsv("D:/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Dados pra valer dos Antimicrobianos/Tetracycline effect RESULTS/table_exported-feature-table/reads_ct_d3.tsv")

View(reads_ct_d3)

#reads_ct_d6.tsv

library(readr)

reads_ct_d6 <- read_tsv("D:/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Dados pra valer dos Antimicrobianos/Tetracycline effect RESULTS/table_exported-feature-table/reads_ct_d6.tsv")

View(reads_ct_d6)

#reads_ct_d9.tsv

library(readr)

reads_ct_d9 <- read_tsv("D:/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Dados pra valer dos Antimicrobianos/Tetracycline effect RESULTS/table_exported-feature-table/reads_ct_d9.tsv")

View(reads_ct_d9)

#Shannon Index

#Two way anova

anova <- aov(shannon ~ Treat_FCT * Day, data=shannon_ct)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

#ratio_FB

ratio_FB<- (reads_ct$ratio_FB)
```

```
head(ratio_FB)

Treat<- (reads_ct$Treat_FCT)

head(Treat)

dados=data.frame(Treat,ratio_FB)

head(dados)

media=tapply(dados$ratio_FB,dados$Treat,mean)

media

anova <- aov(ratio_FB  ~ Treat_FCT * Day, data=reads_ct)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

#Two way anova

#Total time

#Two way anova

anova <- aov(total  ~ Treat_FCT * Day, data=reads_ct)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

# Lactobacillus

#Two way anova

anova <- aov(Lactobacillus  ~ Treat_FCT * Day, data=reads_ct)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

# Gilliamella

#Two way anova

anova <- aov(Gilliamella  ~ Treat_FCT * Day, data=reads_ct)
```

```
summary(anova)

tukey_ <- LTukey(anova, which="Treat", conf.level=0.95)

# Snodgrassella

#Two way anova

anova <- aov(Snodgrassella  ~ Treat_FCT * Day, data=reads_ct)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

# Rhizobiaceae

#Two way anova

anova <- aov(Rhizobiaceae ~ Treat_FCT * Day, data=reads_ct)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

# Commensalibacter

#Two way anova

anova <- aov(Commensalibacter ~ Treat_FCT * Day, data=reads_ct)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

# Bifidobacterium

#Two way anova

anova <- aov(Bifidobacterium ~ Treat_FCT * Day, data=reads_ct)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

#Apibacter

#Two way anova
```

```
anova <- aov(Apibacter ~ Treat_FCT * Day, data=reads_ct)
summary(anova)
tukey <- LTukey(anova, which="Treat", conf.level=0.95)
# Fructobacillus
#Two way anova
anova <- aov(Fructobacillus ~ Treat_FCT * Day, data=reads_ct)
summary(anova)
tukey <- LTukey(anova, which="Treat", conf.level=0.95)
# Bombella
#Two way anova
anova <- aov(Bombella ~ Treat_FCT * Day, data=reads_ct)
summary(anova)
tukey <- LTukey(anova, which="Treat", conf.level=0.95)
# Idiomarina
#Two way anova
anova <- aov(Idiomarina ~ Treat_FCT * Day, data=reads_ct)
summary(anova)
tukey <- LTukey(anova, which="Treat", conf.level=0.95)
# Enterobacteriaceae
#Two way anova
anova <- aov(Enterobacteriaceae ~ Treat_FCT * Day, data=reads_ct)
summary(anova)
tukey <- LTukey(anova, which="Treat", conf.level=0.95)
# Gammaproteobacteria
```

```
#Two way anova
anova <- aov(Gammaproteobacteria ~ Treat_FCT * Day, data=reads_ct)
summary(anova)
tukey <- LTukey(anova, which="Treat", conf.level=0.95)
```

CAPÍTULO 4 - “The effects of zinc and laboratory emerge date on honey bee gut microbiota”

Zinc and laboratory emerge date impact honey bee gut microbiota and survival

Abstract: Honey bees (*Apis mellifera*) may be exposed to a wide variety of chemicals in the environment, including pesticides, antibiotics, and metals. Zinc, for example, is commonly included in fertilizers, pesticides, and feed additives, and is found in agricultural runoff. Honey bees can be exposed to zinc directly or indirectly by consuming zinc-contaminated nectar and pollen. However, there is a paucity of studies addressing the putative effects of zinc on honey bee´s health. In this study, we tested the effects of zinc on honey bee survivorship and gut microbiota. To evaluate survivorship, we exposed bees to six concentrations of zinc (0, 50, 100, 250, 500, or 1000 mg/L) and assessed survival daily for 10 days. To evaluate effects of zinc on gut microbiota, we exposed bees to 5 or 100 mg/L zinc. Bees were sampled before (day 0) and after zinc exposure (days 3, 6, and 9). Abdominal contents underwent DNA extraction and 16S rRNA sequencing (V3-V4) on an Illumina MiSeq. Sequences were filtered and processed through QIIME2 and DADA2. Zinc treatment had minimal effects on bacterial DNA concentrations and absolute cell counts while emerge date (the date a bee emerged from the brood comb) had a significant effect with decreased bacterial concentrations and cell counts observed at later emerge dates. Survival was only minimally impacted (>89% survival) at zinc concentrations up to 100 mg/L. Zinc had limited effects on overall gut microbial composition, diversity, and taxonomic abundances, with the greatest differences noted in the bee group exposed to the higher concentration of zinc (100 mg/L). In this group, several beneficial taxa (*Lactobacillus,* Rhizobiaceae (*Bartonella* sp.), *Gilliamella*) were found at reduced abundances, while *Paenibacillus,* a potentially pathogenic taxa, was found at increased abundances. This suggests that zinc exposure, even at relatively low levels, may negatively impact honey bee health, even if survivorship is not dramatically impacted. Notably, emerge date effects were also observed in microbial composition. These results demonstrate the need to include assessments of honey bee gut microbiota in addition to other metrics of honey bee health and survivorship when evaluating the potential effects of agrochemicals on honey bees

Keywords: Honey bee, Gut Microbiota, Zinc, Emerge Day, Survival, Agrochemical

Zinco e data de emergência em laboratório impactam a microbiota intestinal e a sobrevivência das abelhas

Resumo: As abelhas estão expostas a uma ampla variedade de produtos químicos antropogênicos no meio ambiente, incluindo pesticidas, antibióticos, poluentes do ar, aditivos plásticos e metais. O zinco, por exemplo, é comumente incluído em fertilizantes e pesticidas e encontrado no escoamento agrícola. As abelhas podem ser expostas ao zinco direta ou indiretamente pelo consumo de néctar e pólen contaminados com zinco. No entanto, o impacto do zinco na saúde das abelhas não foi bem explorado. Neste estudo, testamos os efeitos do zinco na sobrevivência das abelhas e na microbiota intestinal. Para avaliar a sobrevivência, expusemos as abelhas a sete concentrações de zinco (0, 50, 100, 250, 500 ou 1000 mg/L) e avaliamos a sobrevivência diariamente por 10 dias. A sobrevivência foi apenas minimamente impactada (>89% de sobrevivência) em concentrações de zinco até 100 mg/L. Para avaliar os efeitos do zinco na microbiota intestinal, expusemos as abelhas a 5 ou 100 mg/L de zinco. As abelhas foram amostradas antes (dia 0) e após a exposição ao zinco (dias 3, 6 e 9). O conteúdo abdominal foi submetido à extração de DNA e sequenciamento de 16S rRNA (V3-V4) em um Illumina MiSeq. As sequências foram filtradas e processadas através de QIIME2 e DADA2. O tratamento com zinco teve efeitos mínimos nas concentrações de DNA bacteriano e contagens absolutas de células, enquanto a data de emergência (a data em que uma abelha emergiu do favo de cria) teve um efeito significativo com a diminuição das concentrações bacterianas e contagens de células observadas em datas de emergência posteriores. O zinco teve efeitos limitados na composição microbiana geral do intestino, diversidade e abundâncias taxonômicas, com as maiores diferenças observadas no grupo de abelhas expostas à maior concentração de zinco (100 mg/L). Neste grupo, vários táxons benéficos (*Lactobacillu*s, Rhizobiaceae (*Bartonella* sp.), *Gilliamella*) foram encontrados em abundâncias reduzidas, e *Paenibacillus*, um táxon potencialmente patogênico, foi encontrado em abundâncias aumentadas. Isso sugere que a exposição ao zinco, mesmo em níveis relativamente baixos, pode afetar negativamente a saúde das abelhas, mesmo que a sobrevivência não seja drasticamente afetada. Notavelmente, os efeitos da data de emergência também foram observados na composição microbiana. Esses resultados destacam uma clara necessidade de controle para o dia de emergência em experimentos de abelhas em laboratório. Esses resultados também demonstram a necessidade de incluir avaliações da microbiota intestinal das abelhas, além de outras métricas de saúde e sobrevivência das abelhas ao avaliar os efeitos potenciais dos agroquímicos nas abelhas.

Palavras-chave: Abelha, Microbiota Intestinal, Zinco, Emerge Day, Sobrevivência, agroquímicos

1. INTRODUCTION

Honey bees (*Apis mellifera*) are regularly exposed to a variety of chemicals in the environment, including pesticides, antibiotics, air pollutants, plastic additives, and metals (ROSZKO *et al.*, 2016; SOARES *et al.*, 2021; SOLAYMAN *et al.*, 2016). Exposure to substances like metals can occur via direct contact with airborne metals (COSTA *et al.*, 2019) or by consuming nectar and pollen contaminated with metals (LEITA *et al.*, 1996; QUINN *et al.*, 2011). Although honey bee hives have long served as biomonitors of metal pollution (BROMENSHENK *et al.*, 1985; LEITA *et al.*, 1996), the effects of metals on honey bee health is relatively understudied. This is a major area of concern because metals, like zinc, are commonly found in fertilizers and foliar sprays, and can bioaccumulate, leading to toxicity in honey bees (Mortvedt and Gilkes, 1993; Montalvo *et al*., 2016; Hesketh *et al*., 2016; Hladun *et al*., 2016). Honey bees are economically important as food-producing animals and as pollinators across the planet (GISDER; GENERSCH, 2017; HUNG *et al.*, 2018; KEVAN; VIANA, 2003; MICHENER, 2022).

In recent years, a limited number of laboratory studies have shown that metals can negatively impact honey bee health including lifespan (DI *et al.*, 2020, 2016; HESKETH *et al.*, 2016), cognitive ability (BURDEN *et al.*, 2016, 2019; HLADUN *et al.*, 2012; MONCHANIN *et al.*, 2021; SØVIK *et al.*, 2015; VINET; ZHEDANOV, 2010), and brood development (DI *et al.*, 2020, 2016; HLADUN *et al.*, 2013, 2016). Metals also alter honey bee expression of genes involved in stress response and metal detoxification (NIKOLIĆ *et al.*, 2019, 2016; PURAĆ *et al.*, 2019). Moreover, metal exposure results in more dead pupae within capped cells, lower worker weights, and increased metal accumulation in body tissue (BROMENSHENK *et al.*, 1991; HLADUN *et al.*, 2016). While certain metals (ex. cadmium and lead) have no known role within the body, others (ex. magnesium and zinc) are essential micronutrients that play key physiological processes in bees. However, excess exposure to essential metals, like zinc, can also result in toxic effects (MILIVOJEVIĆ *et al.*, 2015).

Zinc is frequently included in fertilizers (MONTALVO *et al.*, 2016; MORTVEDT; GILKES, 1993), and has been proposed as an active ingredient for plant antimicrobials (NARANJO *et al.*, 2020). Zinc oxide nanoparticles have received attention as biosafe options for agricultural applications (MOSTAFA; ALMOAMMAR; ABD-ELSALAM, 2019; RAJPUT

et al., 2018). Additionally, zinc oxide is incorporated as a growth promoter in animal feed (MOYNAHAN, 1979). Zinc is frequently utilized in swine feed as zinc oxide (ZnO), ranging from 125 to 3,000 mg/kg feed, exceeding the recommended dietary intake of 50 to 125 mg/kg feed (DĘBSKI, 2016; LÓPEZ-ALONSO, 2012). Excess zinc is then excreted in animal waste, which can lead to zinc soil contamination (NOLLET *et al.*, 2007). Zinc can also bioaccumulate from soil into plants (BALAFREJ *et al.*, 2020) and potentially into higher trophic levels, including florivores such as honey bees (BUTT *et al.*, 2018; XUN *et al.*, 2017). Humans also excrete zinc (3-19 mg of zinc per day in feces, and 0.6-1.6 mg in urine; Drinker *et al.*, 1927), which can make its way into agricultural fields via recycled wastewater (GUPTA *et al.*, 2019). Notably, zinc has been employed heavily as a supplement for the treatment and prevention of COVID-19, which could potentially translate into higher zinc levels in wastewater (GORDON; HARDIGAN, 2021; MICHOS; CAINZOS-ACHIRICA, 2021).

Honey bees have a wide foraging range (up to 10 km^2; Beekman and Ratnieks, 2000) and may be exposed to zinc in fertilizers, pesticides, soils, plants, agricultural runoff, or recycled wastewater during foraging. However, the effects of zinc on honey bees is largely unknown. Importantly, metal exposure may affect the gut microbiome of exposed hosts (SYROMYATNIKOV *et al.*, 2020), and microbiota play a critical role in bee health (Hamdi *et al.*, 2011; Engel *et al.*, 2016; Raymann and Moran, 2018; Dosch *et al.*, 2021). Certain microbes influence pesticide tolerance (WU, Yuqi *et al.*, 2020), and inhibit the growth of fungal pathogens (ENGEL *et al.*, 2016; MILLER; SMITH; NEWTON, 2021). The effects of pesticides on honey bee gut microbiota have been investigated in multiple studies (CUESTA-MATÉ *et al.*, 2021; DIAZ *et al.*, 2019; HOTCHKISS; POULAIN; FORREST, 2022; KAKUMANU *et al.*, 2016; MOTTA; RAYMANN; MORAN, 2018; NOGRADO *et al.*, 2019; PARIS *et al.*, 2020; RAYMANN *et al.*, 2018), but the effects of metals, which can have antimicrobial properties (LEMIRE; HARRISON; TURNER, 2013; LI, Yuan Ping *et al.*, 2021), are largely unexplored (but see Rothman *et al.*, 2019b).

Considering the current knowledge available, we hypothesize that both survival and gut microbial communities are negatively impacted by zinc exposure. Moreover, these are dose-dependent effects, with longer exposure periods and at higher concentrations of zinc leading to more pronounced deleterious effects. In the present study, we experimentally evaluated the effects of zinc exposure on the survival and gut microbial community of adult honey bees.

2. METHODS

2.1. EXPERIMENT DESIGN

The experiment was conducted at the Ohio State University Honey Bee Research Laboratory at the Ohio Agricultural Research and Development Center (OARDC) in Wooster, OH (USA) between July 2020-October 2020. All honey bee hives used in this study were managed according to standard beekeeping practices (HONEY BEE HEALTH COALITION, 2019). The experimental design is presented in **Figure 1**.

2.2. ZINC ORAL TOXICITY ASSAYS

Zinc oral toxicity assays were conducted in July and August of 2020. A 1,000 mg/L stock solution of zinc was prepared by dissolving 112.4 mg of anhydrous zinc acetate (Sigma-Aldrich, St. Louis, MO) into 40 mL of 50% (w/w) sucrose solution. This stock solution was serially diluted with a 50% sucrose solution to yield experimental solutions containing 50, 100, 250, 500, or 1,000 mg/L of zinc (or 40.6, 81.3, 203.3, 406.5, and 813 mg/kg). A control solution with no added zinc was also prepared (**Supplementary Table 1**).

Assays followed standard protocols for assessing chronic oral toxicity in adult honey bees (OECD, 2017). Brood frames were collected from three healthy, queenright hives and stored in a frame box in a laboratory incubator (60-80% RH, 34°C). Newly emerged adult bees were collected from the brood frames daily to ensure that all bees present on a given day were < 24 h old. For each trial, unanesthetized adult bees were brushed into a plastic bin and divided into groups of 17-23 bees (mean: 20.4, standard deviation: 1.2). All adult bees assigned to each treatment group emerged from their cells in the brood frame on the same day. Groups were then transferred into 177 cm^3 paper cups (Uniq Brand, Gilbert, AZ; **Figure 1**) and assigned to one of six treatment groups (0, 50, 100, 250, 500, or 1000 mg/L zinc). Bees were fed *ad libitum* from 1.5 mL microcentrifuge tubes that were modified to serve as feeders. Tubes were checked daily and refilled as needed. Surviving bees were counted every 24 hours (+/- 1.5 hours) for 10 days. Five replicates were performed for each treatment group. Replicates were omitted if their respective control groups (0 mg/L zinc) had $\leq$ 85% survival by the end of the 10-day trial (OECD, 2017). This resulted in each treatment group containing bees from 1-3 hives and 3-5 replicates (**Supplementary Table 1**).

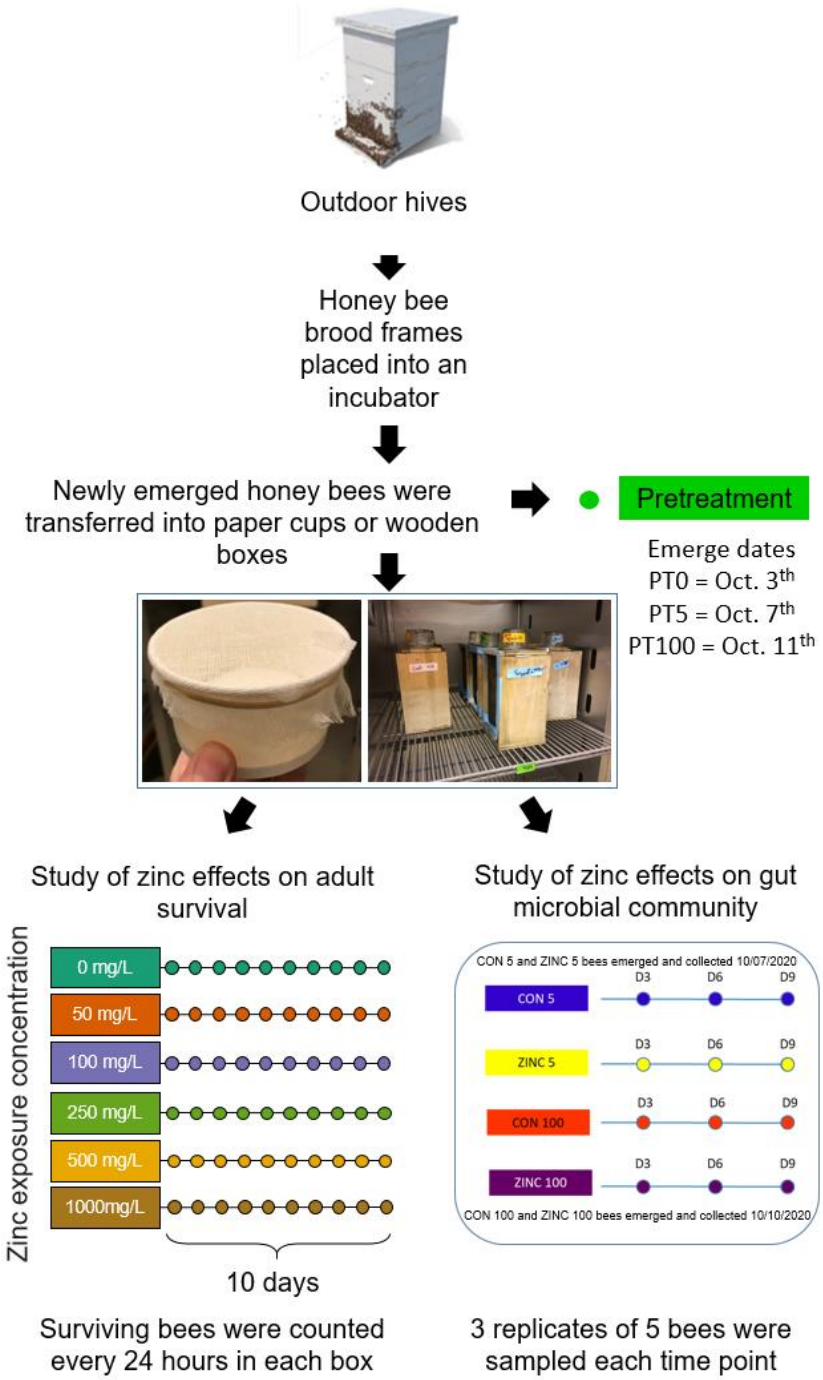


Figure *1*. Experimental design. Honey bee brood frames were placed into a lab incubator. Newly emerged honey bees were collected daily. To evaluate the effects of zinc on the gut microbial community (right), bees were divided into wooden/mesh boxes and fed ad libitum with a sucrose-zinc solution at 5 mg/L (ZINC 5) or 100 mg/L (ZINC 100). Each treatment group was paired with a respective control group (CON 5 or CON 100) containing bees that emerged on the same date as the treatment group but that received no zinc (0 mg/L). Fifteen bees were collected from each box on Day 0 (Pretreatment - PT), Day 3 (D3), Day 6 (D6), and Day 9 (D9) for microbial analysis.

Analysis of oral toxicity data

To select an appropriate dose-response model, the US Environmental Protection Agency's Benchmark Dose Software (DAVIS, J. Allen; GIFT; ZHAO, 2011; USEPA, 2020) was used. This software recommended the probit model, which had the lowest Akaike Information Criterion among 12 competing models. Subsequently, a probit model of survival at day 10 over each concentration was generated with the *glm* function in the R package 'stats' (R CORE TEAM, 2021). Additionally, models of survival over time were generated for each treatment group. These models were used to estimate the duration of exposure resulting in 50% mortality for each concentration ("lethal effect times," or LT50s) with the *LC_probit* function in the R package 'ecotox' (HLINA *et al.*, 2021).

To compare rates of survival between treatment groups, Kaplan-Meier survival estimates for each treatment group were calculated with the *survfit* function in the R package 'survival' (THERNEAU, 2021). Rates of survival between treatment groups were compared using the *pairwise_survdiff* function with a Bonferroni correction for multiple comparisons. This function performs multiple pairwise log-rank tests between the survival estimates of each treatment group.

Zinc effects on gut microbiota

Gut microbiota experiments were performed in October of 2020. Honey bee brood frames were taken from a single outdoor hive and placed into an incubator with controlled temperature and humidity (60-80% RH, 34°C). On each day of the experiment, newly emerged adult bees were collected from the brood frames, transferred into wooden/mesh boxes (~1650 cm^3) and provisioned with inverted glass feeding jars containing a 50% (w/w) sucrose solution with zinc at 5 mg/L (ZINC 5) or 100 mg/L (ZINC 100) (**Figure 1**). Boxes and jars were cleaned prior to this study by soaking into 30% bleach and rinsing thoroughly. The selected zinc concentrations (5 and 100 mg/L, or 4.07 and 81.3 mg/kg) were chosen to span the range of zinc concentrations previously reported in honey (SOLAYMAN *et al.*, 2016). Notably, higher zinc concentrations (325 and 592 mg/kg) have been measured in bee propolis and pollen provisions, respectively (LEITA *et al.*, 1996; MOROŃ *et al.*, 2012). Each zinc treatment group was paired with a respective control group (CON 5 or CON 100) containing bees that emerged on the same date as the treatment group but that received no zinc (0 mg/L). Fifteen bees were collected from each box on Day 0 (Pretreatment - PT), Day 3 (D3), Day 6 (D6), and Day 9 (D9) for gut

microbial analysis. Bees were briefly anaesthetized at -20°C before being individually transferred into sterile 1.5 mL microcentrifuge tubes containing 70% ethanol for preservation. Fifteen bees were sampled from each treatment and control group at each timepoint. Samples were then transported to the Ohio State University College of Veterinary Medicine in Columbus, Ohio for processing and gut microbial community analysis.

DNA extraction, library preparation, and sequencing

Using sterile technique, whole abdominal contents were dissected out of each honey bee. To achieve adequate DNA concentrations for sequencing, contents of five bees were pooled into a single tube for DNA extraction, which was performed using the Qiagen® PowerFecal® Pro DNA Isolation Kit, (Qiagen, Germany) following the manufacturer's protocol with one alteration: A FastPrep-24 5G beat beater (MP Biomedicals, USA) with a setting of 6 m/s for 40s was used in place of the vortex adapter. The final elution step was performed in 50 μl to maximize DNA concentration. After extraction, DNA was assessed for concentration, purity, and integrity using the a fluorometer (Qubit 4, Thermo Fisher, USA) and a microvolume spectrophotometer (NanoDrop™, Thermo Fisher, USA).

Sequencing and library preparation (16S rRNA) was performed by Novogene Technology Co. Ltd. (Beijing, China). 16S rRNA amplicon library was constructed from each sample using primers 341F: 5'-CCTAYGGGRBGCASCAG-3′ and 806R: 5'-GGACTACNNGGGTATCTAAT-3′ targeting the V3-V4 region of the 16S rRNA gene (JIA *et al.*, 2017). PCR reaction conditions were as follows: initial denaturation at 95°C for 3 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30s and a final extension at 72°C for 5 min. Barcodes were added to the primer sequences. The sequencing libraries were quantified by fluorometry (Qubit2.0, Life Invitrogen) and an Agilent Bioanalyzer 2100 system prior to sequencing. An Illumina HiSeq 2500 platform was employed to generate 2 x 250 bp paired-end reads. Negative controls including blanks from the extraction kit and samples from each of the feeds also underwent extraction and sequencing.

Bacterial DNA quantification

Bacterial DNA concentrations were quantified through qPCR: Bacterial DNA was amplified using 16S rRNA universal bacterial primers and probes according to Nadkarni *et al.* (2002) using a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA, USA). For the reaction, 300 nM of forward primer (5' – TCCTACGGGAGGCAGCAGT – 3'), 300 nM of reverse primer (5' – GGACTACCAGGGTATCTAATCCTGTT – 3'), and 175 nM of probe ((6FAM) – 5' – CGTATTACCGCGGCTGCTGGCAC – 3' – (TAMRA)) were added. Cycling parameters were as follows: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and an annealing and extension step of 60 °C for 1 min (NADKARNI *et al.*, 2002). qPCR was performed in triplicate and to be included in analyses, at least two replicates per sample had to amplify. Samples containing replicates with greater than 3% variation were removed from analysis. Following qPCR, cycle thresholds were $\log_{10}$-transformed using the following equation based on a standard curve generated using an *Escherichia coli* isolate (MROFCHAK *et al.*, 2021): $y = -5.329x + 36.504$ in which y is the cycle threshold and x is the $\log_{10}$-transformed DNA concentration. The antilog of each sample was then used to calculate the bacterial DNA concentration in each sample.

Sequence Data Processing and Statistical analysis

The raw demultiplexed paired-end sequences were processed using QIIME 2-2020.2 (BOLYEN *et al.*, 2019). Paired-end reads were filtered, denoised, and truncated to a length of 248 base pairs, and then parsed for non-chimeric sequences using DADA2, producing Amplicon Sequence Variants (ASV) (CALLAHAN *et al.*, 2016). Sequences were aligned using "qiime fragment-insertion sepp" for phylogenetic analysis (MATSEN *et al.*, 2012). Taxonomy was assigned in QIIME2 using SILVA version 132, with a 99% similarity threshold using the full length 16S rRNA gene classifier (QUAST *et al.*, 2013; YILMAZ *et al.*, 2014). Negative control samples were examined for potential contaminant taxa and reads were then subjected to in-silico decontamination using the Decontam R package version 1.12.0 (DAVIS, Nicole M. *et al.*, 2018).

Microbial diversity (alpha diversity) was assessed using observed features (richness), Shannon (richness and abundance), Faith's PD (phylogenetic diversity) and Pielou's (evenness) diversity indices. ANOVAs were used to compare diversity between groups using R version 4.1.0

(RIPLEY, 2001). After testing for normality using a Shapiro-Wilk test, the means were compared using Tukey or Kruskal-Wallis tests at 5% probability.

Microbial composition (beta diversity) was evaluated using Bray-Curtis, Jaccard, and weighted and unweighted UniFrac distances in QIIME 2-2020.2. Microbial community similarity and dissimilarity were visualized with principle coordinate analysis (PCoA) plots using the Emperor plugin 2020.2.0 (VÁZQUEZ-BAEZA *et al.*, 2017). PERMANOVAs were employed as recommended (ANDERSON, Marti J, 2001) to test for differences in microbial composition between experimental groups: (Pretreatment (PT), zinc at 5 mg/L (ZINC 5), zinc at 100 mg/L (ZINC 100), and their respective matched control groups that received 0 mg/L zinc (CON 5, CON 100). Microbial communities were also compared over time (Day 0 – Pretreatment (PT), and Days 3 (D3), 6 (D6), and 9 (D9)).

Taxa that were differentially abundant between the treatment groups were identified using an analysis of composition of microbiomes (ANCOM) (MANDAL *et al.*, 2015). We also used the "qiime feature-table core-features" command to identify core taxa present in 100% of the samples. The relative and absolute abundances of differentially abundant microbes were compared by treatment using one-way ANOVAs followed by Tukey or Kruskal-Wallis Rank Sum tests and pairwise comparisons using pairwise Wilcoxon Rank Sum Tests. Normality of data was assessed by the Shapiro-Wilk test. A *P*-value < 0.05 was used in the statistical tests for significance.

3. RESULTS

3.1. ZINC EFFECTS ON HONEY BEE SURVIVAL

Honey bee survival exhibited a clear dose-response relationship with zinc concentration (log-rank test, $p < 0.0001$, $\chi^2 = 1073$, df = 5, **Figure 2, Supplementary Figure 1**). At 0, 50, and 100 mg/L zinc, mean rates of survival were 99%, 91%, and 89%, respectively. Survival decreased steeply at 250 mg/L zinc exposure and was 0% at 1000 mg/L (**Supplementary Table 2**). Accordingly, LT50s (lethal effect times to reach 50% mortality) decreased with increasing zinc concentrations (**Supplementary Table 3**). Pairwise comparisons between treatment groups revealed statistically significant differences in overall survival between all groups ($p << 0.05$) except the 50 and 100 mg/L groups ($p \approx 1$) (**Supplementary Table 4**). Similarly, the 95% confidence intervals of probit LT50 estimates only overlapped for the 50 and 100 mg/L groups (**Supplementary Table 3**). Based on these survivorship results, we chose to test the effects of two field-relevant concentrations of zinc (5 mg/L and 100 mg/L) on the honey bee gut

microbiota. While zinc concentrations up to 100 mg/L had relatively small effects on survival, gut microbial community shifts could impact honey bee health in other ways.

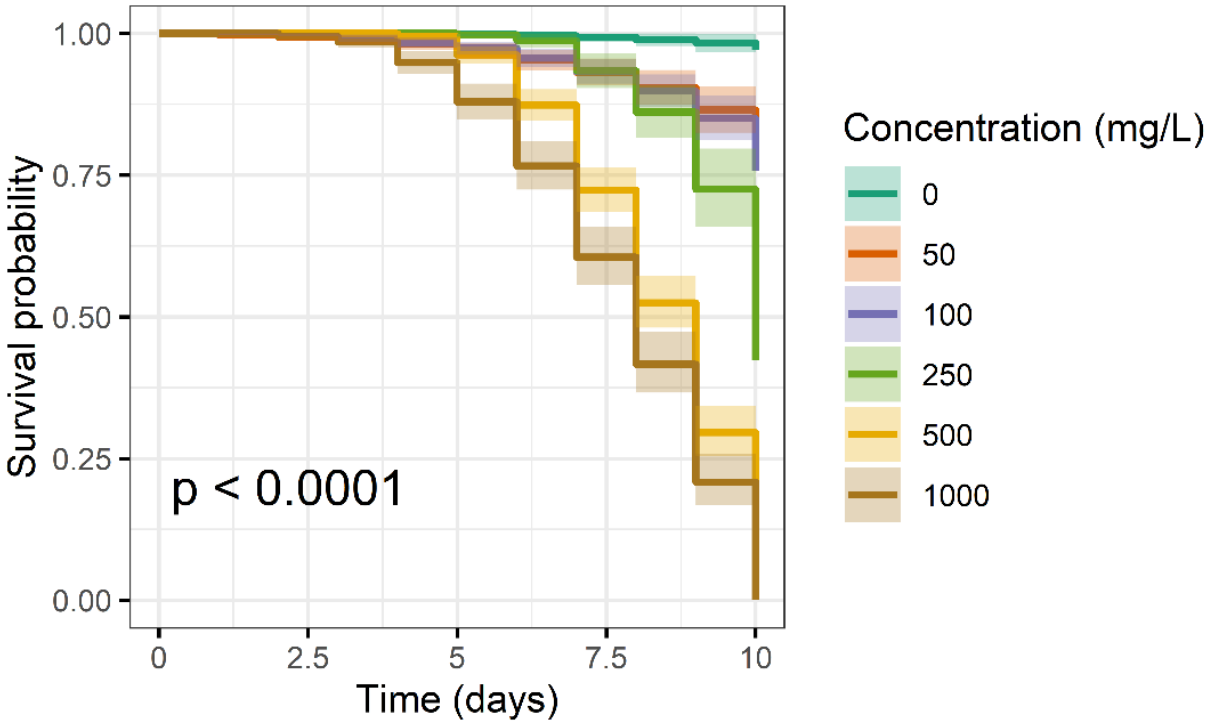


Figure *2*. Kaplan-Meier curves of honey bee survival at varying zinc concentrations over 10 days. Zinc concentrations included 0 (negative control), 50, 100, 250, 500, and 1000 mg/L. Increased mortality was observed with increasing zinc concentrations. Lines and shaded regions represent median survival predictions and 95% confidence regions, respectively. Across all groups, zinc concentration had a statistically significant effect on survival (log-rank test, $p < 0.0001$, $\chi 2 = 1073$, $df = 5$). When focusing on just the field-relevant concentrations (0, 50, and 100 mg/L), concentration still had a statistically significant effect on survival (log-rank test, $p < 0.0001$, $\chi 2 = 45.9$, $df = 2$).

3.2. ZINC EFFECTS ON HONEY BEE BACTERIAL DNA CONCENTRATION AND ABSOLUTE CELL COUNTS

To assess the effects of zinc on overall gut bacterial load, we quantified total bacterial DNA concentrations and absolute bacterial cell counts via qPCR. Five samples exhibited greater than 3% variation in cycle threshold values between all replicates and were removed from analyses. This included two ZINC 5 samples from day 6, one CON 5 sample from day 6 and day 9, and one ZINC 100 sample from the day 3. In cases where only one of the three replicates exceeded 3% variation in cycle threshold value, the replicate with > 3% variation was excluded from analysis, and the remaining two replicates were retained and averaged. Cycle threshold values were then converted to bacterial DNA concentrations based on an equation

generated from an *Escherichia coli* standard curve in our laboratory (MROFCHAK *et al.*, 2021).

Quantifiable bacterial DNA concentrations ranged from 0.0004 ng/ul to 5.87 ng/ul (**Supplementary Table 5**). Concentrations and absolute cell counts differed significantly by treatment (PT, ZINC 5, ZINC 100, CON 5, CON 100) (Bacterial concentration: Kruskal Wallis = 14.7, df = 4, p = 0.005; absolute cell count: Kruskal Wallis = 14.7, df = 4, p = 0.005), but no pairwise comparisons were significant (Wilcoxon rank sum exact test, all p > 0.05; **Figure 3**). Interestingly, both CON 5 and ZINC 5 groups had higher concentrations compared to CON 100 and ZINC 100 groups. CON 5 and ZINC 5 bees all emerged on the same date (October 7, 2021), which was 4 days before all CON 100 and ZINC 100 bees, which emerged on October 11, 2021. This led us to wonder if gut bacterial DNA concentrations decreased over time under laboratory conditions, and if bees with later emerge days had lower overall microbial loads. To test this, we examined bees in the PT group.

When emerge dates of PT bees were considered (PT 0 = bees that emerged on Oct. 3; PT 5 = bees that emerged on Oct. 7th, the same date as CON 5 and ZINC 5 bees; PT 100 = bees that emerged on Oct. 11th, the same date as CON 100 and ZINC 100 bees), we observed a clear decline in DNA concentrations and absolute cell counts over time by emerge date; although, these differences were still not significant (Bacterial DNA concentration: Kruskal-Wallis chi-squared = 4.5714, df = 2, p-value = 0.1017; absolute cell count: Kruskal-Wallis chi-squared = 4.57, df = 2, p-value = 0.102; **Figure 3c,d**). We then averaged data from all bees that emerged on Oct. 7th (PT5, CON 5, ZINC 5), and all bees that emerged on Oct. 11th (PT100, CON 100, ZINC 100) and, in that case, we observed a significant difference in bacterial DNA concentrations and absolute cell counts between emerge day (Bacterial DNA concentration: Kruskal-Wallis chi-squared = 6.15, df = 1, p-value = 0.013; Absolute cell count: Kruskal-Wallis chi-squared = 6.15, df = 1, p-value = 0.013; **Figure 3e,f**).

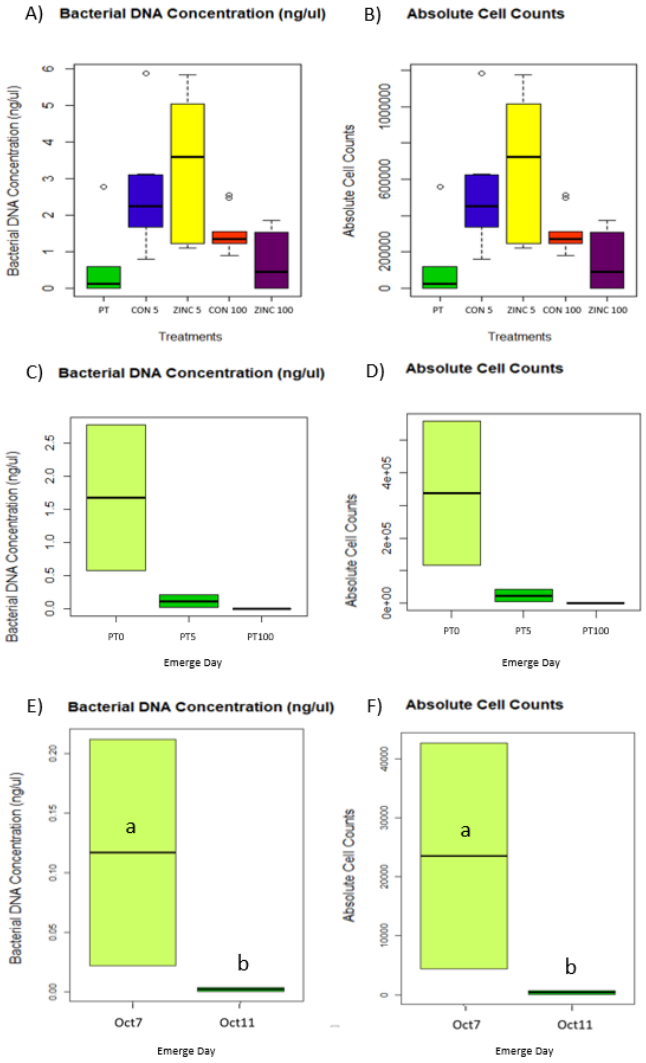


Figure *3*. Bacterial DNA concentrations and absolute cell counts. A) Bacterial DNA concentrations and B) absolute cell counts differed significantly across treatment groups (Kruskal-Wallis, p = 0.005). However, no pairwise comparisons were significant (Wilcoxon Rank Sums, p > 0.05). C) Pretreatment (PT) bacterial DNA concentrations and D) absolute cell counts showed a non-significant decrease over time by emerge day (Bacterial DNA concentration: Kruskal-Wallis chi-squared = 4.57, df = 2, p-value = 0.102; absolute cell count: Kruskal-Wallis chi-squared = 4.57, df = 2, p-value = 0.102). When we combined all bees that emerged on the same day across all treatments and times, we found that E) bacterial DNA concentrations and F) absolute cell counts were significantly greater in bees that emerged on October 7th as compared to October 11th (Bacterial DNA concentrations Kruskal-Wallis chi-squared = 6.15, df = 1, p-value = 0.013; absolute cell counts Kruskal-Wallis chi-squared = 6.1501, df = 1, p-value = 0.013); Box plot shows outliers, first and third quartiles (lower and upper edges), and highest, lowest and median values (horizontal black dash). PT = All Pretreatment (Day 0) bees including those that emerged on Oct. 3, 7, and 11; PT0 = Pretreatment bees that emerged on Oct. 3; PT5 = Pretreatment bees that emerged on Oct 7th – the same day as CON 5 and ZINC 5 bees; PT100 = Pretreatment bees that emerged on Oct. 11th. – the same day as CON 100 and ZINC 100 bees); CON 5 = Bees that emerged on the same day as ZINC 5 bees but treated with no zinc; ZINC 5 = Bees treated with 5 mg/L zinc; CON 100 = Bees that emerged on the same day as ZINC 100 bees but treated with no zinc; ZINC 100 = Bees treated with 100 mg/L zinc.

3.3. 16S rRNA SEQUENCING

We obtained a total of 4,854,562 raw reads across all samples. Samples averaged 112,896 reads per sample and ranged from 3,848 to 107,692 reads. After the denoising process, 4,165,574 (85.86%) reads were retained for downstream analyses. Five putative contaminant taxa (*Romboutsia* sp. CE17, *Methylobacterium* sp., *Cutibacterium acnes* strain 3265, *Methylorubrum extorquens* strain B44 and Uncultured bacterium clone A1435) were identified and removed using the decontam R package (RIPLEY, 2001) (**Supplementary Table 6**). Reads identified as chloroplasts, mitochondria, unassigned, and eukaryota were also removed from all samples. In total, reads were classified into 1,746 amplicon sequence variants (ASVs) which aligned to 413 different taxa. Samples with fewer than 3,000 reads were removed. This resulted in removal of a single sample which was a negative control.

3.4. ZINC EFFECTS ON HONEY BEE GUT MICROBIAL COMPOSITION AND DIVERSITY

Bee gut microbial composition was significantly different across treatment groups (PT, ZINC 5, ZINC 100, CON 5, CON 100) but not by time (D0/PT, D3, D6, D9) (PERMANOVA: Treatment - Bray Curtis R^2= 0.280, p = 0.001; Jaccard Index: R^2= 0.198, p = 0.001; Time - Bray Curtis R^2= 0.026, p = 0.202; Jaccard Index: R^2= 0.020, p = 0.202; **Figure 4, Supplementary Figure 2, Supplementary Tables 7-10**). The Bray Curtis distance metrics revealed that CON 100 and ZINC 100 groups did not differ significantly from each other in terms of microbial composition, and neither did CON 5 or ZINC 5 groups. However, both CON 100 and ZINC 100 groups differed significantly from CON 5 and ZINC 5 groups (**Supplementary Table 9**). These results again suggested an “emerge day” effect and indicated that emerge day and not zinc was driving the main differences observed between groups. We then analyzed microbial composition by emerge day (Oct. 7 vs. Oct. 11) and found significant differences between groups across all indices (PERMANOVA: Bray Curtis R^2= 0.17, p = 0.001; Jaccard R^2= 0.08, p = 0.001; Unweighted Unifrac R^2= 0.08, p = 0.00; Weighted Unifrac R^2= 0.17, p = 0.001).

To compare microbial diversity across groups, we used Shannon and Pielou’s Indices after first testing data for normality using the Shapiro Wilk Test. Microbial diversity did not differ significantly by treatment groups or by time (Two way ANOVA: Treatment - Shannon Index p = 0.183; Pielou’s Index p = 0.226; Time (D0, D3, D6, D9) - Shannon Index p = 0.105;

Pielou's Index p = 0.08; **Figure 5a,b**; **Supplementary Figure 3a,b)**. To test for emerge day effects, we also compared microbial diversity of PT bees by emerge date and found no significant differences (Emerge Day - Shannon Index p = 0.671; Pielou's Index p = 0.952 **Figure 5c,d; Supplementary Figure 3c,d**). Further, we compared microbial diversity of all bees that emerged on Oct. 7 (PT5, CON 5, ZINC 5) to all bees that emerged on Oct. 11 (PT100, CON 100, ZINC 100) and again found no significant differences (ANOVA: Treatment - Shannon Index p = 0.216; Pielou's Index p = 0.376; **Figure 5e,f**).

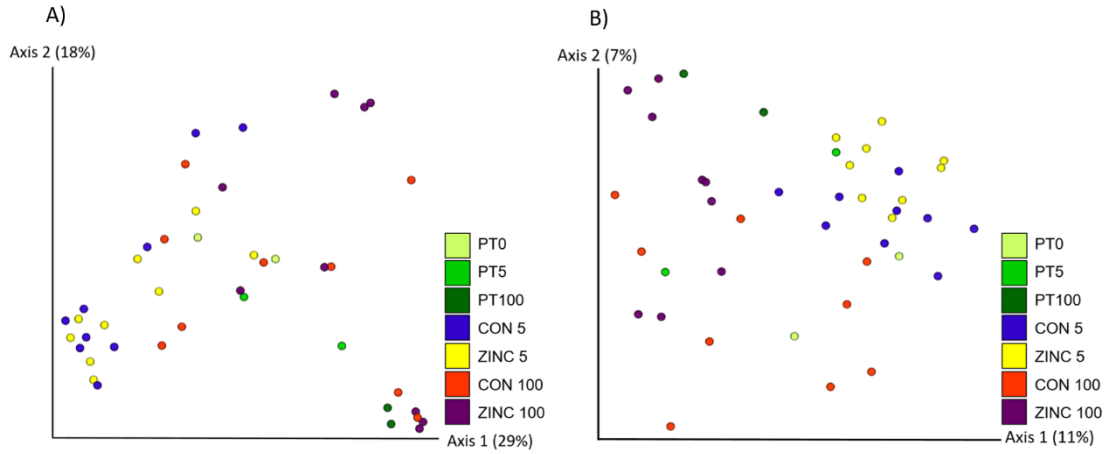


Figure *4*. Bee gut microbial composition by treatment. Bee gut microbial composition differed significantly by treatment based on A) Bray Curtis (PERMANOVA: p = 0.001) and B) Jaccard distance matrices (PERMANOVA: p = 0.001). These differences were largely driven by emerge day as opposed to zinc treatment (also see Supplementary Tables 9,10). PT0 = Pretreatment bees that emerged on Oct. 3; PT5 = Pretreatment bees that emerged on Oct 7th – the same day as CON 5 and ZINC 5 bees; PT100 = Pretreatment bees that emerged on Oct. 11th. – the same day as CON 100 and ZINC 100 bees); CON 5 = Bees that emerged on the same day as ZINC 5 bees but treated with no zinc; ZINC 5 = Bees treated with 5 mg/L zinc; CON 100 = Bees that emerged on the same day as ZINC 100 bees but treated with no zinc; ZINC 100 = Bees treated with 100 mg/L zinc.

3.5. CORE MICROBIOTA AND DIFFERENTIALLY ABUNDANT TAXA

A core microbiota analysis was performed to identify core taxa defined here as taxa present in 100% of the samples across all treatments and times. Four core taxa were identified including: *Lactobacillus, Bifidobacterium, Commensalibacter* and a taxon in the Rhizobiaceae family (*Bartonella* sp.) (**Supplementary Material Table 11**). These taxa accounted for 8% of all taxa in the dataset. *Gilliamella* and *Snodgrassella* were also detected in the majority of samples and are considered core microbiota in honey bees (KEŠNEROVÁ *et al.*, 2020; KWONG; MORAN, 2016) (**Supplementary Figure 4**).

Six taxa (at the genera level) were identified as differentially abundant between treatment groups by ANCOM: *Lactobacillus, Gilliamella, Paenibacillus,* and three taxa from the families Rhizobiaceae (*Bartonella* sp.), Enterobacteriaceae and Microbacteriaceae (**Figure 6**; **Supplementary Figure 5**; **Supplementary Table 12**). Significantly decreased abundances of Enterobacteriaceae and increased abundances in *Paenibacillus* and Microbacteriaceae were observed in the ZINC 100 group compared to the CON 100 group (Kruskal-Wallis test on absolute abundances: Enterobacteriaceae p = 0.0003; Microbacteriaceae p = 0.0001; **Figure 6, c,e,f**; Relative abundances: Enterobacteriaceae p = 0.00007; *Paenibacillus* p = 0.0005; Microbacteriaceae p = 0.0007; **Supplementary Figure 5, c,e,f**). For *Lactobacillus,* the Rhizobiaceae taxa (*Bartonella* sp.), and *Gilliamella*, there were slight but non-significant decreases in relative and absolute abundances of these taxa in the zinc treated groups (ZINC 5 and ZINC 100). Notably, all CON 5 and ZINC 5 bees (emerge date: Oct. 7) contained greater relative abundances of *Lactobacillus,* the Rhizobiaceae taxa (*Bartonella* sp.), and *Gilliamella* as compared to all CON 100 and ZINC 100 bees (emerge date Oct 11), suggesting unique emerge day impacts on some microbial taxa distinct from the effects of zinc.

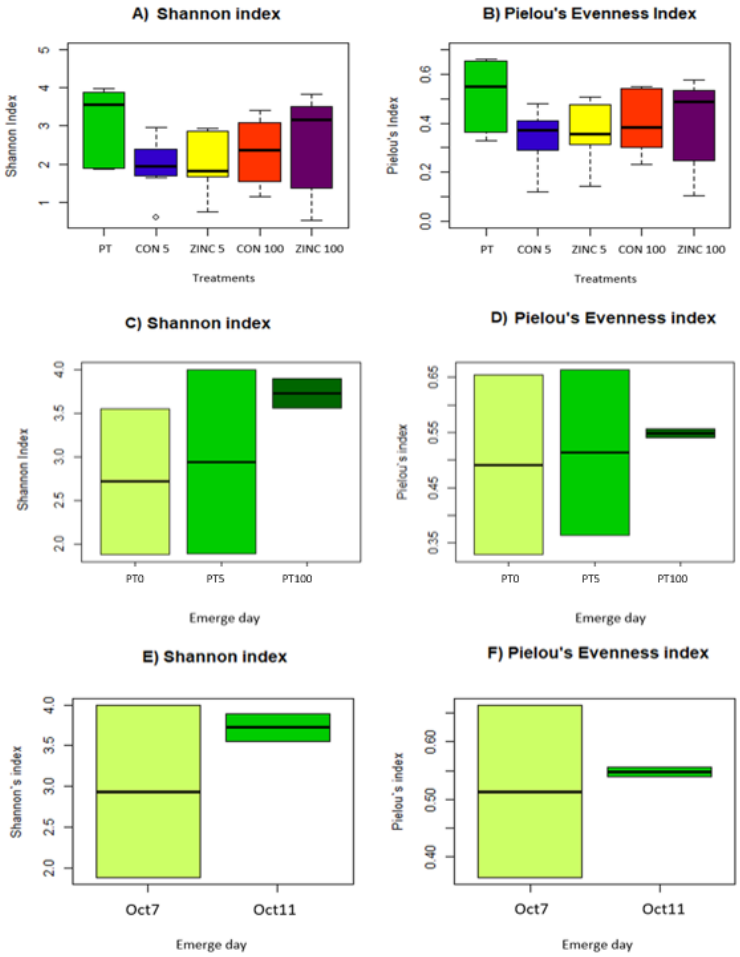


Figure 5. Microbial diversity by treatment. Gut microbial diversity and evenness did not differ significantly by treatment (**A,B**) or by emerge day (**C,D**) as measured by the Shannon (**A,C**) or Pielou's evenness index (**B,D**) (ANOVA: Treatment - Shannon Index p = 0.216; Pielou's Index p = 0.211; Emerge Day - Shannon Index p = 0.671; Pielou's Index p = 0.952. When we combined all bees that emerged on the same day across all treatments and times, we still found no significant difference in microbial diversity by emerge day (**E,F**) (ANOVA: Emerge day - Shannon Index p = 0.216; Pielou's Index p = 0.376). Box plots show outliers, first and third quartiles (lower and upper edges), and highest, lowest and median values (horizontal black dash). PT = All Pretreatment (Day 0) bees including those that emerged on Oct. 3, 7, and 11; PT0 = Pretreatment bees that emerged on Oct. 3; PT 5 = Pretreatment bees that emerged on Oct 7th – the same day as CON 5 and ZINC 5 bees; PT 100 = Pretreatment bees that emerged on Oct. 11$^{th.}$ – the same day as CON 100 and ZINC 100 bees); CON 5 = Bees that emerged on the same day as ZINC 5 bees but treated with no zinc; ZINC 5 = Bees treated with 5 mg/L zinc; CON 100 = Bees that emerged on the same day as ZINC 100 bees but treated with no zinc; ZINC 100 = Bees treated with 100 mg/L zinc.

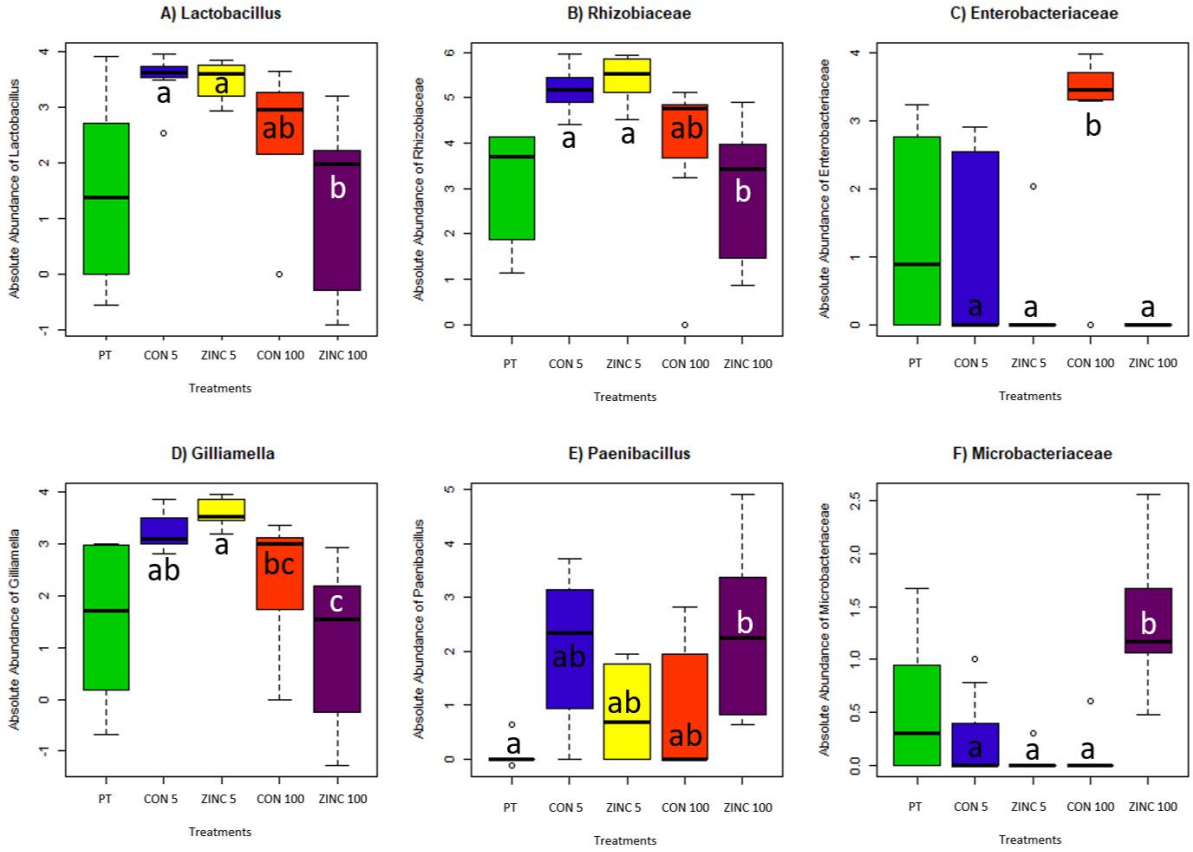


Figure 6. Differentially abundant microbiota by treatment. Absolute abundances of bacteria that were differentially abundant (ANCOM) at the L7 (roughly species) level between groups. Each species is listed at the lowest taxonomic level that could be identified based on reference sequences: A) Lactobacillus, B) Bifidobacterium, C) Rhizobiaceae, D) Gilliamella, E) Tyzzerella, F) Streptomyces, G) Paenibacillus, H) Enterobacteriaceae, I) Proteobacteria. Box plot shows outliers, first and third quartiles (lower and upper edges), and highest, lowest and median values (horizontal black dash). Groups that share the same letter do not differ significantly (Wilcoxon rank sum test with continuity correction). PT = All Pretreatment (Day 0) bees including those that emerged on Oct. 3, 7, and 11; CON 5 = Bees that emerged on the same day as ZINC 5 bees but treated with no zinc; ZINC 5 = Bees treated with 5 mg/L zinc; CON 100 = Bees that emerged on the same day as ZINC 100 bees but treated with no zinc; ZINC 100 = Bees treated with 100 mg/L zinc.

4. DISCUSSION

Our results demonstrate that low level zinc exposure had limited impacts on honey bee survival and overall gut microbial composition and diversity over time. However, we observed significant shifts in a few core and non-core bacterial abundances by treatment and emerge day. These zinc-induced gut microbial changes have potentially negative implications for honey bee nutrient metabolism and pathogen resistance. Importantly, the gut microbial community effects observed by emerge day indicate that emerge day must be accounted for as a variable in laboratory-based experiments involving honey bees.

4.1. ZINC AND HONEY BEE SURVIVAL

Each concentration of zinc tested in the oral toxicity assays produced a significant, negative effect on honey bee survival. Notably, this included zinc at concentrations of 50 and 100 mg/L, which are within the range of zinc concentrations reported from honey (Solayman *et al*., 2016). Zhang *et al*. (2015) reported similarly decreased survival of adult worker bees when fed zinc at concentrations of 45 mg/kg. Interestingly, they also observed increased longevity when honey bees were fed zinc at lower concentrations (30 mg/kg), which also resulted in increased antioxidant activity of the enzyme Cu-Zn superoxide dismutase (Cu-Zn SOD). Taken together, this suggests that honey bees could benefit from added zinc when zinc concentrations are low (i.e. ≤ 30 mg/kg), but zinc can have detrimental effects on adult honey bees at higher concentrations. Notably, while organic zinc acetate was used in this study, different formulations of zinc (e.g. zinc-methionine, zinc sulfate, zinc oxide, zinc nanoparticles) can yield different and highly variable results (De Barros *et al*. 2021). For example, a previous study reported a 20% reduction in survival and a 30% reduction in brain weight of honey bees exposed to just 0.8 mg/mL zinc oxide for ten days, indicating that zinc oxide may be significantly more toxic than zinc acetate (Milivojević *et al*. 2015).

4.2. ZINC, EMERGE DATE, AND HONEY BEE GUT MICROBIOTA

Zinc treatment (5 mg/L or 100 mg/L) had minimal effects on honey bee gut bacterial DNA concentrations and absolute cell counts while emerge date had a more pronounced, although still non-significant, effect with decreased bacterial loads and cell counts at later emerge dates. One previous study demonstrated that bees with reduced bacterial loads exhibited alterations in weight gain, sugar sensitivity, and metabolite profiles (ZHENG *et al.*, 2017). This suggests that altered bacterial loads, potentially due to laboratory emerge day, could also have critical effects on honey bee health distinct from any experimental treatments being tested in the lab.

Zinc had minimal effects on overall gut microbial composition and diversity (**Figure 4, 5**). Although non-significant, a slightly larger shift in microbial composition was observed in the higher zinc exposure group (ZINC 100) compared to its control (CON 100) while essentially no shift was observed in the ZINC 5 group compared to its control (CON 5). Emerge day, on the other hand, had significant effects on microbial composition (**Supplementary Tables 7, 8, 9 and10**).

Among the differentially abundant taxa identified between treatment groups, Enterobacteriaceae was found at high abundances in the CON 100 group as compared to ZINC 100 group and compared to the CON 5, ZINC 5, and PT groups. Some taxa within the *Enterobacteriaceae* family can be considered pathogens in honey bees (SABREE; HANSEN; MORAN, 2012). Additionally *Enterobacteriaceae* have been linked with dysbiosis and increased mortality in honey bees, and they have been negatively correlated with Lactobacillaceae, which are considered beneficial bacteria that stimulate the honey bee innate immune system (BLEAU *et al.*, 2020; BUDGE *et al.*, 2016; KWONG; MORAN, 2016). Zinc oxide also reduced digesta Enterobacteriaceae numbers and improved gut integrity in weaned piglets (WANG, Wei *et al.*, 2019). One possibility to explain the high abundances of Enterobacteriaceae in the CON 100 but not ZINC 100 group is that Enterobacteriaceae expansion is supported at later emerge days but zinc exposure limited Enterobacteriaceae growth in the ZINC 100 group.

Although the ZINC 100 group did not show increased abundances of Enterobacteriacea, we did observe significant shifts in other microbial abundances including an unidentified *Paenibacillus* species that was increased in the ZINC 100 group as compared the CON 100 group. Some members of the *Paenibacillus* genus, such as *P. larvae*, the causative agent of American foulbrood (AFB), are considered honey bee pathogens (GRADY *et al.*, 2016). *P. larvae* spores are commonly found in hives, and are vectored by adult honey bees that are resistant to infection but can transmit the spores to new brood (RIESSBERGER-GALLÉ; VON DER OHE; CRAILSHEIM, 2001). Exposure to high concentrations of zinc may allow *Paenibacillus* species like *P. larvae* to proliferate in the honey bee gut, which could pose increased risks for infection and transmission of diseases like AFB.

A Microbacteriaceae species was also found at significantly increased abundances in the ZINC 100 group as compared to the CON 100 group and the PT, CON 5, and ZINC 5 groups. Microbacteriaceae belong to the Actinobacteria family which is generally found at low relative abundances in the bee gut (KAČÁNIOVÁ *et al.*, 2020; KELLER; GRIMMER; STEFFAN-DEWENTER, 2013; PRADO *et al.*, 2023; ROTHMAN; ANDRIKOPOULOS; *et al.*, 2019). Interestingly, one Microbacteriaceae species, isolated from a willow tree growing in soil heavily contaminated with zinc and cadmium, demonstrated heavy metal resistance (CORRETTO *et al.*, 2017). This suggests that bacteria within this family may be uniquely suited to survive or even grow at high zinc concentrations. Future efforts involving isolation and genome sequencing of the honey bee Microbacteriacea are required to confirm this.

Decreased abundances of *Lactobacillus*, Rhizobiaceae (*Bartonella* sp.), and *Gilliamella* were observed both in ZINC 100 bees as compared to CON 100 bees and in the Oct. 11 emerge day bees as compared to the Oct. 7 emerge day bees. These differences were not significant but hint at potential effects of zinc and emerge day on these taxa, which play important roles in bee and hive health. *Lactobacillus* bacteria convert pollen into nutrients more accessible to the bee host (KEŠNEROVÁ *et al.*, 2017). Rhizobiaceae (*Bartonella* sp.) taxa are consistently found at greater abundances in healthy bees relative to bees from collapsing colonies; although, their function has yet to be fully defined (CORNMAN *et al.*, 2012). Finally, *Gillamella* species produce a biofilm on the gut wall that aids in pathogen defense (Engel *et al*., 2012).

In this study, zinc had limited effects on overall gut microbial composition, diversity, and taxonomic abundances, with the greatest differences noted particularly in the groups exposed to the higher concentration of zinc (100 mg/L). Effects linked to zinc included decreased abundances of several taxa including Enterobacteriacea (significant), and *Lactobacillus*, Rhizobiaceae (*Bartonella* sp.), and *Gilliamella* (all non-significant). Zinc has antibacterial properties that may have driven some of these decreases (ALMOUDI *et al.*, 2018). However, significant increases in other taxa, including *Paenibacillus* and Microbacteriacea, were also observed in the ZINC 100 group. The *Paenibacillus* taxa could represent a potential pathogen bloom resulting from altered microbiota, while the Microbacteriacea enrichment could be associated with zinc resistance in this taxon. Bees exposed to 100 mg/L zinc also demonstrated increased mortality, indicating that overall, zinc exposure at this field-relevant concentration negatively impacted bee health. These negative effects could be the result of zinc toxicity directly on host (bee) cells, or they may be mediated through changes in gut microbiota that alter other aspects of health such as immune dynamics and pathogen resistance (BLEAU *et al.*, 2020; BUDGE *et al.*, 2016; KWONG; MANCENIDO; MORAN, 2017; MILLER; SMITH; NEWTON, 2021; POWELL *et al.*, 2016). It is known that zinc oxide nanoparticles are toxic for *Culex pipiens* larvae, retarding the growth and development (IBRAHIM; THABET; ALI, 2023). Importantly, zinc, in various formulations (KOŁODZIEJCZAK-RADZIMSKA; JESIONOWSKI, 2014; NARANJO *et al.*, 2020), continues to be incorporated into bactericides designed for application on plants. These products could have serious negative impacts on honey bees, and continued evaluation of safe dosing ranges and formulations should include honey bee gut microbial analyses.

Independent of zinc, emerge day clearly affects gut microbial composition, microbial load, and, to a lesser degree, in taxonomic abundances. Honey bees that emerged on different

days were likely exposed to differing pre- and probiotic elements on the surface of the brood frame due to aging of the wax, changes over time in colony residues (bee bread, feces, etc.), lack of new bacteria typically introduced by other bees moving within the hive, or the removal of residues by previously-emerged bees. These fidings are supported by Anderson *et al*. (2022) which notes that prior to eclosion, bees have minimal gut microbiota, and they are primarily colonized after emergence from the brood comb and through exposure to hive materials. This was an unexpected finding, and it highlights a clear need to control for emerge day in laboratory experiments like this one. Additional studies are needed to determine the type and source of emerge day effects, as well as the potential health impacts of these effects. Determining if emerge day also impacts microbiota under natural (non-laboratory) conditions will also be important.

Continuing studies on agrochemical exposure that include evaluations of the gut microbiota as part of the studies on honey bee health, fitness, behavior, immune response, and disease susceptibility are needed as honey bee colonies continue to collapse at unprecedent rates (SÁNCHEZ-BAYO; WYCKHUYS, 2019). Understanding how chemicals like zinc affect bees is essential to guide agricultural practices that effectively support ecosystem health.

Data Availability Statement

16S rRNA sequencing data are publicly available at NCBI PRJNA916867. Raw survivorship data is publicly available via FigShare: 10.6084/m9.figshare.21692435

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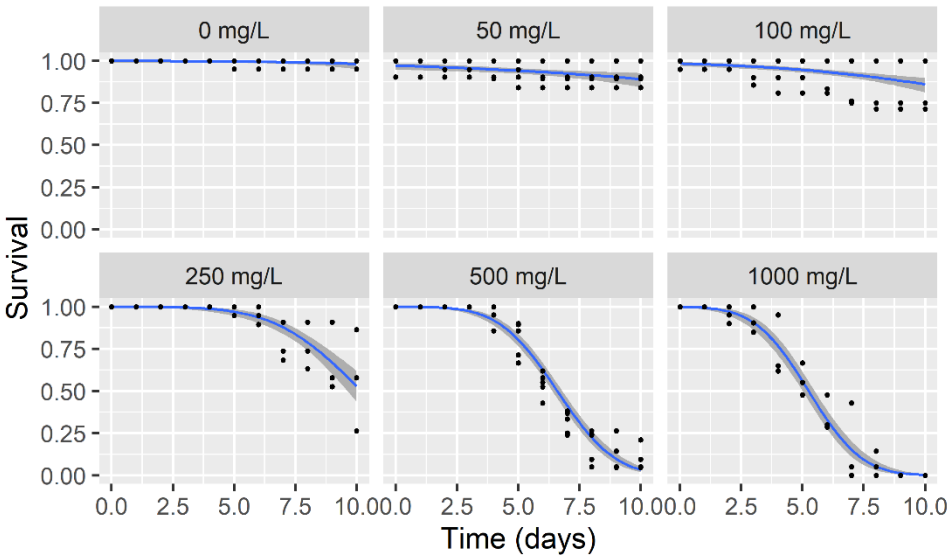
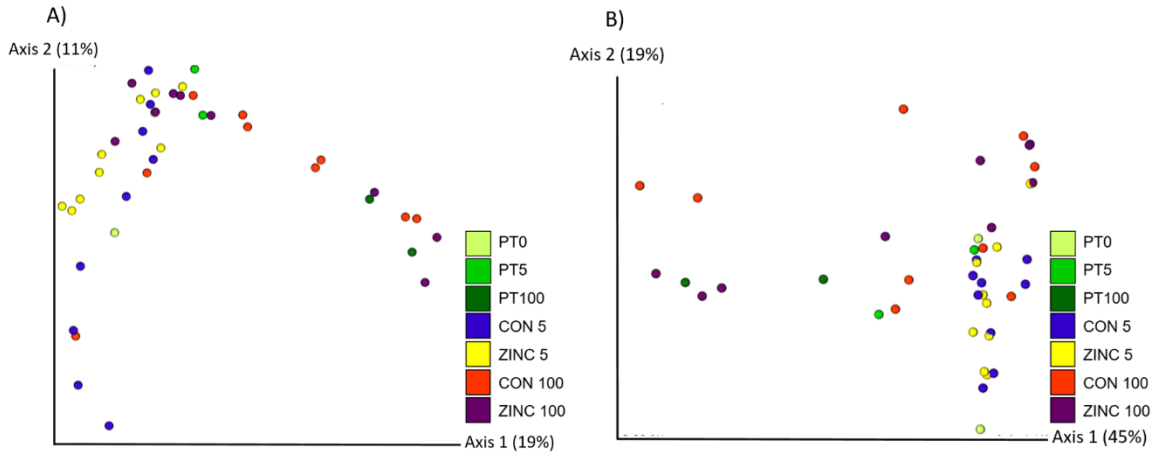
SUPPLEMENTARY MATERIAL

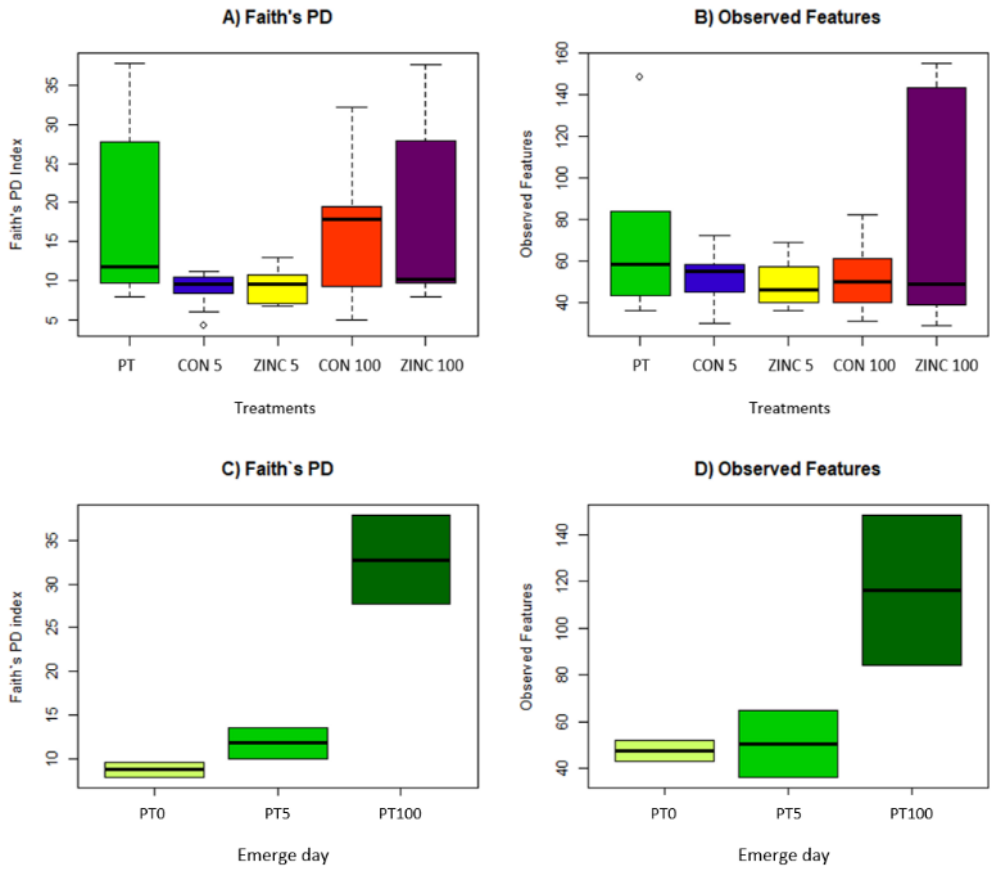
Supplementary Figures



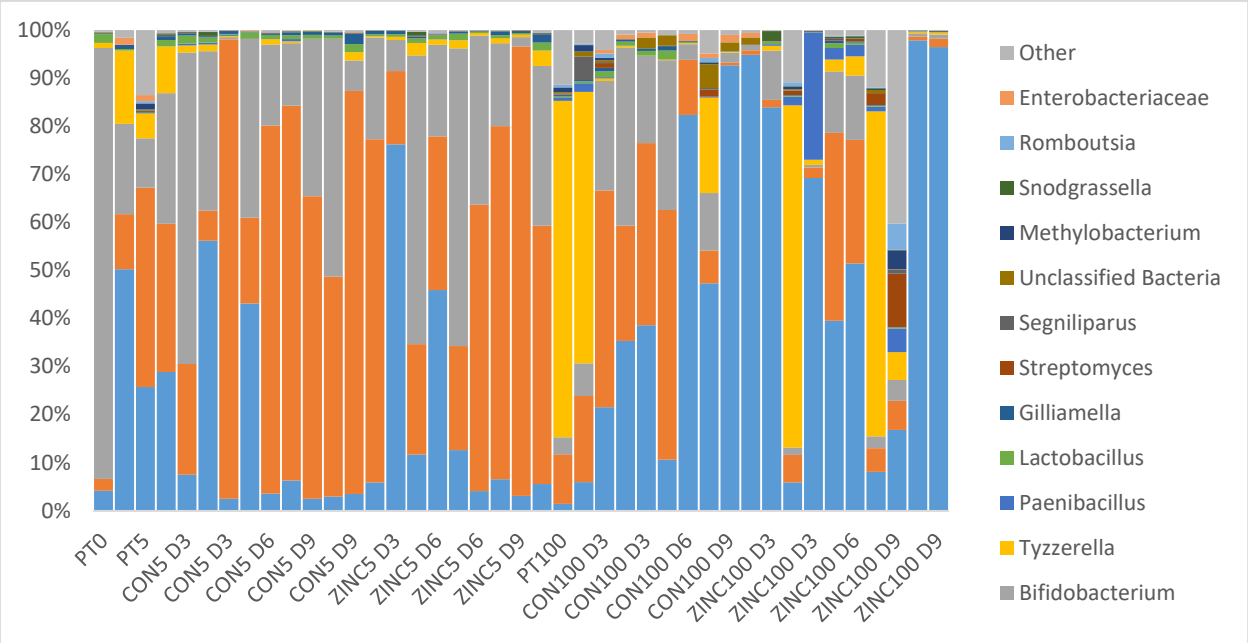
Supplementary material. Figure. 1 Survival rates of adult honey bees fed zinc at varying concentrations over ten days. Zinc concentrations included 0 (negative control), 50, 100, 250, 500, and 1000 mg/L. Points represent rates of honey bee survival. Lines and shaded regions represent the median survival predictions and 95% confidence regions, respectively, of probit models fitted to each dataset. Zinc exposure had a statistically significant effect on survival (likelihood ratio test, $p < 2e\text{-}16$, $df = 22$). When focusing on just the field-relevant concentrations (0, 50, and 100 mg/L), exposure still had a statistically significant effect on survival (likelihood ratio test, $p = 0.007$, $df = 11$).



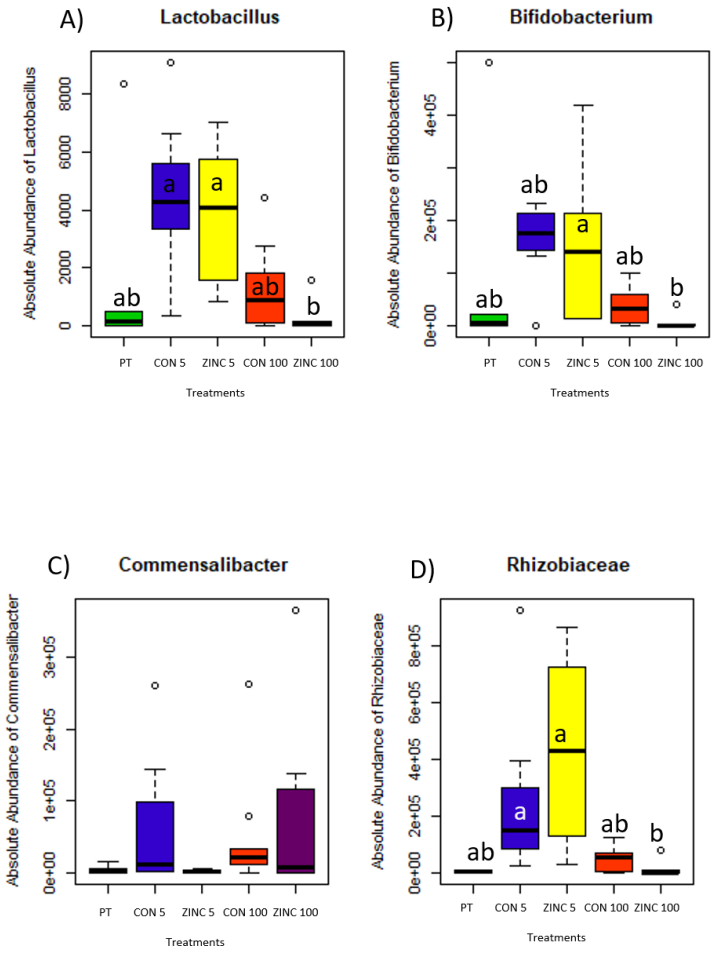
Supplementary material. Figure. 2 Gut microbial composition by treatment. Bee gut microbial composition differed significantly by treatment based on A) unweighted UniFrac (PERMANOVA: p = 0.001) and B) weighted UniFrac distance matrices (PERMANOVA: p = 0.006). These differences were largely driven by emerge day as opposed to zinc treatment (also see Supplementary Tables 9,10). PT0 = Pretreatment bees that emerged on Oct. 3; PT5 = Pretreatment bees that emerged on Oct 7th – the same day as CON 5 and ZINC 5 bees; PT100 = Pretreatment bees that emerged on Oct. 11th. – the same day as CON 100 and ZINC 100 bees); CON 5 = Bees that emerged on the same day as ZINC 5 bees but treated with no zinc; ZINC 5 = Bees treated with 5 mg/L zinc; CON 100 = Bees that emerged on the same day as ZINC 100 bees but treated with no zinc; ZINC 100 = Bees treated with 100 mg/L zinc.



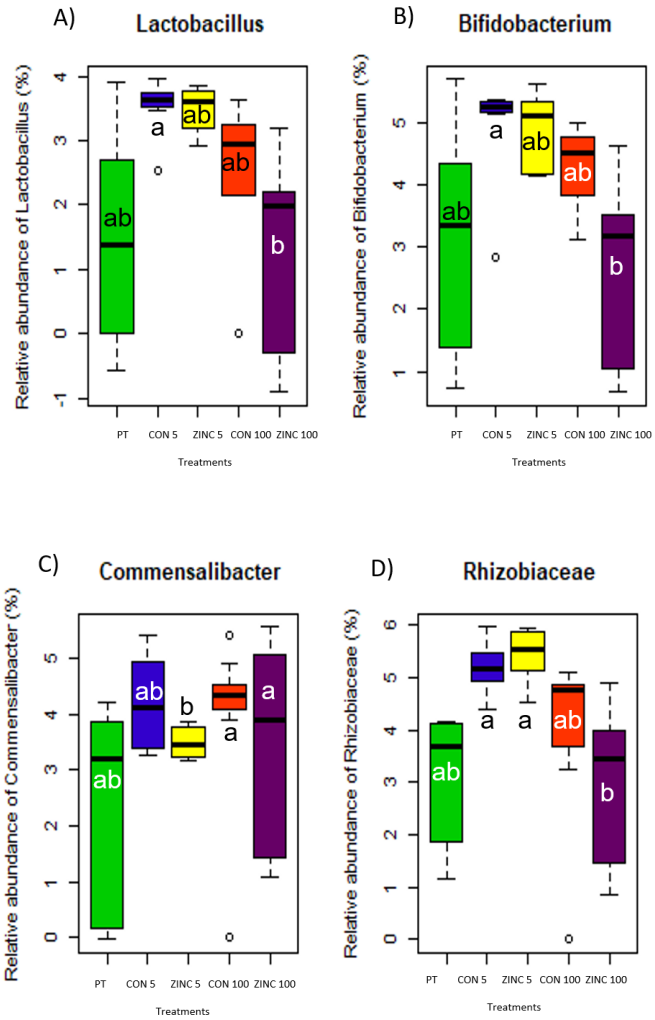
Supplementary material. Figure. 3 Microbial diversity by treatment. Gut microbial diversity did not differ significantly in honey bees by treatment or emerge day as measured by A, C) Faith's PD (Kruskal Wallis, p = 0.17) and B, D) Observed Features (Kruskal Wallis, p = 0.9). An increase in microbial diversity, albeit non-significant, is observed by emerge day. Box plots show outliers, first and third quartiles (lower and upper edges), and highest, lowest and median values (horizontal black dash). PT = All Pretreatment (Day 0) bees including those that emerged on Oct. 3, 7, and 11; PT0 = Pretreatment bees that emerged on Oct. 3; PT5 = Pretreatment bees that emerged on Oct 7th – the same day as CON 5 and ZINC 5 bees; PT100 = Pretreatment bees that emerged on Oct. 11th. – the same day as CON 100 and ZINC 100 bees); CON 5 = Bees that emerged on the same day as ZINC 5 bees but treated with no zinc; ZINC 5 = Bees treated with 5 mg/L zinc; CON 100 = Bees that emerged on the same day as ZINC 100 bees but treated with no zinc; ZINC 100 = Bees treated with 100 mg/L zinc.



Supplementary material. Figure. 4 Taxanomic abundances by sample and treatment group. Named taxa were found at >0.1% abundance across all samples. The remaining taxa (<1% abundance) were combined under “Other.”



Supplementary material. Figure. 5 Absolute abundances of core microbiota that were present in 100% of all samples by treatment at the level 7 taxonomy: A) Lactobacillus, B) Bifidobacterium, C) Commensalibacter and D) Rhizobiaceae. Box plot shows outliers, first and third quartiles (lower and upper edges), and highest, lowest and median values (horizontal black dash) for Control (Con) and Zinc groups. (ANOVA: *$p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$). Means followed by the same letter are not significantly different at the 95% confidence level (Wilcoxon Rank Sum Test; 95% Bonferroni confidence intervals for standard deviations).



Supplementary material. Figure. 6 Relative abundances of core microbiota that were present in 100% of all samples by treatment at the level 7 taxonomy: A) Lactobacillus, B) Bifidobacterium, C) Commensalibacter and D) Rhizobiaceae. Box plot shows outliers, first and third quartiles (lower and upper edges), and highest, lowest and median values (horizontal black dash) for Control (Con) and Zinc groups. (ANOVA: *p < 0.05; ** p < 0.01 and *** p < 0.001). Means followed by the same letter are not significantly different at the 95% confidence level (Tukey's test).

Supplementary Tables

Supplementary material. Table 1 - **Zinc oral toxicity assays**. The number of bees, replicates, and hives in each treatment group of the zinc oral toxicity trials. The final number of hives varied between 1-3 after omitting replicates with control groups that exhibited survival < 85% (OECD, 2017).

Zinc Concentration (mg/L)	**Number of Bees across all Replicates**	**Replicates (cups)**	**Hives**
0 (negative control)	85	4	3
50	80	4	3
100	101	5	3
250	60	3	2
500	102	5	2
1000	62	3	1

Supplementary material. Table 2 - Mean rates of survival observed during oral toxicity assays after 10 days.

Zinc Concentration (mg/L)	**Mean Percent Survival ± SE**
0	99 ± 1
50	91 ± 3
100	89 ± 7
250	57 ± 17
500	10 ± 3
1000	0 ± 0

Supplementary material. Table 3 - Honey bee survival duration estimates based on zinc exposure. LT50 is the estimated lethal effect times to reach 50% mortality. The LT50 decreased (fewer days) with increasing zinc concentrations.

Zinc Concentration (mg/L)	**LT50 Estimate (days)**	**Confidence Limits**	**Standard Error**	**χ^2 Goodness-of-Fit Statistic**	**df**
50	24.89	18.05 - 48.13	5.37	43.92	42
100	19.39	14.35 - 38.7	2.48	134.31	53
250	10.20	9.51 - 11.29	0.34	43.87	31
500	6.59	6.37 - 6.82	0.1	65.01	53
1000	5.25	4.95 - 5.55	0.12	43.36	31

Supplementary material. Table 4 - P-values from pairwise log-rank tests of differences in the overall rates of survival observed between treatment groups receiving varying zinc concentrations. All pairwise comparisons were significant ($p << 0.05$) except the comparison between the 50 and 100 mg/L groups.

	Zinc Concentration (mg/L)				
	0	**50**	**100**	**250**	**500**
50	2.33E-09	NA	NA	NA	NA
100	2.23E-10	~1	NA	NA	NA
250	2.32E-21	0.002	0.003	NA	NA
500	3.03E-90	4.77E-56	4.61E-65	5.45E-31	NA
1000	2.99E-112	2.38E-74	1.74E-86	4.94E-46	2.75E-05

Supplementary material. Table 5 - Total and bacterial DNA concentrations and read counts. Total and bacterial DNA concentrations and number of 16S reads in each sample. (16S read counts are reported both before and after running DADA2. PT = All Pretreatment (Day 0) bees including those that emerged on Oct. 3, 7, and 11; PT0 = Pretreatment bees that emerged on Oct. 3; PT 5 = Pretreatment bees that emerged on Oct 7th – the same day as CON 5 and ZINC 5 bees; PT 100 = Pretreatment bees that emerged on Oct. 11th. – the same day as CON 100 and ZINC 100 bees); CON 5 = Bees that emerged on the same day as ZINC 5 bees but treated with no zinc; ZINC 5 = Bees treated with 5 mg/L zinc; CON 100 = Bees that emerged on the same day as ZINC 100 bees but treated with no zinc; ZINC 100 = Bees treated with 100 mg/L zinc; D3 = Day 3, D6 = Day 6, D9 = Day 9. * = Excluded from analyses as variation between all replicates was greater than 3%.

Sample ID	**Group**	**Zinc Conc. (mg/L)**	**Emerge Day**	**Time**	**Cycle Threshold Values**	**Total DNA Conc. (ng/ul)**	**Bacterial DNA Conc. (ng/ul)**	**Percent Bacterial DNA**	**Number of 16S Reads (raw)**	**Number of 16S Reads (post DADA2)**	**Total Absolute Cell Counts**	**Retained in analyses**
PT1	PT / PT0	0	10/03/20	Pretreatment	18.16	141	2.77	1.96	115,704	20,752	558,131	Yes
PT2	PT / PT0	0	10/03/20	Pretreatment	21.77	117	0.58	0.50	93,268	4,580	117,514	Yes
PT3	PT / PT5	0	10/07/20	Pretreatment	29.36	136	0.02	0.02	94,952	31,368	4,419	Yes

PT4	PT / PT5	0	10/07/20	Pretreatment	24.11	140	0.21	0.15	117,458	7,365	42,718	Yes
PT5	PT / PT100	0	10/11/20	Pretreatment	33.60	140	0.00	0.00	129,983	68,083	707	Yes
PT6	PT / PT100	0	10/11/20	Pretreatment	38.71	157	0.00	0.00	137,281	88,988	78	Yes
A7	ZINC 5	5	10/07/20	D3	16.78	229	5.03	2.20	91,953	39,853	1,013,624	Yes
A8	ZINC 5	5	10/07/20	D3	20.32	128	1.09	0.85	88,601	21,952	219,924	Yes
A9	ZINC 5	5	10/07/20	D3	17.65	195	3.46	1.77	132,705	36,577	696,909	Yes
A10	ZINC 5	5	10/07/20	D6	19.45	144	1.58	1.10	146,690	47,571	384,943	No *
A11	ZINC 5	5	10/07/20	D6	18.97	167	1.95	1.17	122,443	29,375	280,759	No *
A12	ZINC 5	5	10/07/20	D6	18.76	219	2.14	0.98	132,757	84,203	430,497	Yes

A13	ZINC 5	5	10/07/20	D9	16.44	214	5.82	2.72	128,878	40,413	1,173,826	Yes
A14	ZINC 5	5	10/07/20	D9	17.48	192	3.71	1.93	135,517	61,750	748,191	Yes
A15	ZINC 5	5	10/07/20	D9	20.07	174	1.21	0.70	114,409	6,452	244,561	Yes
B16	ZINC 100	100	10/11/20	D3	18.17	154	2.76	1.79	113,731	28,476	615,873	No *
B17	ZINC 100	100	10/11/20	D3	35.59	124	0.00	0.00	106,334	56,532	299	Yes
B18	ZINC 100	100	10/11/20	D3	19.54	155	1.53	0.99	137,343	105,651	308,074	Yes
B19	ZINC 100	100	10/11/20	D6	24.66	166	0.17	0.10	77,309	4,544	33,627	Yes
B20	ZINC 100	100	10/11/20	D6	19.54	177	1.53	0.86	67,922	3,848	307,770	Yes
B21	ZINC 100	100	10/11/20	D6	32.86	162	0.00	0.00	120,927	32,924	975	Yes

B22	ZINC 100	100	10/11/20	D9	37.87	182	0.00	0.00	123,715	69,452	112	Yes
B23	ZINC 100	100	10/11/20	D9	19.08	176	1.86	1.06	126,685	101,403	375,157	Yes
B24	ZINC 100	100	10/11/20	D9	21.25	183	0.73	0.40	135,415	107,692	146,927	Yes
C25	CON 5	0	10/07/20	D3	19.84	144	1.34	0.93	105,481	25,491	269,872	Yes
C26	CON 5	0	10/07/20	D3	18.92	180	1.99	1.11	97,260	24,495	401,622	Yes
C27	CON 5	0	10/07/20	D3	21.08	136	0.79	0.58	98,363	44,490	158,272	Yes
C28	CON 5	0	10/07/20	D6	17.92	176	3.08	1.75	129,716	47,728	620,423	Yes
C29	CON 5	0	10/07/20	D6	16.23	179	6.38	3.56	112,603	49,089	1,495,437	No *
C30	CON 5	0	10/07/20	D6	16.42	191	5.87	3.08	125,249	59,330	1,184,372	Yes

C31	CON 5	0	10/07/20	D9	17.89	222	3.12	1.40	79,066	31,636	628,082	Yes
C32	CON 5	0	10/07/20	D9	18.66	183	2.24	1.22	91,428	36,709	450,635	Yes
C33	CON 5	0	10/07/20	D9	20.60	162	0.96	0.59	103,253	15,614	192,676	No *
D34	CON 100	0	10/11/20	D3	19.92	167	1.29	0.77	119,104	5,379	260,604	Yes
D35	CON 100	0	10/11/20	D3	19.84	147	1.34	0.91	108,771	7,940	269,888	Yes
D36	CON 100	0	10/11/20	D3	20.72	195	0.92	0.47	121,383	10,367	184,862	Yes
D37	CON 100	0	10/11/20	D6	20.05	157	1.22	0.78	109,498	6,820	246,472	Yes
D38	CON 100	0	10/11/20	D6	18.35	255	2.55	1.00	104,853	23,899	515,108	Yes
D39	CON 100	0	10/11/20	D6	19.62	185	1.47	0.80	139,057	10,041	297,290	Yes

D40	CON 100	0	10/11/20	D9	19.52	140	1.54	1.10	131,680	23,263	310,587	Yes
D41	CON 100	0	10/11/20	D9	18.42	149	2.47	1.66	133,753	43,962	498,253	Yes
D42	CON 100	0	10/11/20	D9	20.77	167	0.90	0.54	148,812	5,324	180,869	Yes

Supplementary material. Table 6 - Taxa that were identified by decontam as potential contaminants. These taxa were bioinformatically removed prior to analyses.

Taxa ID	**Description**
354acc009b4d5743f420af8925691527	Romboutsia sp. CE17 chromosome, complete genome
302f68ec5342caa417fa82f6bd6a6eeb	Methylobacterium sp. SO-204 gene for 16S ribosomal RNA, partial sequence
05993181c81cb6e09bf67c0093fb2595	Cutibacterium acnes strain 3265 16S ribosomal RNA gene, partial sequence
9fcd9366dd83ecf153270aa8c8d4e609	Methylorubrum extorquens strain B44 16S ribosomal RNA gene, partial sequence
b8ecf6ec5cc4215ab3954b72f41f0912	Uncultured bacterium clone A1435 16S ribosomal RNA gene, partial sequence

Supplementary material. Table 7 - Evaluating effects of treatment (ZINC vs. CONTROL) and time (days) on gut microbial composition (Bray Curtis -PERMANOVA). Only treatment was significant.

	Df	**SumsOfSqs**	**MeanSqs**	**F.Model**	**R2**	**Pr(>F)**
Treatment	4	3.262155	0.815539	3.718791	0.280026	0.001
Time	1	0.308681	0.308681	1.407563	0.026497	0.201
Treatment:Time	3	0.841663	0.280554	1.279305	0.072249	0.203
Residuals	33	7.236970	0.219302	NaN	0.621227	NaN
Total	41	11.649469	NaN	NaN	1.000000	NaN

Supplementary material. Table 8 - Evaluating effects of treatment (ZINC vs. CONTROL) and time (days) on gut microbial composition (Jacaard -PERMANOVA). Only treatment was significant.

	Df	**SumsOfSqs**	**MeanSqs**	**F.Model**	**R2**	**Pr(>F)**
Treatment	4	2.702407	0.675602	2.310244	0.198455	0.001
Time	1	0.285827	0.285827	0.977395	0.020990	0.509
Treatment:Time	3	0.978558	0.326186	1.115404	0.071862	0.146
Residuals	33	9.650435	0.292437	NaN	0.708693	NaN
Total	41	13.617227	NaN	NaN	1.000000	NaN

Supplementary material. Table 9 - Bray Curtis PERMANOVA pairwise comparisons. Notably, CON 100 and ZINC 100 groups do not differ. CON 5 and ZINC 5 groups also do not differ. However, CON 100 and ZINC 100 groups differ significantly from CON 5 and ZINC 5 groups. Treatment groups included: PT = Pretreatment (Day 0), CON 5 = Bees that emerged on the same day as ZINC 5 bees but treated with no zinc, ZINC 5 = Bees treated with 5 mg/L zinc, CON 100 = Bees that emerged on the same day as ZINC 100 bees but treated with no zinc, ZINC 100 = Bees treated with 100 mg/L zinc.

Group 1	**Group 2**	**Sample size**	**Permutations**	**pseudo-F**	**p-value**	**q-value**
ZINC 100	PT	15	999	1.94	0.07	0.088
ZINC 100	CON 100	18	999	1.68	0.175	0.194
ZINC 100	CON 5	18	999	6.26	0.001	0.003
ZINC 5	ZINC 100	18	999	6.5	0.001	0.003
ZINC 5	PT	15	999	4.15	0.001	0.003
ZINC 5	CON 100	18	999	3.57	0.006	0.010
ZINC 5	CON 5	18	999	0.79	0.514	0.514
PT	CON 100	15	999	1.0	0.039	0.056
PT	CON 5	15	999	5.05	0.001	0.003

CON 5	CON 100	18	999	4.35	0.004	0.008

Supplementary material. Table 10 - Jaccard PERMANOVA pairwise comparisons. All groups differed significantly. PT = Pretreatment (Day 0), CON 5 = Bees that emerged on the same day as ZINC 5 bees but treated with no zinc, ZINC 5 = Bees treated with 5 mg/L zinc, CON 100 = Bees that emerged on the same day as ZINC 100 bees but treated with no zinc, ZINC 100 = Bees treated with 100 mg/L zinc.

Group 1	**Group 2**	**Sample size**	**Permutations**	**pseudo-F**	**p-value**	**q-value**
ZINC 100	PT	15	999	1.56	0.004	0.004
ZINC 100	CON 100	18	999	2.17	0.001	0.001
ZINC 100	CON 5	18	999	2.68	0.001	0.001
ZINC 5	ZINC 100	18	999	3.34	0.001	0.001
ZINC 5	PT	15	999	2.08	0.001	0.001
ZINC 5	CON 100	18	999	3.21	0.001	0.001
ZINC 5	CON 5	18	999	1.88	0.001	0.001
PT	CON 100	15	999	1.47	0.022	0.022
PT	CON 5	15	999	1.89	0.001	0.001
CON 5	CON 100	18	999	2.48	0.001	0.001

Supplementary material Table 11 - Core microbiota. L7 – or roughly species level taxa - that were present in 100% of samples across all treatment groups.

Feature ID
D_1_Proteobacteria;D_2_Alphaproteobacteria;D_3_Rhizobiales;D_4_Rhizobiaceae
D_1_Actinobacteria;D_2_Actinobacteria;D_3_Bifidobacteriales;D_4_Bifidobacteriaceae; D_5_Bifidobacterium
D_1_Proteobacteria;D_2_Alphaproteobacteria;D_3_Acetobacterales;D_4_Acetobacteraceae; D_5_Commensalibacter
D_1_Firmicutes;D_2_Bacilli;D_3_Lactobacillales;D_4_Lactobacillaceae;D_5_Lactoba cillus

Supplementary material. Table 12 - Differentially abundant taxa (ANCOM) at the genera (L7) level by treatment.

Feature ID		**W**
D_0_Bacteria;D_1_Firmicutes;D_2_Bacilli;D_3_Lactobacillales;D_4_Lactobacillaceae;D_5_Lactobacillus	Decreased in PT100	409
D_0_Bacteria;D_1_Proteobacteria;D_2_Alphaproteobacteria;D_3_Rhizobiales;D_4_Rhizobiaceae	Decreased in PT100	409
D_0_Bacteria;D_1_Proteobacteria;D_2_Gammaproteobacteria;D_3_Enterobacteriales;D_4_Enterobacteriaceae	Increased in CON100	409
D_0_Bacteria;D_1_Proteobacteria;D_2_Gammaproteobacteria;D_3_Orbales;D_4_Orbaceae;D_5_Gilliamella	Decreased in ZINC100	408
D_0_Bacteria;D_1_Firmicutes;D_2_Bacilli;D_3_Bacillales;D_4_Paenibacillaceae;D_5_Paenibacillus	Increased in ZINC100	408
D_0_Bacteria;D_1_Actinobacteria;D_2_Actinobacteria;D_3_Micrococcales;D_4_Microbacteriaceae_	Increased in ZINC100	375

QIIME and R Scripts

Script 1 – QIIME2 script

```
qiime metadata tabulate \
  --m-input-file metadata.tsv \
  --o-visualization metadata.qzv


#import decontam table
biom convert -i dada2_table_decontam.tsv -o dada2_table_decontam.biom --to-hdf5
qiime tools import \
  --input-path dada2_table_decontam.biom \
  --type 'FeatureTable[Frequency]' \
  --input-format BIOMV210Format \
  --output-path dada2_table.qza
qiime metadata tabulate \
  --m-input-file ./dada2_stats.qza  \
  --o-visualization ./dada2_stats.qzv
qiime feature-table summarize \
  --i-table ./dada2_table.qza \
  --m-sample-metadata-file ./metadata.tsv \
  --o-visualization ./dada2_table.qzv


 #Generating a phylogenetic tree for diversity analysis
```

```
qiime fragment-insertion sepp \
  --i-representative-sequences ./dada2_rep_set.qza \
  --i-reference-database sepp-refs-gg-13-8.qza \
  --o-tree ./tree.qza \
  --o-placements ./tree_placements.qza \
  --p-threads 4  # update to a higher number if you can

  #######

  ##I have chosen I have chosen “p-min-depth 3000  and --p-max-depth 107666”  based on min and max the maximum sample total frequency of the feature_table  dada2_table.qzv
qiime diversity alpha-rarefaction \
--i-table ./dada2_table.qza \
--m-metadata-file ./metadata.tsv \
--o-visualization ./alpha_rarefaction_curves.qzv \
--p-min-depth 3000 \
--p-max-depth 107666
 #Diversity analysis

 #I have shosen p-sampling-depth 5000 based on the min Feature Count on dada2_table.qzv
qiime diversity core-metrics-phylogenetic \
  --i-table ./dada2_table.qza \
  --i-phylogeny ./tree.qza \
  --m-metadata-file ./metadata.tsv \
  --p-sampling-depth 3000 \
```

```
  --output-dir ./core-metrics-results_EDE


   #Alpha Rarefaction group significance (after core metrics)
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-results/shannon_vector.qza \
  --m-metadata-file metadata.tsv \
  --o-visualization core-metrics-results/shannon_vector-group-significance.qzv


  #Alpha diversity
qiime diversity alpha-group-significance \
  --i-alpha-diversity ./core-metrics-results/faith_pd_vector.qza \
  --m-metadata-file ./metadata.tsv \
  --o-visualization ./core-metrics-results/faiths_pd_statistics.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results/evenness_vector.qza \
 --m-metadata-file ./metadata.tsv \
 --o-visualization ./core-metrics-results/evenness_statistics.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results/shannon_vector.qza \
 --m-metadata-file ./metadata.tsv \
 --o-visualization ./core-metrics-results/shannon_statistics.qzv
qiime diversity alpha-group-significance \
 --i-alpha-diversity ./core-metrics-results/observed_otus_vector.qza \
 --m-metadata-file ./metadata.tsv \
```

```
 --o-visualization ./core-metrics-results/observed_otus_statistic.qzv


  #alpha-correlation for continuous covariate
qiime diversity alpha-correlation \
--i-alpha-diversity core-metrics-results/evenness_vector.qza \
--m-metadata-file metadata.tsv \
--p-method spearman \
--output-dir alpha_correlation/
qiime diversity alpha-correlation \
--i-alpha-diversity core-metrics-results/shannon_vector.qza \
--m-metadata-file metadata.tsv \
--p-method shannon \
--output-dir alpha_correlation_shannon/
qiime diversity alpha-correlation \
--i-alpha-diversity core-metrics-results/shannon_vector.qza \
--m-metadata-file metadata.tsv \
--p-method pearson \
--output-dir alpha_correlation_pearson/
  #Alpha diversity longitudinal anova
qiime longitudinal anova \
 --m-metadata-file ./core-metrics-results/faith_pd_vector.qza \
 --m-metadata-file ./metadata.tsv \
 --p-formula 'faith_pd ~ Treat * Day' \
 --o-visualization ./core-metrics-results/faiths_pd_anova.qzv
```

```
#For Treat_LD
qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --m-metadata-column Treat_LD \
 --o-visualization core-metrics-results/unweighted-unifrac-Treat_LD-significance.qzv \
 --p-pairwise
qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --m-metadata-column Treat_LD \
 --o-visualization core-metrics-results/weighted-unifrac-Treat_LD-significance.qzv \
 --p-pairwise

qiime diversity beta-group-significance \
--i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza \
--m-metadata-file metadata.tsv \
--p-pairwise \
--m-metadata-column Treat_LD \
--o-visualization core-metrics-results/bray_curtis-Treat_LD-significance_pairwise.qzv

qiime diversity beta-group-significance \
--i-distance-matrix core-metrics-results/jaccard_distance_matrix.qza \
```

```
 --m-metadata-file metadata.tsv \
 --p-pairwise \
 --m-metadata-column Treat_LD \
 --o-visualization core-metrics-results/jaccard-Treat_LD-significance_pairwise.qzv
 #For Treat
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column Treat \
  --o-visualization core-metrics-results/unweighted-unifrac-Treat-significance.qzv \
  --p-pairwise
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column Treat \
  --o-visualization core-metrics-results/weighted-unifrac-Treat-significance.qzv \
  --p-pairwise


qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --p-pairwise \
 --m-metadata-column Treat \
 --o-visualization core-metrics-results/bray_curtis-Treat-significance_pairwise.qzv
```

```
qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/jaccard_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --p-pairwise \
 --m-metadata-column Treat \
 --o-visualization core-metrics-results/jaccard-Treat-significance_pairwise.qzv

 #For Day
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column Day \
  --o-visualization core-metrics-results/unweighted-unifrac-Day-significance.qzv \
  --p-pairwise
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column Day \
  --o-visualization core-metrics-results/weighted-unifrac-Day-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza \
```

```
 --m-metadata-file metadata.tsv \
 --p-pairwise \
 --m-metadata-column Day \
 --o-visualization core-metrics-results/bray_curtis-Day-significance_pairwise.qzv

qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/jaccard_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --p-pairwise \
 --m-metadata-column Day \
 --o-visualization core-metrics-results/jaccard-Day-significance_pairwise.qzv

  #Adonis to look at a multivariate model

qiime diversity adonis \
  --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --o-visualization core-metrics-results/bray_curtis_adonis.qzv \
  --p-formula Treat*Day\
qiime diversity adonis \
  --i-distance-matrix core-metrics-results/jaccard_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --o-visualization core-metrics-results/jaccard_adonis.qzv \
  --p-formula Treat*Day\
```

```
qiime diversity adonis \
  --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --o-visualization core-metrics-results/unweighted_adonis.qzv \
  --p-formula Treat*Day\

qiime diversity adonis \
  --i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --o-visualization core-metrics-results/weighted_adonis.qzv \
  --p-formula Treat*Day\
####EMERGE DAY EFFECT
qiime diversity adonis \
  --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza \
  --m-metadata-file metadata_EDE.tsv \
  --o-visualization core-metrics-results/bray_curtis_adonis_EDE.qzv \
  --p-formula Treat\

qiime diversity adonis \
  --i-distance-matrix core-metrics-results/jaccard_distance_matrix.qza \
  --m-metadata-file metadata_EDE.tsv \
  --o-visualization core-metrics-results/jaccard_adonis_EDE.qzv \
  --p-formula Treat\
```

```
qiime diversity adonis \
  --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata_EDE.tsv \
  --o-visualization core-metrics-results/unweighted_adonis_EDE.qzv \
  --p-formula Treat\

qiime diversity adonis \
  --i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata_EDE.tsv \
  --o-visualization core-metrics-results/weighted_adonis_EDE.qzv \
  --p-formula Treat\

 ###############Beta gorup significance

qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --p-pairwise \
 --m-metadata-column Treat \
 --o-visualization core-metrics-results/bray_curtis-treatment-significance_pairwise_Treat.qzv

qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/jaccard_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
```

```
 --p-pairwise \
 --m-metadata-column Treat \
 --o-visualization core-metrics-results/jaccard-treatment-significance_pairwise_Treat.qzv

qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --p-pairwise \
 --m-metadata-column Treat \
 --o-visualization core-metrics-results/unweighted_unifrac_treatment-
significance_pairwise_Treat.qzv

 qiime diversity beta-group-significance \
 --i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza \
 --m-metadata-file metadata.tsv \
 --p-pairwise \
 --m-metadata-column Treat \
 --o-visualization core-metrics-results/weighted_unifrac_-significance_pairwise_Treat.qzv
  #Taxonomic classification
    #alinhar e classificar
qiime feature-classifier classify-sklearn \
  --i-reads ./dada2_rep_set.qza \
  --i-classifier ./silva-132-99-nb-classifier.qza \
  --o-classification ./taxonomy.qza
qiime metadata tabulate \
```

```
  --m-input-file ./taxonomy.qza \
  --o-visualization ./taxonomy.qzv
qiime feature-table tabulate-seqs \
  --i-data ./dada2_rep_set.qza \
  --o-visualization ./dada2_rep_set.qzv

###############Filter the table of ASVs
#Mitochondria
qiime taxa filter-table \
--i-table dada2_table.qza \
--i-taxonomy taxonomy.qza \
--p-exclude      Mitochondria \
--o-filtered-table table-no-mitochondria.qza
#Chloroplast
qiime taxa filter-table \
 --i-table table-no-mitochondria.qza \
 --i-taxonomy taxonomy.qza \
 --p-exclude     Chloroplast \
 --o-filtered-table table-no-chloroplast.qza
#Unassigned
qiime taxa filter-table \
 --i-table table-no-chloroplast.qza \
 --i-taxonomy taxonomy.qza \
 --p-exclude     Unassigned \
```

```
 --o-filtered-table table-no-Unassigned.qza
#Eukaryota
qiime taxa filter-table \
 --i-table table-no-Unassigned.qza \
 --i-taxonomy taxonomy.qza \
 --p-exclude     Eukaryota \
 --o-filtered-table table-final.qza
  #reatribuir taxonomia
qiime feature-table filter-seqs \
 --i-data dada2_rep_set.qza \
 --i-table table-final.qza \
 --o-filtered-data seqs-final
qiime feature-classifier classify-sklearn \
 --i-reads ./seqs-final.qza \
 --i-classifier ./silva-132-99-nb-classifier.qza \
 --o-classification ./taxonomyfinal.qza
qiime metadata tabulate \
 --m-input-file ./taxonomyfinal.qza \
 --o-visualization ./taxonomyfinal.qzv

 #Taxonomy barchart (5000 based on the min Feature Count on dada2_table.qzv
qiime feature-table filter-samples \
 --i-table ./table-final.qza \
 --p-min-frequency 3000 \
```

```
 --o-filtered-table ./table_2kfiltered.qza
qiime taxa barplot \
 --i-table ./table_2kfiltered.qza \
 --i-taxonomy ./taxonomyfinal.qza \
 --m-metadata-file ./metadata.tsv \
 --o-visualization ./taxa_barplotfiltered.qzv
  #genereates the table with the number of asv per sample

  # table with taxonomy
qiime taxa collapse \
  --i-table table-final.qza \
  --i-taxonomy taxonomy.qza \
  --p-level 7 \
  --o-collapsed-table table-l7.qza

qiime tools export \
 --input-path table-l7.qza \
 --output-path table_exported-feature-table
cd table_exported-feature-table
biom convert --to-tsv -i feature-table.biom -o bee_zinc_table.tsv
qiime feature-table filter-features \
  --i-table ./table_2kfiltered.qza \
  --p-min-frequency 50 \
  --p-min-samples 3 \
```

```
  --o-filtered-table ./table_2k_abund.qza


  #Differential abundance with ANCOM
qiime composition add-pseudocount \
  --i-table table_2k_abund.qza\
  --o-composition-table comp-table.qza
#Ancon treat_LD
qiime composition ancom \
  --i-table comp-table.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column Treat_LD \
  --o-visualization ancom-subject.qzv


#Ancon Treat_LD   #Ancon Treat_LD##### make metadatas with different possibilities floraxtet, flora, ctrl..
qiime composition ancom \
  --i-table comp-table.qza \
  --m-metadata-file metadata_NO_PT.tsv \
  --m-metadata-column Treat \
  --o-visualization ancom-subject_NO_PT.qzv


  #core features
qiime feature-table core-features \
--i-table table_2k_abund.qza \
 --o-visualization core-features
```

```
#Longitudinal analysis
#PCoA-based analyses
qiime longitudinal volatility \
--m-metadata-file ./metadata.tsv \
--m-metadata-file ./core-metrics-results/unweighted_unifrac_pcoa_results.qza \
--p-state-column time_hours \
--p-individual-id-column samples \
--p-default-group-column 'Treat_LD' \
--p-default-metric 'Axis 2' \
--o-visualization ./pc_vol.qzv
#Machine-learning classifiers for predicting sample characteristics
qiime sample-classifier classify-samples \
--i-table ./table_2k_abund.qza \
--m-metadata-file ./metadata.tsv \
--m-metadata-column Treat_LD \
--p-random-state 666 \
--p-n-jobs 1 \
--output-dir ./sample-classifier-results/
qiime sample-classifier heatmap \
 --i-table ./table_2k_abund.qza \
 --i-importance ./sample-classifier-results/feature_importance.qza \
 --m-sample-metadata-file metadata.tsv \
 --m-sample-metadata-column Treat_LD \
 --p-group-samples \
```

```
 --p-feature-count 100 \
 --o-heatmap ./sample-classifier-results/heatmap100.qzv \
 --o-filtered-table ./sample-classifier-results/filtered-table100.qza
```

Script 2 - R studio Post hoc analysis

```
library(readr)
library(laercio)
require(laercio)
#install.packages("dplyr")
require("dplyr")
library("dplyr")
library(readr)
par(mfrow = c(1,2), oma = c(2,1,1,1))
par(mfrow = c(2,5), oma = c(2,1,1,1))
#importdata
#Relative Abundances and Diversity (reads_cz)
library(readr)
reads_cz <-
read_delim("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/reads_cz.txt",
                   delim = "\t", escape_double = FALSE,
                   trim_ws = TRUE)
reads_cz_N5 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/reads_cz.txt")
```

```
reads_cz_N5 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/reads_cz_N5.csv")

reads_cz_N100 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/reads_cz_N100.csv")

reads_cz_N5_d3 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/reads_cz_N5_d3.csv")

reads_cz_N5_d6 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/reads_cz_N5_d6.csv")

reads_cz_N5_d9 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/reads_cz_N5_d9.csv")

reads_cz_N100_d3 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/reads_cz_N100_d3.csv")

reads_cz_N100_d6 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/reads_cz_N100_d6.csv")

reads_cz_N100_d9 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/reads_cz_N100_d9.csv")

#Absolute abundances and qPCR

qpcr_cz <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_CZ.csv")
```

```
qpcr_cz_L5 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_cz_L5.csv")

qpcr_cz_L100 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_cz_L100.csv")

qpcr_cz_L5_d3 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_cz_L5_d3.csv")

qpcr_cz_L5_d6 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_cz_L5_d6.csv")

qpcr_cz_L5_d9 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_cz_L5_d9.csv")

qpcr_cz_L100_d3 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_cz_L100_d3.csv")

qpcr_cz_L100_d6 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_cz_L100_d6.csv")

qpcr_cz_L100_d9 <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_cz_L100_d9.csv")

qpcr_cz_zin <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_cz_zin.csv")

qpcr_cz_CTRL <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_cz_CTRL.csv")
```

```
#log

qpcr_log <- read_csv("importing_tables/qpcr_log.csv")

View(qpcr_log)

#LOG for plots

par(mfrow = c(1,1), oma = c(2,1,1,1))

boxplot(bacterial_DNA_concentration ~ Treat, data=qpcr_log, cex = 1,  main=" Bacterial DNA Concentration (ng/ul)", xlab="Treatments", ylab="Bacterial DNA Concentration (ng/ul)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(percent_bacterial_DNA ~ Treat, data=qpcr_log, cex = 1,  main=" Bacterial DNA Concentration (ng/ul)", xlab="Treatments", ylab="Percent Bacterial DNA", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Absolute_cell_counts_per_sample ~ Treat, data=qpcr_log, cex = 1,  main=" Absolute Cell Counts", xlab="Treatments", ylab="Absolute Cell Counts", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(sixteen_s_raw_reads ~ Treat, data=qpcr_log, cex = 1,  main=" Sixteen S raw reads", xlab="Treatments", ylab="Raw reads", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(sixteen_s_denoised_reads ~ Treat, data=qpcr_log, cex = 1,  main=" sixteen S denoised reads", xlab="Treatments", ylab="Raw reads", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

#Absolute abundance (BEFORE LOG)

#Only all ANCOM

par(mfrow = c(2,5), oma = c(2,1,1,1))

boxplot(Lactobacillus ~ Treat, data=qpcr_cz, cex = 1,  main="A) Lactobacillus", xlab="Treatments", ylab="Absolute Abundance of Lactobacillus", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))
```

```
boxplot(Bifidobacterium ~ Treat, data=qpcr_cz, cex = 1,  main="Bifidobacterium",
xlab="Treatments", ylab="Absolute Abundance of Bifidobacterium", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Tyzzerella ~ Treat, data=qpcr_cz, cex = 1,  main=" Tyzzerella", xlab="Treatments",
ylab="Absolute Abundance of Tyzzerella", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Proteobacteria ~ Treat, data=qpcr_cz, cex = 1,  main=" Proteobacteria",
xlab="Treatments", ylab="Absolute Abundance of Proteobacteria", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Enterobacteriaceae ~ Treat, data=qpcr_cz, cex = 1,  main=" Enterobacteriaceae",
xlab="Treatments", ylab="Absolute Abundance of Enterobacteriaceae", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Rhizobiaceae ~ Treat, data=qpcr_cz, cex = 1,  main=" Rhizobiaceae",
xlab="Treatments", ylab="Absolute Abundance of Rhizobiaceae", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Gilliamella ~ Treat, data=qpcr_cz, cex = 1,  main=" Gilliamella", xlab="Treatments",
ylab="Absolute Abundance of Gilliamella", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Streptomyces ~ Treat, data=qpcr_cz, cex = 1,  main=" Streptomyces",
xlab="Treatments", ylab="Absolute Abundance of Streptomyces", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Paenibacillus ~ Treat, data=qpcr_cz, cex = 1,  main=" Paenibacillus",
xlab="Treatments", ylab="Absolute Abundance of Paenibacillus", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#Absolute abundance of the microbe (LOG)

par(mfrow = c(2,4), oma = c(2,1,1,1))

#Core
```

```
boxplot(Lactobacillus ~ Treat, data=qpcr_log, cex = 1,  main=" Lactobacillus",
xlab="Treatments", ylab="Absolute Abundance of Lactobacillus", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Bifidobacterium ~ Treat, data=qpcr_log, cex = 1,  main="Bifidobacterium",
xlab="Treatments", ylab="Absolute Abundance of Bifidobacterium", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Commensalibacter ~ Treat, data=qpcr_log, cex = 1,  main=" Commensalibacter",
xlab="Treatments", ylab="Absolute Abundance of Commensalibacter", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Rhizobiaceae ~ Treat, data=qpcr_log, cex = 1,  main=" Rhizobiaceae",
xlab="Treatments", ylab="Absolute Abundance of Rhizobiaceae", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#Ancom

par(mfrow = c(2,3), oma = c(2,1,1,1))

boxplot(Tyzzerella ~ Treat, data=qpcr_log, cex = 1,  main=" Tyzzerella", xlab="Treatments",
ylab="Absolute Abundance of Tyzzerella", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Streptomyces ~ Treat, data=qpcr_log, cex = 1,  main=" Streptomyces",
xlab="Treatments", ylab="Absolute Abundance of Streptomyces", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Paenibacillus ~ Treat, data=qpcr_log, cex = 1,  main=" Paenibacillus",
xlab="Treatments", ylab="Absolute Abundance of Paenibacillus", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Gilliamella ~ Treat, data=qpcr_log, cex = 1,  main=" Gilliamella",
xlab="Treatments", ylab="Absolute Abundance of Gilliamella", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Enterobacteriaceae ~ Treat, data=qpcr_log, cex = 1,  main=" Enterobacteriaceae",
xlab="Treatments", ylab="Absolute Abundance of Enterobacteriaceae", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))
```

```
boxplot(Proteobacteria ~ Treat, data=qpcr_log, cex = 1,  main=" Proteobacteria",
xlab="Treatments", ylab="Absolute Abundance of Proteobacteria", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#Only all ANCOM

par(mfrow = c(2,5), oma = c(2,1,1,1))

boxplot(Lactobacillus ~ Treat, data=qpcr_log, cex = 1,  main="A) Lactobacillus",
xlab="Treatments", ylab="Absolute Abundance of Lactobacillus", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Bifidobacterium ~ Treat, data=qpcr_log, cex = 1,  main="Bifidobacterium",
xlab="Treatments", ylab="Absolute Abundance of Bifidobacterium", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Tyzzerella ~ Treat, data=qpcr_log, cex = 1,  main=" Tyzzerella", xlab="Treatments",
ylab="Absolute Abundance of Tyzzerella", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Proteobacteria ~ Treat, data=qpcr_log, cex = 1,  main=" Proteobacteria",
xlab="Treatments", ylab="Absolute Abundance of Proteobacteria", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Enterobacteriaceae ~ Treat, data=qpcr_log, cex = 1,  main=" Enterobacteriaceae",
xlab="Treatments", ylab="Absolute Abundance of Enterobacteriaceae", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Rhizobiaceae ~ Treat, data=qpcr_log, cex = 1,  main=" Rhizobiaceae",
xlab="Treatments", ylab="Absolute Abundance of Rhizobiaceae", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Gilliamella ~ Treat, data=qpcr_log, cex = 1,  main=" Gilliamella",
xlab="Treatments", ylab="Absolute Abundance of Gilliamella", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Streptomyces ~ Treat, data=qpcr_log, cex = 1,  main=" Streptomyces",
xlab="Treatments", ylab="Absolute Abundance of Streptomyces", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))
```

```
boxplot(Paenibacillus ~ Treat, data=qpcr_log, cex = 1,  main=" Paenibacillus",
xlab="Treatments", ylab="Absolute Abundance of Paenibacillus", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#Relative abundances boxplots

boxplot(Bifidobacterium ~ Treat, data=reads_cz, cex = 1, ylim=c(0,100), main=" Total
Bifidobacterium",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Streptomyces ~ Treat, data=reads_cz, cex = 1,  ylim=c(0,100),main=" Total
Streptomyces",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Paenibacillus ~ Treat, data=reads_cz, cex = 1,  ylim=c(0,100),main=" Total
Paenibacillus",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Lactobacillus ~ Treat, data=reads_cz, cex = 1, ylim=c(0,100),main=" Total
Lactobacillus",xlab="Treatments",pairwise.wilcox.test, ylab="Relative abundance (%)", col =
c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Tyzzerella ~ Treat, data=reads_cz, cex = 1, ylim=c(0,100), main=" Total
Tyzzerella",xlab="Treatments", ylab="Relative abundance (%)",col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Commensalibacter ~ Treat, data=reads_cz, cex = 1, ylim=c(0,100),main=" Total
Commensalibacter",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Rhizobiaceae ~ Treat, data=reads_cz, cex = 1, ylim=c(0,100),main=" Total
Rhizobiaceae",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Gilliamella ~ Treat, data=reads_cz, cex = 1,  ylim=c(0,100),main=" Total
Gilliamella",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))
```

```
boxplot(Enterobacteriaceae ~ Treat, data=reads_cz, cex = 1,  ylim=c(0,100),main=" Total Enterobacteriaceae",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Proteobacteria ~ Treat, data=reads_cz, cex = 1, ylim = c(0, 100),main=" Total Proteobacteria",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

#Relative only ANCON

par(mfrow = c(2,5), oma = c(2,1,1,1))

boxplot(Lactobacillus ~ Treat, data=reads_cz, cex = 1,main=" Total Lactobacillus",xlab="Treatments",pairwise.wilcox.test, ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Bifidobacterium ~ Treat, data=reads_cz, cex = 1, main=" Total Bifidobacterium",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Rhizobiaceae ~ Treat, data=reads_cz, cex = 1,main=" Total Rhizobiaceae",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Gilliamella ~ Treat, data=reads_cz, cex = 1,main=" Total Gilliamella",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Tyzzerella ~ Treat, data=reads_cz, cex = 1, main=" Total Tyzzerella",xlab="Treatments", ylab="Relative abundance (%)",col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Streptomyces ~ Treat, data=reads_cz, cex = 1,main=" Total Streptomyces",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Paenibacillus ~ Treat, data=reads_cz, cex = 1,main=" Total Paenibacillus",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))
```

```
boxplot(Enterobacteriaceae ~ Treat, data=reads_cz, cex = 1,main=" Total Enterobacteriaceae",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Proteobacteria ~ Treat, data=reads_cz, cex = 1,main=" Total Proteobacteria",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

#Plots Relative LOG

#Relative abundances log

reads_cz_log <- read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/reads_cz_log.csv")

#Only all ANCOM

par(mfrow = c(2,5), oma = c(2,1,1,1))

boxplot(Bifidobacterium ~ Treat, data=reads_cz_log, cex = 1,  main=" Total Bifidobacterium",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Streptomyces ~ Treat, data=reads_cz_log, cex = 1,  main=" Total Streptomyces",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Paenibacillus ~ Treat, data=reads_cz_log, cex = 1,  main=" Total Paenibacillus",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Lactobacillus ~ Treat, data=reads_cz_log, cex = 1, main=" Total Lactobacillus",xlab="Treatments",pairwise.wilcox.test, ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Tyzzerella ~ Treat, data=reads_cz_log, cex = 1,  main=" Total Tyzzerella",xlab="Treatments", ylab="Relative abundance (%)",col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))
```

```
boxplot(Commensalibacter ~ Treat, data=reads_cz_log, cex = 1, main=" Total Commensalibacter",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Rhizobiaceae ~ Treat, data=reads_cz_log, cex = 1, main=" Total Rhizobiaceae",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Gilliamella ~ Treat, data=reads_cz_log, cex = 1,  main=" Total Gilliamella",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Enterobacteriaceae ~ Treat, data=reads_cz_log, cex = 1,  main=" Total Enterobacteriaceae",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Proteobacteria ~ Treat, data=reads_cz_log, cex = 1, ylim = c(0, 100),main=" Total Proteobacteria",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

#Core

par(mfrow = c(1,4), oma = c(2,1,1,1))

boxplot(Lactobacillus ~ Treat, data=reads_cz_log, cex = 1, main=" Total Lactobacillus",xlab="Treatments",pairwise.wilcox.test, ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Bifidobacterium ~ Treat, data=reads_cz_log, cex = 1,  main=" Total Bifidobacterium",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Commensalibacter ~ Treat, data=reads_cz_log, cex = 1, main=" Total Commensalibacter",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Rhizobiaceae ~ Treat, data=reads_cz_log, cex = 1, main=" Total Rhizobiaceae",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))
```

```
#Ancom

par(mfrow = c(2,3), oma = c(2,1,1,1))

boxplot(Tyzzerella ~ Treat, data=reads_cz_log, cex = 1,  main=" Total
Tyzzerella",xlab="Treatments", ylab="Relative abundance (%)",col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Streptomyces ~ Treat, data=reads_cz_log, cex = 1,  main=" Total
Streptomyces",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Paenibacillus ~ Treat, data=reads_cz_log, cex = 1,  main=" Total
Paenibacillus",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Gilliamella ~ Treat, data=reads_cz_log, cex = 1,  main=" Total
Gilliamella",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Enterobacteriaceae ~ Treat, data=reads_cz_log, cex = 1,  main=" Total
Enterobacteriaceae",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Proteobacteria ~ Treat, data=reads_cz_log, cex = 1, ylim = c(0, 100),main=" Total
Proteobacteria",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#absolute abudances of the microbes

# Lactobacillus

#anova All

shapiro.test(qpcr_cz$Lactobacillus) #not signifcant, data are normal

#kruskal.test

kruskal.test(Lactobacillus~ Treat, data = qpcr_cz)

pairwise.wilcox.test(qpcr_cz $ Lactobacillus, qpcr_cz $Treat, p.adj = "bonferroni",
exact=FALSE)
```

```
# Bifidobacterium

#anova All

shapiro.test(qpcr_cz$Bifidobacterium) #not signifcant, data are normal

#kruskal.test

kruskal.test(Bifidobacterium~ Treat, data = qpcr_cz)

pairwise.wilcox.test(qpcr_cz $ Bifidobacterium, qpcr_cz $Treat, p.adj = "bonferroni",
exact=FALSE)

# Rhizobiaceae

#anova All

shapiro.test(qpcr_cz$Rhizobiaceae) #not signifcant, data are normal

#kruskal.test

kruskal.test(Rhizobiaceae~ Treat, data = qpcr_cz)

pairwise.wilcox.test(qpcr_cz $ Rhizobiaceae, qpcr_cz $Treat, p.adj = "bonferroni",
exact=FALSE)

# Gilliamella

#anova All

shapiro.test(qpcr_cz$Gilliamella) #not signifcant, data are normal

#kruskal.test

kruskal.test(Gilliamella~ Treat, data = qpcr_cz)

pairwise.wilcox.test(qpcr_cz $ Gilliamella, qpcr_cz $Treat, p.adj = "bonferroni",
exact=FALSE)

# Tyzzerella

#anova All

shapiro.test(qpcr_cz$Tyzzerella) #not signifcant, data are normal

#kruskal.test
```

```
kruskal.test(Tyzzerella~ Treat, data = qpcr_cz)

pairwise.wilcox.test(qpcr_cz $ Tyzzerella, qpcr_cz $Treat, p.adj = "bonferroni",
exact=FALSE)

# Streptomyces

#anova All

shapiro.test(qpcr_cz$Streptomyces) #not signifcant, data are normal

#kruskal.test

kruskal.test(Streptomyces~ Treat, data = qpcr_cz)

pairwise.wilcox.test(qpcr_cz $ Streptomyces, qpcr_cz $Treat, p.adj = "bonferroni",
exact=FALSE)

# Commensalibacter

#anova All

shapiro.test(qpcr_cz$Commensalibacter) #not signifcant, data are normal

#kruskal.test

kruskal.test(Commensalibacter~ Treat, data = qpcr_cz)

pairwise.wilcox.test(qpcr_cz $ Commensalibacter, qpcr_cz $Treat, p.adj = "bonferroni",
exact=FALSE)

# Enterobacteriaceae

#anova All

shapiro.test(qpcr_cz$Enterobacteriaceae) #not signifcant, data are normal

#kruskal.test

kruskal.test(Enterobacteriaceae~ Treat, data = qpcr_cz)

pairwise.wilcox.test(qpcr_cz $ Enterobacteriaceae, qpcr_cz $Treat, p.adj = "bonferroni",
exact=FALSE)

# Proteobacteria

#anova All
```

```
shapiro.test(qpcr_cz$Proteobacteria) #not signifcant, data are normal

#kruskal.test

kruskal.test(Proteobacteria~ Treat, data = qpcr_cz)

pairwise.wilcox.test(qpcr_cz $ Proteobacteria, qpcr_cz $Treat, p.adj = "bonferroni",
exact=FALSE)

#LOG for plots

par(mfrow = c(1,1), oma = c(2,1,1,1))

boxplot(bacterial_DNA_concentration ~ Treat, data=qpcr_log, cex = 1,  main=" Bacterial
DNA Concentration (ng/ul)", xlab="Treatments", ylab="Bacterial DNA Concentration
(ng/ul)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(percent_bacterial_DNA ~ Treat, data=qpcr_log, cex = 1,  main=" Bacterial DNA
Concentration (ng/ul)", xlab="Treatments", ylab="Percent Bacterial DNA", col =
c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Absolute_cell_counts_per_sample ~ Treat, data=qpcr_log, cex = 1,  main=" Absolute
Cell Counts", xlab="Treatments", ylab="Absolute Cell Counts", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(sixteen_s_raw_reads ~ Treat, data=qpcr_log, cex = 1,  main=" Sixteen S raw reads",
xlab="Treatments", ylab="Raw reads", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(sixteen_s_denoised_reads ~ Treat, data=qpcr_log, cex = 1,  main=" sixteen S
denoised reads", xlab="Treatments", ylab="Raw reads", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#Absolute abundance of the microbe ()

par(mfrow = c(2,4), oma = c(2,1,1,1))

#Core

boxplot(Lactobacillus ~ Treat, data=qpcr_log, cex = 1,  main=" Lactobacillus",
xlab="Treatments", ylab="Absolute Abundance of Lactobacillus", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))
```

```
boxplot(Bifidobacterium ~ Treat, data=qpcr_log, cex = 1,  main="Bifidobacterium",
xlab="Treatments", ylab="Absolute Abundance of Bifidobacterium", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Commensalibacter ~ Treat, data=qpcr_log, cex = 1,  main=" Commensalibacter",
xlab="Treatments", ylab="Absolute Abundance of Commensalibacter", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Rhizobiaceae ~ Treat, data=qpcr_log, cex = 1,  main=" Rhizobiaceae",
xlab="Treatments", ylab="Absolute Abundance of Rhizobiaceae", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#Ancom

par(mfrow = c(2,3), oma = c(2,1,1,1))

boxplot(Tyzzerella ~ Treat, data=qpcr_log, cex = 1,  main=" Tyzzerella", xlab="Treatments",
ylab="Absolute Abundance of Tyzzerella", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Streptomyces ~ Treat, data=qpcr_log, cex = 1,  main=" Streptomyces",
xlab="Treatments", ylab="Absolute Abundance of Streptomyces", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Paenibacillus ~ Treat, data=qpcr_log, cex = 1,  main=" Paenibacillus",
xlab="Treatments", ylab="Absolute Abundance of Paenibacillus", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Gilliamella ~ Treat, data=qpcr_log, cex = 1,  main=" Gilliamella",
xlab="Treatments", ylab="Absolute Abundance of Gilliamella", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Enterobacteriaceae ~ Treat, data=qpcr_log, cex = 1,  main=" Enterobacteriaceae",
xlab="Treatments", ylab="Absolute Abundance of Enterobacteriaceae", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Proteobacteria ~ Treat, data=qpcr_log, cex = 1,  main=" Proteobacteria",
xlab="Treatments", ylab="Absolute Abundance of Proteobacteria", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))
```

```
#Only all ANCOM

par(mfrow = c(2,5), oma = c(2,1,1,1))

boxplot(Lactobacillus ~ Treat, data=qpcr_log, cex = 1,  main="A) Lactobacillus",
xlab="Treatments", ylab="Absolute Abundance of Lactobacillus", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Bifidobacterium ~ Treat, data=qpcr_log, cex = 1,  main="Bifidobacterium",
xlab="Treatments", ylab="Absolute Abundance of Bifidobacterium", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Rhizobiaceae ~ Treat, data=qpcr_log, cex = 1,  main=" Rhizobiaceae",
xlab="Treatments", ylab="Absolute Abundance of Rhizobiaceae", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Gilliamella ~ Treat, data=qpcr_log, cex = 1,  main=" Gilliamella",
xlab="Treatments", ylab="Absolute Abundance of Gilliamella", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Tyzzerella ~ Treat, data=qpcr_log, cex = 1,  main=" Tyzzerella", xlab="Treatments",
ylab="Absolute Abundance of Tyzzerella", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Streptomyces ~ Treat, data=qpcr_log, cex = 1,  main=" Streptomyces",
xlab="Treatments", ylab="Absolute Abundance of Streptomyces", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Paenibacillus ~ Treat, data=qpcr_log, cex = 1,  main=" Paenibacillus",
xlab="Treatments", ylab="Absolute Abundance of Paenibacillus", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Enterobacteriaceae ~ Treat, data=qpcr_log, cex = 1,  main=" Enterobacteriaceae",
xlab="Treatments", ylab="Absolute Abundance of Enterobacteriaceae", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Proteobacteria ~ Treat, data=qpcr_log, cex = 1,  main=" Proteobacteria",
xlab="Treatments", ylab="Absolute Abundance of Proteobacteria", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))
```

```
#only 1c 1 control

#importdata

reads_cz_1C <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/reads_cz_1C.csv")

View(reads_cz_1C)

#Diversity

#Shannon

shapiro.test(reads_cz_1C$shannon) #not signifcant, data are normal

#ANOVA

anova <- aov(shannon ~ Treat, data= reads_cz_1C)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

#Pielou

shapiro.test(reads_cz_1C$pielou) #not signifcant, data are normal

#ANOVA

anova <- aov(pielou ~ Treat, data= reads_cz_1C)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

# Faith

#anova All

shapiro.test(reads_cz_1C$faith) # signifcant, data are not normal

#kruskal.test

kruskal.test(faith~ Treat, data = reads_cz_1C)
```

```
pairwise.wilcox.test(reads_cz_1C $ faith, reads_cz_1C $Treat, p.adj = "bonferroni", exact=FALSE)

# Observed_otus

#anova All

shapiro.test(reads_cz_1C$observed_otus) #signifcant, data are not normal

#kruskal.test

kruskal.test(observed_otus~ Treat, data = reads_cz_1C)

pairwise.wilcox.test(reads_cz_1C$observed_otus,reads_cz_1C$Treat, p.adj = "bonferroni", exact=FALSE)

#Box plots 1C

#Diversity

par(mfrow = c(1,2), oma = c(2,1,1,1))

boxplot(shannon ~ Treat, data=reads_cz_1C, cex = 1,main="Shannon's Index",xlab="Treatments",pairwise.wilcox.test, ylab="Shannon's Index", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(faith ~ Treat, data=reads_cz_1C, cex = 1,main="Faith's PD",xlab="Treatments", ylab="Faith's PD", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

par(mfrow = c(1,2), oma = c(2,1,1,1))

boxplot(observed_otus ~ Treat, data=reads_cz_1C, cex = 1, main="Observed Features",xlab="Treatments", ylab="Observed Features", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(pielou ~ Treat, data=reads_cz_1C, cex = 1,main="Pielou's Evenness Index",xlab="Treatments", ylab="Pielou's Evenness Index", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

#Relative Abundances C1

# Lactobacillus

shapiro.test(reads_cz_1C$Lactobacillus) #not signifcant, data are normal
```

```
kruskal.test(Lactobacillus ~ Treat, data = reads_cz_1C)

pairwise.wilcox.test(reads_cz_1C$Lactobacillus, reads_cz_1C$Treat, paired= FALSE, p.adj = "bonferroni", exact=FALSE)

# Bifidobacterium

shapiro.test(reads_cz_1C$Bifidobacterium) #not signifcant, data are normal

kruskal.test(Bifidobacterium ~ Treat, data = reads_cz_1C)

pairwise.wilcox.test(reads_cz_1C$Bifidobacterium, reads_cz_1C$Treat, paired= FALSE, p.adj = "bonferroni", exact=FALSE)

# Rhizobiaceae

shapiro.test(reads_cz_1C$Rhizobiaceae) #not signifcant, data are normal

kruskal.test(Rhizobiaceae~ Treat, data = reads_cz_1C)

pairwise.wilcox.test(reads_cz_1C$Rhizobiaceae, reads_cz_1C$Treat, paired= FALSE, p.adj = "bonferroni", exact=FALSE)

# Gilliamella

shapiro.test(reads_cz_1C$Gilliamella) #not signifcant, data are normal

kruskal.test(Gilliamella~ Treat, data = reads_cz_1C)

pairwise.wilcox.test(reads_cz_1C$Gilliamella, reads_cz_1C$Treat, paired= FALSE, p.adj = "bonferroni", exact=FALSE)

# Tyzzerella

shapiro.test(reads_cz_1C$Tyzzerella) #not signifcant, data are normal

kruskal.test(Tyzzerella ~ Treat, data=reads_cz_1C)

pairwise.wilcox.test(reads_cz_1C $ Tyzzerella, reads_cz_1C $Treat, p.adj = "bonferroni", exact=FALSE)

# Streptomyces

shapiro.test(reads_cz_1C$Streptomyces) #not signifcant, data are normal

kruskal.test(Streptomyces ~ Treat, data = reads_cz_1C)
```

```
pairwise.wilcox.test(reads_cz_1C$Streptomyces, reads_cz_1C$Treat, paired= FALSE, p.adj = "bonferroni", exact=FALSE)

# Paenibacillus

shapiro.test(reads_cz_1C$Paenibacillus) #not signifcant, data are normal

kruskal.test(Paenibacillus ~ Treat, data = reads_cz_1C)

pairwise.wilcox.test(reads_cz_1C$Paenibacillus, reads_cz_1C$Treat, paired= FALSE, p.adj = "bonferroni", exact=FALSE)

# Enterobacteriaceae

shapiro.test(reads_cz_1C$Enterobacteriaceae) #not signifcant, data are normal

kruskal.test(Enterobacteriaceae~ Treat, data = reads_cz_1C)

pairwise.wilcox.test(reads_cz_1C$Enterobacteriaceae, reads_cz_1C$Treat, paired= FALSE, p.adj = "bonferroni", exact=FALSE)

# Proteobacteria

shapiro.test(reads_cz_1C$Proteobacteria) #not signifcant, data are normal

kruskal.test(Proteobacteria~ Treat, data = reads_cz_1C)

pairwise.wilcox.test(reads_cz_1C$Proteobacteria, reads_cz_1C$Treat, paired= FALSE, p.adj = "bonferroni", exact=FALSE)

#Relative abundances 1C

#Only all ANCOM

par(mfrow = c(2,5), oma = c(2,1,1,1))

boxplot(Lactobacillus ~ Treat, data=reads_cz_1C, cex = 1,main=" Total Lactobacillus",xlab="Treatments",pairwise.wilcox.test, ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Bifidobacterium ~ Treat, data=reads_cz_1C, cex = 1, main=" Total Bifidobacterium",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))
```

```
boxplot(Rhizobiaceae ~ Treat, data=reads_cz_1C, cex = 1,main=" Total Rhizobiaceae",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Gilliamella ~ Treat, data=reads_cz_1C, cex = 1,main=" Total Gilliamella",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Tyzzerella ~ Treat, data=reads_cz_1C, cex = 1, main=" Total Tyzzerella",xlab="Treatments", ylab="Relative abundance (%)",col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Streptomyces ~ Treat, data=reads_cz_1C, cex = 1,main=" Total Streptomyces",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Paenibacillus ~ Treat, data=reads_cz_1C, cex = 1,main=" Total Paenibacillus",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Enterobacteriaceae ~ Treat, data=reads_cz_1C, cex = 1,main=" Total Enterobacteriaceae",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Proteobacteria ~ Treat, data=reads_cz_1C, cex = 1,main=" Total Proteobacteria",xlab="Treatments", ylab="Relative abundance (%)", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

#importdata

#Absolute Abundances and Diversity (reads_cz_1C)

qpcr_cz_1C <- read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_cz_1C.csv")

View(qpcr_cz_1C)

# Lactobacillus

shapiro.test(qpcr_cz_1C$Lactobacillus) #not signifcant, data are normal
```

```
kruskal.test(Lactobacillus ~ Treat, data = qpcr_cz_1C)

pairwise.wilcox.test(qpcr_cz_1C$Lactobacillus, qpcr_cz_1C$Treat, paired= FALSE, p.adj =
"bonferroni", exact=FALSE)

# Bifidobacterium

shapiro.test(qpcr_cz_1C$Bifidobacterium) #not signifcant, data are normal

kruskal.test(Bifidobacterium ~ Treat, data = qpcr_cz_1C)

pairwise.wilcox.test(qpcr_cz_1C$Bifidobacterium, qpcr_cz_1C$Treat, paired= FALSE, p.adj
= "bonferroni", exact=FALSE)

# Rhizobiaceae

shapiro.test(qpcr_cz_1C$Rhizobiaceae) #not signifcant, data are normal

kruskal.test(Rhizobiaceae~ Treat, data = qpcr_cz_1C)

pairwise.wilcox.test(qpcr_cz_1C$Rhizobiaceae, qpcr_cz_1C$Treat, paired= FALSE, p.adj =
"bonferroni", exact=FALSE)

# Gilliamella

shapiro.test(qpcr_cz_1C$Gilliamella) #not signifcant, data are normal

kruskal.test(Gilliamella~ Treat, data = qpcr_cz_1C)

pairwise.wilcox.test(qpcr_cz_1C$Gilliamella, qpcr_cz_1C$Treat, paired= FALSE, p.adj =
"bonferroni", exact=FALSE)

# Tyzzerella

shapiro.test(qpcr_cz_1C$Tyzzerella) #not signifcant, data are normal

kruskal.test(Tyzzerella ~ Treat, data=qpcr_cz_1C)

pairwise.wilcox.test(qpcr_cz_1C $ Tyzzerella, qpcr_cz_1C $Treat, p.adj = "bonferroni",
exact=FALSE)

# Streptomyces

shapiro.test(qpcr_cz_1C$Streptomyces) #not signifcant, data are normal

kruskal.test(Streptomyces ~ Treat, data = qpcr_cz_1C)
```

```
pairwise.wilcox.test(qpcr_cz_1C$Streptomyces, qpcr_cz_1C$Treat, paired= FALSE, p.adj = "bonferroni", exact=FALSE)

# Paenibacillus

shapiro.test(qpcr_cz_1C$Paenibacillus) #not signifcant, data are normal

kruskal.test(Paenibacillus ~ Treat, data = qpcr_cz_1C)

pairwise.wilcox.test(qpcr_cz_1C$Paenibacillus, qpcr_cz_1C$Treat, paired= FALSE, p.adj = "bonferroni", exact=FALSE)

# Enterobacteriaceae

shapiro.test(qpcr_cz_1C$Enterobacteriaceae) #not signifcant, data are normal

kruskal.test(Enterobacteriaceae~ Treat, data = qpcr_cz_1C)

pairwise.wilcox.test(qpcr_cz_1C$Enterobacteriaceae, qpcr_cz_1C$Treat, paired= FALSE, p.adj = "bonferroni", exact=FALSE)

# Proteobacteria

shapiro.test(qpcr_cz_1C$Proteobacteria) #not signifcant, data are normal

kruskal.test(Proteobacteria~ Treat, data = qpcr_cz_1C)

pairwise.wilcox.test(qpcr_cz_1C$Proteobacteria, qpcr_cz_1C$Treat, paired= FALSE, p.adj = "bonferroni", exact=FALSE)

#boxplots absolute abundance 1C

#Only all ANCOM

par(mfrow = c(2,5), oma = c(2,1,1,1))

boxplot(Lactobacillus ~ Treat, data=qpcr_cz_1C, cex = 1,  main="A) Lactobacillus", xlab="Treatments", ylab="Absolute Abundance of Lactobacillus", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Bifidobacterium ~ Treat, data=qpcr_cz_1C, cex = 1,  main="B) Bifidobacterium", xlab="Treatments", ylab="Absolute Abundance of Bifidobacterium", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))
```

```
boxplot(Rhizobiaceae ~ Treat, data=qpcr_cz_1C, cex = 1,  main=" C) Rhizobiaceae",
xlab="Treatments", ylab="Absolute Abundance of Rhizobiaceae", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Gilliamella ~ Treat, data=qpcr_cz_1C, cex = 1,  main=" D) Gilliamella",
xlab="Treatments", ylab="Absolute Abundance of Gilliamella", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Tyzzerella ~ Treat, data=qpcr_cz_1C, cex = 1,  main=" E) Tyzzerella",
xlab="Treatments", ylab="Absolute Abundance of Tyzzerella", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Streptomyces ~ Treat, data=qpcr_cz_1C, cex = 1,  main=" F) Streptomyces",
xlab="Treatments", ylab="Absolute Abundance of Streptomyces", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Paenibacillus ~ Treat, data=qpcr_cz_1C, cex = 1,  main=" G) Paenibacillus",
xlab="Treatments", ylab="Absolute Abundance of Paenibacillus", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Enterobacteriaceae ~ Treat, data=qpcr_cz_1C, cex = 1,  main=" H)
Enterobacteriaceae", xlab="Treatments", ylab="Absolute Abundance of Enterobacteriaceae",
col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Proteobacteria ~ Treat, data=qpcr_cz_1C, cex = 1,  main=" I) Proteobacteria",
xlab="Treatments", ylab="Absolute Abundance of Proteobacteria", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#Absolute Abundances and Diversity (qpcr_log_1C)

qpcr_log_1C <-
read_csv("C:/Users/kilme/OneDrive/Documentos/DOUTORADO/PRINT/ESTUDY/COmput
ationalanalises/Zinc/R_zinc/importing_tables/qpcr_log_1C.csv")

View(qpcr_log_1C)

boxplot(Lactobacillus ~ Treat, data=qpcr_log_1C, cex = 1,  main="A) Lactobacillus",
xlab="Treatments", ylab="Absolute Abundance of Lactobacillus", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))
```

```
boxplot(Bifidobacterium ~ Treat, data=qpcr_log_1C, cex = 1,  main="B) Bifidobacterium", xlab="Treatments", ylab="Absolute Abundance of Bifidobacterium", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Rhizobiaceae ~ Treat, data=qpcr_log_1C, cex = 1,  main=" C) Rhizobiaceae", xlab="Treatments", ylab="Absolute Abundance of Rhizobiaceae", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Gilliamella ~ Treat, data=qpcr_log_1C, cex = 1,  main=" D) Gilliamella", xlab="Treatments", ylab="Absolute Abundance of Gilliamella", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Tyzzerella ~ Treat, data=qpcr_log_1C, cex = 1,  main=" E) Tyzzerella", xlab="Treatments", ylab="Absolute Abundance of Tyzzerella", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Streptomyces ~ Treat, data=qpcr_log_1C, cex = 1,  main=" F) Streptomyces", xlab="Treatments", ylab="Absolute Abundance of Streptomyces", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Paenibacillus ~ Treat, data=qpcr_log_1C, cex = 1,  main=" G) Paenibacillus", xlab="Treatments", ylab="Absolute Abundance of Paenibacillus", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Enterobacteriaceae ~ Treat, data=qpcr_log_1C, cex = 1,  main=" H) Enterobacteriaceae", xlab="Treatments", ylab="Absolute Abundance of Enterobacteriaceae", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Proteobacteria ~ Treat, data=qpcr_log_1C, cex = 1,  main=" I) Proteobacteria", xlab="Treatments", ylab="Absolute Abundance of Proteobacteria", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

#taxa sem pt ABsolute

library(readr)

qpcr_cz_NOPT <-
read_delim("~/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_cz_NO_PT.txt",
```

```
"\t", escape_double = FALSE, trim_ws = TRUE)

View(qpcr_cz_NO_PT)

#absolute abudances of the microbes SEM CONSIDERAR OS PTS NAS STATISTICAS###################################

#Lactobacillus

#Rhizobiaceae

#Enterobacteriaceae

#Gilliamella

#Paenibacillus

#Microbacteriaceae

# Lactobacillus

#anova All

shapiro.test(qpcr_cz_NOPT$Lactobacillus) #not signifcant, data are normal

#kruskal.test

kruskal.test(Lactobacillus~ Treat, data = qpcr_cz_NOPT)

pairwise.wilcox.test(qpcr_cz_NOPT $ Lactobacillus, qpcr_cz_NOPT $Treat, p.adj = "bonferroni", exact=FALSE)

# Rhizobiaceae

#anova All

shapiro.test(qpcr_cz_NOPT$Rhizobiaceae) #not signifcant, data are normal

#kruskal.test

kruskal.test(Rhizobiaceae~ Treat, data = qpcr_cz_NOPT)

pairwise.wilcox.test(qpcr_cz_NOPT $ Rhizobiaceae, qpcr_cz_NOPT $Treat, p.adj = "bonferroni", exact=FALSE)

# Enterobacteriaceae
```

```
#anova All

shapiro.test(qpcr_cz_NOPT$Enterobacteriaceae) #not signifcant, data are normal

#kruskal.test

kruskal.test(Enterobacteriaceae~ Treat, data = qpcr_cz_NOPT)

pairwise.wilcox.test(qpcr_cz_NOPT $ Enterobacteriaceae, qpcr_cz_NOPT $Treat, p.adj =
"bonferroni", exact=FALSE)

# Gilliamella

#anova All

shapiro.test(qpcr_cz_NOPT$Gilliamella) #not signifcant, data are normal

#kruskal.test

kruskal.test(Gilliamella~ Treat, data = qpcr_cz_NOPT)

pairwise.wilcox.test(qpcr_cz_NOPT $ Gilliamella, qpcr_cz_NOPT $Treat, p.adj =
"bonferroni", exact=FALSE)

# Paenibacillus

#anova All

shapiro.test(qpcr_cz_NOPT$Paenibacillus) #not signifcant, data are normal

#kruskal.test

kruskal.test(Paenibacillus~ Treat, data = qpcr_cz_NOPT)

pairwise.wilcox.test(qpcr_cz_NOPT $ Paenibacillus, qpcr_cz_NOPT $Treat, p.adj =
"bonferroni", exact=FALSE)

# Microbacteriaceae

#anova All

shapiro.test(qpcr_cz_NOPT$Microbacteriaceae) #not signifcant, data are normal

#kruskal.test

kruskal.test(Microbacteriaceae~ Treat, data = qpcr_cz_NOPT)
```

```
pairwise.wilcox.test(qpcr_cz_NOPT $ Microbacteriaceae, qpcr_cz_NOPT $Treat, p.adj =
"bonferroni", exact=FALSE)

#Barplots

library(readr)

qpcr_cz_NOPT_log <-
read_delim("~/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/import
ing_tables/qpcr_cz_NOPT_log.txt",
                    "\t", escape_double = FALSE, trim_ws = TRUE)

View(qpcr_cz_NOPT_log)

par(mfrow = c(2,3), oma = c(2,1,1,1))

boxplot(Lactobacillus ~ Treat, data=qpcr_cz_NOPT_log, cex = 1,  main="A) Lactobacillus",
xlab="Treatments", ylab="Absolute Abundance of Lactobacillus", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Rhizobiaceae ~ Treat, data=qpcr_cz_NOPT_log, cex = 1,  main=" B) Rhizobiaceae",
xlab="Treatments", ylab="Absolute Abundance of Rhizobiaceae", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Enterobacteriaceae ~ Treat, data=qpcr_cz_NOPT_log, cex = 1,  main=" C)
Enterobacteriaceae", xlab="Treatments", ylab="Absolute Abundance of Enterobacteriaceae",
col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Gilliamella ~ Treat, data=qpcr_cz_NOPT_log, cex = 1,  main=" D) Gilliamella",
xlab="Treatments", ylab="Absolute Abundance of Gilliamella", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Paenibacillus ~ Treat, data=qpcr_cz_NOPT_log, cex = 1,  main=" E) Paenibacillus",
xlab="Treatments", ylab="Absolute Abundance of Paenibacillus", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(Microbacteriaceae ~ Treat, data=qpcr_cz_NOPT_log, cex = 1,  main="F)
Microbacteriaceae", xlab="Treatments", ylab="Absolute Abundance of Microbacteriaceae",
col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

###oct7 (PT5,Con5,zinc5) and all bees that emerged on oct11( Pt100, Con100, Zinc100)
```

```
library(readr)

qpcr_cz_ED <-
read_delim("~/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_cz_ED.txt",
                   delim = "\t", escape_double = FALSE,
                   trim_ws = TRUE)

View(qpcr_cz_ED)

qpcr_log_ED <-
read_delim("~/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_log_ED.txt",
                  delim = "\t", escape_double = FALSE,
                  trim_ws = TRUE)

View(qpcr_log_ED)

par(mfrow = c(1,2), oma = c(2,1,1,1))

# Bacterial DNA Concentration (ng/ul)

#anova All

shapiro.test(qpcr_cz_ED$bacterial_DNA_concentration) #not signifcant, data are normal

#kruskal.test

kruskal.test(bacterial_DNA_concentration ~ Treat_ED, data = qpcr_cz_ED)

pairwise.wilcox.test(qpcr_cz_ED $ bacterial_DNA_concentration, qpcr_cz_ED $Treat_ED,
p.adj = "bonferroni")

boxplot(bacterial_DNA_concentration ~ Treat_ED, data= qpcr_log_ED, cex = 1,
main="Bacterial DNA Concentration (ng/ul)", xlab="Treatments", ylab="Bacterial DNA
Concentration (ng/ul) ", col = c("#ccff66","#00CC00", "#006600"))

# # Absolute Cell Counts

#anova All
```

```
shapiro.test(qpcr_cz_ED$Absolute_cell_counts_per_sample) #not signifcant, data are normal

#kruskal.test

kruskal.test(Absolute_cell_counts_per_sample~ Treat_ED, data = qpcr_cz_ED)

pairwise.wilcox.test(qpcr_cz_ED $ Absolute_cell_counts_per_sample, qpcr_cz_ED
$Treat_ED, p.adj = "bonferroni")

boxplot(Absolute_cell_counts_per_sample ~ Treat_ED, data= qpcr_log_ED, cex = 1,  main="
Absolute Cell Counts", xlab="Treatments", ylab="Absolute Cell Counts", col =
c("#ccff66","#00CC00", "#006600"))

###PT vs PT5 vs PT100

par(mfrow = c(1,2), oma = c(2,1,1,1))

# Bacterial DNA Concentration (ng/ul)

#anova All

shapiro.test(qpcr_cz_ED$bacterial_DNA_concentration) #not signifcant, data are normal

#kruskal.test

kruskal.test(bacterial_DNA_concentration~ Abrev, data = qpcr_cz_ED)

pairwise.wilcox.test(qpcr_cz_ED $ bacterial_DNA_concentration, qpcr_cz_ED $Abrev, p.adj
= "bonferroni")

boxplot(bacterial_DNA_concentration ~ Abrev, data= qpcr_cz_ED, cex = 1,  main="Bacterial
DNA Concentration (ng/ul)", xlab="Treatments", ylab="Bacterial DNA Concentration (ng/ul)
", col = c("#ccff66","#00CC00", "#006600"))

# # Absolute Cell Counts

#anova All

shapiro.test(qpcr_cz_ED$Absolute_cell_counts_per_sample) #not signifcant, data are normal

#kruskal.test

kruskal.test(Absolute_cell_counts_per_sample~ Abrev, data = qpcr_cz_ED)
```

```
pairwise.wilcox.test(qpcr_cz_ED $ Absolute_cell_counts_per_sample, qpcr_cz_ED $Abrev, p.adj = "bonferroni")

boxplot(Absolute_cell_counts_per_sample ~ Abrev, data= qpcr_cz_ED, cex = 1,  main=" Absolute Cell Counts", xlab="Treatments", ylab="Absolute Cell Counts", col = c("#ccff66","#00CC00", "#006600"))

###qpcr PTs

qpcr_pt<-
read_delim("~/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/qpcr_pt.txt",
                delim = "\t", escape_double = FALSE,
                trim_ws = TRUE)

View(qpcr_pt)

par(mfrow = c(1,2), oma = c(2,1,1,1))

# Bacterial DNA Concentration (ng/ul)

#anova All

shapiro.test(qpcr_pt$bacterial_DNA_concentration) #not signifcant, data are normal

#kruskal.test

kruskal.test(bacterial_DNA_concentration~ Abrev, data = qpcr_pt)

pairwise.wilcox.test(qpcr_pt$ bacterial_DNA_concentration, qpcr_pt $Abrev, p.adj = "bonferroni")

boxplot(bacterial_DNA_concentration ~ Abrev, data= qpcr_pt, cex = 1,  main="C) Bacterial DNA Concentration (ng/ul)", xlab="Treatments", ylab="Bacterial DNA Concentration (ng/ul) ", col = c("#ccff66","#00CC00", "#006600"))

# # Absolute Cell Counts

#anova All

shapiro.test(qpcr_pt $Absolute_cell_counts_per_sample) #not signifcant, data are normal

#kruskal.test
```

```
kruskal.test(Absolute_cell_counts_per_sample~ Abrev, data = qpcr_pt)

pairwise.wilcox.test(qpcr_pt $ Absolute_cell_counts_per_sample, qpcr_pt $Abrev, p.adj =
"bonferroni")

boxplot(Absolute_cell_counts_per_sample ~ Abrev, data= qpcr_pt, cex = 1,  main="D)
Absolute Cell Counts", xlab="Treatments", ylab="Absolute Cell Counts", col =
c("#ccff66","#00CC00", "#006600"))

#Diversity PT separado

library(readr)

reads_pt <-
read_delim("~/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/import
ing_tables/reads_pt.txt",
                   "\t", escape_double = FALSE, trim_ws = TRUE)

View(reads_pt)

# Shannon

#anova All

shapiro.test(reads_pt$shannon) #not signifcant, data are normal

anova <- aov(shannon ~ Treat, data= reads_pt)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

boxplot(shannon ~ Treat, data=reads_pt, cex = 1, ylim = c(0.5, 5), main=" Shannon's Index",
xlab="Treatments", ylab="Shannon Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

# Pielou

#anova All

shapiro.test(reads_pt$pielou) #not signifcant, data are normal

#kruskal.test
```

```
#kruskal.test(pielou~ Treat, data = reads_pt)

#pairwise.wilcox.test(reads_pt $ pielou, reads_pt $Treat, pp.adj = "bonferroni",
exact=FALSE)

anova <- aov(pielou ~ Treat, data= reads_pt)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

boxplot(pielou ~ Treat, data=reads_pt, cex = 1, ylim = c(0, 0.7), main="Pielou's Evenness
Index",xlab="Treatments", ylab="Pielou's Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#boxplots with pt separetely

par(mfrow = c(2,2), oma = c(2,1,1,1))

boxplot(shannon ~ Treat, data=reads_cz, cex = 1, ylim = c(0.5, 5), main=" A) Shannon's
Index", xlab="Treatments", ylab="Shannon Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(pielou ~ Treat, data=reads_cz, cex = 1, ylim = c(0, 0.7), main="B) Pielou's Evenness
Index",xlab="Treatments", ylab="Pielou's Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(shannon ~ Treat, data=reads_pt, cex = 1,  main="C) Shannon`s index",
xlab="Treatments", ylab="Shannon`s index", col = c("#ccff66","#00CC00", "#006600"))

boxplot(pielou ~ Treat, data=reads_pt, cex = 1,  main="D) Pielou`s Evenness index",
xlab="Treatments", ylab="Pielou`s index", col = c("#ccff66","#00CC00", "#006600"))

# Faith

#anova All

shapiro.test(reads_pt$Faith) #not signifcant, data are normal

anova <- aov(Faith ~ Treat, data= reads_pt)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)
```

```
boxplot(Faith ~ Treat, data=reads_pt, cex = 1, ylim = c(0.5, 5), main=" Faith's PD Index",
xlab="Treatments", ylab=" Faith's PD Index ", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

# Observed_otus

#anova All

shapiro.test(reads_pt$observed_otus) #not signifcant, data are normal

#kruskal.test

#kruskal.test(observed_otus~ Treat, data = reads_pt)

#pairwise.wilcox.test(reads_pt $ observed_otus, reads_pt $Treat, pp.adj = "bonferroni",
exact=FALSE)

anova <- aov(observed_otus ~ Treat, data= reads_pt)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

boxplot(observed_otus ~ Treat, data=reads_pt, cex = 1, ylim = c(0, 0.7), main="Observed
Features",xlab="Treatments", ylab="Pielou's Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#boxplots with pt separetely

par(mfrow = c(2,2), oma = c(2,1,1,1))

boxplot(faith ~ Treat, data=reads_cz, cex = 1, main=" A) Faith's PD ", xlab="Treatments",
ylab=" Faith's PD Index ", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(observed_otus ~ Treat, data=reads_cz, cex = 1, main="B) Observed
Features",xlab="Treatments", ylab="Observed Features", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(faith ~ Treat, data=reads_pt, cex = 1,  main="C) Faith`s PD", xlab="Treatments",
ylab="Faith`s PD index", col = c("#ccff66","#00CC00", "#006600"))

boxplot(observed_otus ~ Treat, data=reads_pt, cex = 1,  main="D) Observed Features",
xlab="Treatments", ylab="Observed Features", col = c("#ccff66","#00CC00", "#006600"))
```

###Diversity on October 7 (PT 5, CON 5, ZINC 5) to all bees that emerged on October 11 (PT 100, CON 100, ZINC 100).

```
# Shannon

#anova All

shapiro.test(reads_cz$shannon) #not signifcant, data are normal

anova <- aov(shannon ~ Treat_ED, data= reads_cz)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

boxplot(shannon ~ Treat_ED, data=reads_cz, cex = 1, ylim = c(0.5, 5), main=" Shannon's Index", xlab="Treatments", ylab="Shannon Index", col = c("#ccff66","#00CC00", "#006600"))

# Pielou

#anova All

shapiro.test(reads_cz$pielou) #not signifcant, data are normal

#kruskal.test

#kruskal.test(pielou~ Treat_ED, data = reads_cz)

#pairwise.wilcox.test(reads_cz $ pielou, reads_cz $Treat_ED, pp.adj = "bonferroni", exact=FALSE)

anova <- aov(pielou ~ Treat_ED, data= reads_cz)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

boxplot(pielou ~ Treat_ED, data=reads_cz, cex = 1, ylim = c(0, 0.7), main="Pielou's Evenness Index",xlab="Treatments", ylab="Pielou's Index",  col = c("#ccff66","#00CC00", "#006600"))

===par(mfrow = c(2,2), oma = c(2,1,1,1))
```

```
boxplot(shannon ~ Treat_ED, data=reads_cz, cex = 1, ylim = c(0.5, 5), main=" A) Shannon's Index", xlab="Treatments", ylab="Shannon Index",  col = c("#ccff66","#00CC00", "#006600"))

boxplot(pielou ~ Treat_ED, data=reads_cz, cex = 1, ylim = c(0, 0.7), main="B) Pielou's Evenness Index",xlab="Treatments", ylab="Pielou's Index",  col = c("#ccff66","#00CC00", "#006600"))

boxplot(shannon ~ Treat_ED, data=reads_cz, cex = 1,  main="C) Shannon`s index", xlab="Treatments", ylab="Shannon`s index", col = c("#ccff66","#00CC00", "#006600"))

boxplot(pielou ~ Treat_ED, data=reads_cz, cex = 1,  main="D) Pielou`s Evenness index", xlab="Treatments", ylab="Pielou`s index", col = c("#ccff66","#00CC00", "#006600"))

# Faith

#anova All

shapiro.test(reads_cz$Faith) #not signifcant, data are normal

anova <- aov(Faith ~ Treat_ED, data= reads_cz)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

boxplot(faith ~ Treat_ED, data=reads_cz, cex = 1, ylim = c(0.5, 5), main=" Faith's PD Index", xlab="Treatments", ylab=" Faith's PD Index ", col = c("#ccff66","#00CC00", "#006600"))

# Observed_otus

#anova All

shapiro.test(reads_cz$observed_otus) #not signifcant, data are normal

#kruskal.test

#kruskal.test(observed_otus~ Treat_ED, data = reads_cz)

#pairwise.wilcox.test(reads_cz $ observed_otus, reads_cz $Treat_ED, pp.adj = "bonferroni", exact=FALSE)

anova <- aov(observed_otus ~ Treat_ED, data= reads_cz)

summary(anova)
```

```
tukey <- LTukey(anova, which="Treat", conf.level=0.95)

boxplot(observed_otus ~ Treat_ED, data=reads_cz, cex = 1, ylim = c(0, 0.7),
main="Observed Features",xlab="Treatments", ylab="Pielou's Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

par(mfrow = c(2,2), oma = c(2,1,1,1))

boxplot(faith ~ Treat_ED, data=reads_cz, cex = 1, main=" A) Faith's PD ",
xlab="Treatments", ylab=" Faith's PD Index ", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(observed_otus ~ Treat_ED, data=reads_cz, cex = 1, main="B) Observed
Features",xlab="Treatments", ylab="Observed Features", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(faith ~ Treat_ED, data=reads_cz, cex = 1,  main="C) Faith`s PD",
xlab="Treatments", ylab="Faith`s PD index", col = c("#ccff66","#00CC00", "#006600"))

boxplot(observed_otus ~ Treat_ED, data=reads_cz, cex = 1,  main="D) Observed Features",
xlab="Treatments", ylab="Observed Features", col = c("#ccff66","#00CC00", "#006600"))

#EDE PT (Cell cont)

library(readr)

edf_pt <-
read_delim("~/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/import
ing_tables/ede_pt.txt",

                "\t", escape_double = FALSE, trim_ws = TRUE)

View(edf_pt)

# Shannon

#anova All

shapiro.test(edf_pt$shannon) #not signifcant, data are normal

anova <- aov(shannon ~ Treat, data= edf_pt)

summary(anova)
```

```
tukey <- LTukey(anova, which="Treat", conf.level=0.95)

boxplot(shannon ~ Treat, data=edf_pt, cex = 1, ylim = c(0.5, 5), main=" Shannon's Index",
xlab="Treatments", ylab="Shannon Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

# Pielou

#anova All

shapiro.test(edf_pt$pielou) #not signifcant, data are normal

#kruskal.test

#kruskal.test(pielou~ Treat, data = edf_pt)

#pairwise.wilcox.test(edf_pt $ pielou, edf_pt $Treat, pp.adj = "bonferroni", exact=FALSE)

anova <- aov(pielou ~ Treat, data= edf_pt)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

boxplot(pielou ~ Treat, data=edf_pt, cex = 1, ylim = c(0, 0.7), main="Pielou's Evenness
Index",xlab="Treatments", ylab="Pielou's Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#boxplots with pt separetely

par(mfrow = c(2,2), oma = c(2,1,1,1))

boxplot(shannon ~ Treat, data=edf_pt, cex = 1, ylim = c(0.5, 5), main=" A) Shannon's Index",
xlab="Treatments", ylab="Shannon Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(pielou ~ Treat, data=edf_pt, cex = 1, ylim = c(0, 0.7), main="B) Pielou's Evenness
Index",xlab="Treatments", ylab="Pielou's Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(shannon ~ Treat, data=edf_pt, cex = 1,  main="C) Shannon`s index",
xlab="Treatments", ylab="Shannon`s index", col = c("#ccff66","#00CC00", "#006600"))
```

```
boxplot(pielou ~ Treat, data=edf_pt, cex = 1,  main="D) Pielou`s Evenness index",
xlab="Treatments", ylab="Pielou`s index", col = c("#ccff66","#00CC00", "#006600"))

# Faith

#anova All

shapiro.test(edf_pt$Faith) #not signifcant, data are normal

anova <- aov(Faith ~ Treat, data= edf_pt)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

boxplot(Faith ~ Treat, data=edf_pt, cex = 1, ylim = c(0.5, 5), main=" Faith's PD Index",
xlab="Treatments", ylab=" Faith's PD Index ", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

# Observed_otus

#anova All

shapiro.test(edf_pt$observed_otus) #not signifcant, data are normal

#kruskal.test

#kruskal.test(observed_otus~ Treat, data = edf_pt)

#pairwise.wilcox.test(edf_pt $ observed_otus, edf_pt $Treat, pp.adj = "bonferroni",
exact=FALSE)

anova <- aov(observed_otus ~ Treat, data= edf_pt)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

boxplot(observed_otus ~ Treat, data=edf_pt, cex = 1, ylim = c(0, 0.7), main="Observed
Features",xlab="Treatments", ylab="Pielou's Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#boxplots with pt separetely

par(mfrow = c(2,2), oma = c(2,1,1,1))
```

```
boxplot(faith ~ Treat, data=edf_pt, cex = 1, main=" A) Faith's PD ", xlab="Treatments", ylab=" Faith's PD Index ", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(observed_otus ~ Treat, data=edf_pt, cex = 1, main="B) Observed Features",xlab="Treatments", ylab="Observed Features", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

boxplot(faith ~ Treat, data=edf_pt, cex = 1,  main="C) Faith`s PD", xlab="Treatments", ylab="Faith`s PD index", col = c("#ccff66","#00CC00", "#006600"))

boxplot(observed_otus ~ Treat, data=edf_pt, cex = 1,  main="D) Observed Features", xlab="Treatments", ylab="Observed Features", col = c("#ccff66","#00CC00", "#006600"))

##EDE_only_PT

library(readr)

EDE_only_PT <- read_delim("~/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/EDE_only_PT.txt",
                          delim = "\t", escape_double = FALSE,
                          trim_ws = TRUE)

View(EDE_only_PT)

# Shannon

#anova All

shapiro.test(EDE_only_PT$shannon) #not signifcant, data are normal

#kruskal.test

kruskal.test(shannon~ abrev, data = EDE_only_PT)

pairwise.wilcox.test(edf_pt $ pielou, edf_pt $Treat, pp.adj = "bonferroni", exact=FALSE)

#anova <- aov(shannon ~ abrev, data= EDE_only_PT)

#summary(anova)

#tukey <- LTukey(anova, which="Treat", conf.level=0.95)
```

```
boxplot(shannon ~ abrev, data=EDE_only_PT, cex = 1, main=" Shannon's Index",
xlab="Treatments", ylab="Shannon Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

# Pielou

#anova All

shapiro.test(EDE_only_PT$pielou) #not signifcant, data are normal

#kruskal.test

#kruskal.test(pielou~ abrev, data = EDE_only_PT)

#pairwise.wilcox.test(EDE_only_PT $ pielou, EDE_only_PT $Treat, pp.adj = "bonferroni",
exact=FALSE)

anova <- aov(pielou ~ abrev, data= EDE_only_PT)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

#boxplot(pielou ~ Treat, data=EDE_only_PT, cex = 1, ylim = c(0, 0.7), main="Pielou's
Evenness Index",xlab="Treatments", ylab="Pielou's Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#boxplots with pt separetely

par(mfrow = c(2,2), oma = c(2,1,1,1))

boxplot(shannon ~ abrev, data=EDE_only_PT, cex = 1, ylim = c(0.5, 5), main=" A)
Shannon's Index", xlab="Treatments", ylab="Shannon Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(pielou ~ abrev, data=EDE_only_PT, cex = 1, ylim = c(0, 0.7), main="B) Pielou's
Evenness Index",xlab="Treatments", ylab="Pielou's Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(shannon ~ abrev, data=EDE_only_PT, cex = 1,  main="E) Shannon index",
xlab="Treatments", ylab="Shannon`s index", col = c("#ccff66","#00CC00", "#006600"))
```

```
boxplot(pielou ~ abrev, data=EDE_only_PT, cex = 1,  main="F) Pielou's Evenness index", xlab="Treatments", ylab="Pielou`s index", col = c("#ccff66","#00CC00", "#006600"))

# Faith

#anova All

shapiro.test(EDE_only_PT$faith) #not signifcant, data are normal

anova <- aov(faith ~ abrev, data= EDE_only_PT)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

boxplot(Faith ~ Treat, data=EDE_only_PT, cex = 1, ylim = c(0.5, 5), main=" Faith's PD Index", xlab="Treatments", ylab=" Faith's PD Index ", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

# Observed_otus

#anova All

shapiro.test(EDE_only_PT$observed_otus) #not signifcant, data are normal

#kruskal.test

#kruskal.test(observed_otus~ Treat, data = EDE_only_PT)

#pairwise.wilcox.test(EDE_only_PT $ observed_otus, EDE_only_PT $Treat, pp.adj = "bonferroni", exact=FALSE)

anova <- aov(observed_otus ~ abrev, data= EDE_only_PT)

summary(anova)

tukey <- LTukey(anova, which="Treat", conf.level=0.95)

boxplot(observed_otus ~ Treat, data=EDE_only_PT, cex = 1, ylim = c(0, 0.7), main="Observed Features",xlab="Treatments", ylab="Pielou's Index", col = c("#00CC00", "#3300cc","#ffff00","#ff3300","#660066"))

#boxplots with pt separetely

par(mfrow = c(2,2), oma = c(2,1,1,1))
```

```
boxplot(faith ~ abrev, data=EDE_only_PT, cex = 1, main=" A) Faith's PD ", xlab="Emerge Day", ylab=" Faith's PD Index ", col = c("#ccff66","#00CC00", "#006600"))

boxplot(observed_otus ~ abrev, data=EDE_only_PT, cex = 1, main="B) Observed Features",xlab="Emerge Day", ylab="Observed Features", col = c("#ccff66","#00CC00", "#006600"))

boxplot(faith ~ abrev, data=EDE_only_PT, cex = 1,  main="C) Faith`s PD", xlab="Emerge Day", ylab="Faith`s PD index", col = c("#ccff66","#00CC00", "#006600"))

boxplot(observed_otus ~ abrev, data=EDE_only_PT, cex = 1,  main="D) Observed Features", xlab="Emerge Day", ylab="Observed Features", col = c("#ccff66","#00CC00", "#006600"))

##reads_EDE all bees emerged on each days (7 and 11)

library(readr)

reads_EDE <-
read_delim("~/DOUTORADO/PRINT/ESTUDY/COmputationalanalises/Zinc/R_zinc/importing_tables/reads_EDE.txt",

                delim = "\t", escape_double = FALSE,

                trim_ws = TRUE)

View(reads_EDE)

# Shannon

#anova All

shapiro.test(reads_EDE$shannon) #not signifcant, data are normal

anova <- aov(shannon ~ abrev, data= reads_EDE)

summary(anova)

tukey <- LTukey(anova, which="abrev", conf.level=0.95)

boxplot(shannon ~ abrev, data=reads_EDE, cex = 1, main=" Shannon's Index",
xlab="abrevments", ylab="Shannon Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

# Pielou
```

```
#anova All

shapiro.test(reads_EDE$pielou) #not signifcant, data are normal

#kruskal.test

#kruskal.test(pielou~ abrev, data = reads_EDE)

#pairwise.wilcox.test(reads_EDE $ pielou, reads_EDE $abrev, pp.adj = "bonferroni",
exact=FALSE)

anova <- aov(pielou ~ abrev, data= reads_EDE)

summary(anova)

tukey <- LTukey(anova, which="abrev", conf.level=0.95)

boxplot(pielou ~ abrev, data=reads_EDE, cex = 1, ylim = c(0, 0.7), main="Pielou's Evenness
Index",xlab="abrevments", ylab="Pielou's Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#boxplots with pt separetely

par(mfrow = c(1,2), oma = c(2,1,1,1))

boxplot(shannon ~ abrev, data=reads_EDE, cex = 1, ylim = c(0.5, 5), main=" A) Shannon's
Index", xlab="Emerge Day", ylab="Shannon Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(pielou ~ abrev, data=reads_EDE, cex = 1, ylim = c(0, 0.7), main="B) Pielou's
Evenness Index",xlab="Emerge Day", ylab="Pielou's Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

boxplot(shannon ~ abrev, data=reads_EDE, cex = 1,  main="C) Shannon index",
xlab="Emerge Day", ylab="Shannon`s index", col = c("#ccff66","#00CC00", "#006600"))

boxplot(pielou ~ abrev, data=reads_EDE, cex = 1,  main="D) Pielou's Evenness index",
xlab="Emerge Day", ylab="Pielou`s index", col = c("#ccff66","#00CC00", "#006600"))

# Faith

#anova All

shapiro.test(reads_EDE$faith) #not signifcant, data are normal
```

```
anova <- aov(faith ~ abrev, data= reads_EDE)

summary(anova)

tukey <- LTukey(anova, which="abrev", conf.level=0.95)

boxplot(Faith ~ abrev, data=reads_EDE, cex = 1, ylim = c(0.5, 5), main=" Faith's PD Index",
xlab="abrevments", ylab=" Faith's PD Index ", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

# Observed_otus

#anova All

shapiro.test(reads_EDE$observed_otus) #not signifcant, data are normal

#kruskal.test

#kruskal.test(observed_otus~ abrev, data = reads_EDE)

#pairwise.wilcox.test(reads_EDE $ observed_otus, reads_EDE $abrev, pp.adj = "bonferroni",
exact=FALSE)

anova <- aov(observed_otus ~ abrev, data= reads_EDE)

summary(anova)

tukey <- LTukey(anova, which="abrev", conf.level=0.95)

boxplot(observed_otus ~ abrev, data=reads_EDE, cex = 1, ylim = c(0, 0.7), main="Observed
Features",xlab="abrevments", ylab="Pielou's Index", col = c("#00CC00",
"#3300cc","#ffff00","#ff3300","#660066"))

#boxplots with pt separetely

par(mfrow = c(2,2), oma = c(2,1,1,1))

boxplot(faith ~ abrev, data=reads_EDE, cex = 1, main=" A) Faith's PD ", xlab="abrevments",
ylab=" Faith's PD Index ", col = c("#ccff66","#00CC00", "#006600"))

boxplot(observed_otus ~ abrev, data=reads_EDE, cex = 1, main="B) Observed
Features",xlab="abrevments", ylab="Observed Features", col = c("#ccff66","#00CC00",
"#006600"))
```

```
boxplot(faith ~ abrev, data=reads_EDE, cex = 1,  main="C) Faith`s PD", xlab="abrevments", ylab="Faith`s PD index", col = c("#ccff66","#00CC00", "#006600"))

boxplot(observed_otus ~ abrev, data=reads_EDE, cex = 1,  main="D) Observed Features", xlab="abrevments", ylab="Observed Features", col = c("#ccff66","#00CC00", "#006600"))
```

Script 3 - Decontam Script

```
#importing data

Zinc.data <- bee_zinc_table_wNEG

View(Zinc.data)

MAT <- as.matrix(Zinc.data)

View(MAT)

# Calling is.matrix() function

is.matrix(MAT)

# Installing things

library(phyloseq); packageVersion("phyloseq")

if (!requireNamespace("BiocManager", quietly = TRUE))

  install.packages("BiocManager")

#browseVignettes("decontam")

library(ggplot2); packageVersion("ggplot2")

library(decontam); packageVersion("decontam")

BiocManager::install("phyloseq")

browseVignettes("phyloseq")

library(phyloseq); packageVersion("phyloseq")

matrix_for_otu <- MAT

class(matrix_for_otu) <- "numeric"

library("phyloseq")
```

```
OTU = otu_table(MAT, taxa_are_rows = FALSE)

head(otu_table(OTU))

sampledata = sample_data(decontam.metadata.Zinc)

sampledata

gplots::venn(list(decontam.metadata.Zinc=rownames(decontam.metadata.Zinc),
featuretable=colnames(bee_zinc_table_wNEG)))

sample_names(sampledata)

sample_names(OTU)

 ################

carbom5 <- phyloseq(OTU,sampledata)

carbom5

sample_names(sampledata)

get_variable(carbom5, "Sample_or_Control")

df <- as.data.frame(sample_data(carbom5)) # Put sample_data into a ggplot-friendly
data.frame

df$LibrarySize <- sample_sums(carbom5)

df <- df[order(df$LibrarySize),]

df$Index <- seq(nrow(df))

ggplot(data=df, aes(x=Index, y=LibrarySize, color=Sample_or_Control)) + geom_point() +
scale_y_continuous(limits=c(0,45000))

sample_data(carbom5)$is.neg <- sample_data(carbom5)$Sample_or_Control == "Control
Sample"

contamdf.prev <- isContaminant(carbom5, method="prevalence", neg="is.neg")

table(contamdf.prev$contaminant)

head(which(contamdf.prev$contaminant))

write.csv(contamdf.prev, file = "contam1_csv")
```

```
contamdf.prev05 <- isContaminant(carbom5, method="prevalence", neg="is.neg",
threshold=0.5)

table(contamdf.prev05$contaminant)

write.csv(contamdf.prev05, file = "comtam2.csv")

#remove taxa

ps.noncontam3 <- prune_taxa(!contamdf.prev05$contaminant, carbom5)

ps.noncontam3

# Extract abundance matrix from the phyloseq object (updated feature table)

OTU2 = as(otu_table(ps.noncontam3), "matrix")

# Coerce to data.frame

OTUdf = as.data.frame(OTU2)

write.csv(OTUdf, file = "export.csv")

??isContaminant

ps<-carbom5

# Make phyloseq object of presence-absence in negative controls and true samples

ps.pa <- transform_sample_counts(ps, function(abund) 1*(abund>0))

ps.pa.neg <- prune_samples(sample_data(ps.pa)$Sample_or_Control == "Control Sample",
ps.pa)

ps.pa.pos <- prune_samples(sample_data(ps.pa)$Sample_or_Control == "True Sample",
ps.pa)

# Make data.frame of prevalence in positive and negative samples

df.pa <- data.frame(pa.pos=taxa_sums(ps.pa.pos), pa.neg=taxa_sums(ps.pa.neg),

                    contaminant=contamdf.prev$contaminant)

ggplot(data=df.pa, aes(x=pa.neg, y=pa.pos, color=contaminant)) + geom_point() +

  xlab("Prevalence)
```

FINAL CONSIDERATIONS

The microbiome of bees plays a critical role in the health of the host, and numerous human activities put the honey bee gut microbiome at risk. It has been found that bees are exposed to a number of dangers that are contributing to the reduction of their populations. Results indicate that the gut microbiota composition of Africanized honey bees is similar to that of European honey bees. In comparison to European bees, Africanized and African bees had larger abundances of some important bacteria, including *Snodgrassella* and *Gilliamell*a. Orbaceae is linked to both African and Africanized honey bees, while *Snodgrassella, Lactobacillus, Bifidobacterium,* and *Apibacter* were related with Africanized bees. These findings may imply a relationship between the gut microbiome composition and disease resistance in Africanized and African honey bees.

Exposure to tetracycline was linked to a decline in the relative abundance of *Bombella* and *Fructobacillus* as well as important core microbiota as *Snodgrassella*, *Gilliamella*, Rhizobiaceae, and *Apibacter*. It is plausible that decreasing abundances of these microorganisms, which are essential for nutrition metabolism and pathogen defense, could have a detrimental effect on bee health. Understanding how agrochemicals, such as antimicrobials, affect honey bees is crucial given the pollination role honey bees have in the world's ecology and economy. While emerge date (the day a bee emerged from the brood comb) had a substantial impact, with lower bacterial concentrations and cell counts reported at later emerge dates, zinc therapy had very minor impacts on bacterial DNA concentrations and absolute cell counts. At zinc concentrations as high as 100 mg/L, survival was only marginally reduced (>89% survival). The bee group subjected to the greater dose of zinc (100 mg/L) showed the biggest alterations in the overall composition, diversity, and taxonomic abundances of the gut microbiome. *Paenibacillus*, a potentially pathogenic taxonomic, was detected in higher abundances in this group while numerous beneficial taxa (*Lactobacillus, Rhizobiaceae, Gilliamella*) were found at lower abundances. This implies that exposure to zinc, even at very modest levels, may have a deleterious effect on honey bee health, even if survivability is not significantly impaired. Notably, emerging date impacts were also seen in the makeup of the microbes. These findings show that when assessing the possible impacts of agrochemicals on honey bees, studies of the gut microbiota as well as other indicators of honey bee health and survival are necessary.